Université de Montréal

Angiopoietin like-2: a pro-inflammatory and pro-oxidative protein that contributes to endothelial dysfunction

par

Carol Yu

Département de Pharmacologie
Faculté de Médicine

Thèse présentée à la Faculté de Médicine
en vue de l'obtention du grade de Doctorat
en Pharmacologie

Août 2014

© Carol Yu, 2014

Université de Montréal

Faculté des études supérieures et postdoctorales

Cette thèse intitulée:

Angiopoietin like-2: a pro-inflammatory and pro-oxidative protein that contributes to endothelial dysfunction

Présentée par:

Carol Yu

a été évaluée par un jury composé des personnes suivantes:

Dre Hélène Girouard, président-rapporteur

Dr Eric Thorin, directeur de recherche

Dr Jean-Sébastien Joyal, membre du jury

Dr Ismail Laher, examinateur externe

Dr Daniel Lamontagne, représentant du doyen de la FES

ABSTRACT

Vascular aging is characterized by changes in the endothelium. Common cardiovascular risk factors, including obesity and hypertension, predispose the endothelium to increased oxidative stress, leading to endothelial dysfunction commonly characterized by diminished nitric oxide bioavailability. Although endothelial function can be a major determinant of cardiovascular risk prediction in patients, individual testing is still limited in clinical settings and thus there is increasing scientific interest in finding better biomarkers.

Angiopoietin like-2 (angptl2), a recently identified protein, is a pro-inflammatory and pro-oxidative protein involved in chronic inflammatory disorders ranging from obesity to atherosclerosis. As inflammation and increased oxidative stress are established underlying mechanisms by which endothelial dysfunction occurs, this work focuses on the role of angptl2 in endothelial dysfunction, a topic that is largely unexplored. Specifically, this work aims to 1) determine the acute effects of angptl2 on endothelial function, 2) characterize endothelial function and contribution of different endothelium-derived relaxing factors in various vascular beds in a newly generated angptl2 knock-down (KD) mouse model, and 3) examine whether the lack of angptl2 expression protects against endothelial dysfunction induced by either a high-fat diet (HFD) or angiotensin II (angII) infusion in mice.

In the first study, we show that a recombinant angptl2 acutely evokes endothelial dysfunction in the femoral artery isolated from wild-type (WT) mice, likely due to increased production of reactive oxygen species. Also in the femoral artery, angptl2 KD mice display better endothelial function compared to WT, which may be a result of greater prostacyclin contribution to vasodilation. After a 3-month HFD, the main respective endothelium-derived relaxing factors in the femoral and mesenteric arteries are preserved in angptl2 KD mice only, which was associated with a better metabolic profile, such as lower total cholesterol-to-high-density lipoprotein and low-density-to-high-density lipoprotein ratios compared to WT mice. After a HFD, KD mice have less triglyceride accumulation in the liver and smaller adipocytes in their mesenteric and epididymal white adipose tissues compared to WT

mice, while inflammatory gene expressions in adipose tissues increase in WT mice only.

In the second study, we reveal that the lack of angptl2 in KD mice results in greater nitric oxide production compared to WT mice in their isolated cerebral arteries. Chronic infusion of pro-inflammatory and pro-oxidative angII results in cerebral endothelial dysfunction only in WT mice, which is acutely ameliorated with either N-acetylcysteine, apocynin, or indomethacin, suggesting increased reactive oxygen species, likely derived from the NADPH oxidases 1/2, and increased cyclooxygenase-derived endothelium-derived contracting factors. In contrast, apocynin reduces cerebral dilation in angII-treated KD mice, suggesting recruitment of a potential compensatory dilatory NADPH oxidase pathway.

These studies are the first to explore angptl2 contribution to endothelial dysfunction in different vascular beds, and strongly suggest that angptl2 can directly impair endothelial function by its pro-inflammatory and pro-oxidative properties. Translating this to the clinical setting, expression levels of angptl2 may be an indicator of endothelial function, and lowering angptl2 levels could become a potential therapeutic approach in the treatment of chronic inflammatory disorders including cardiovascular diseases.

Keywords: Angiopoietin like-2 (angptl2), endothelium-derived relaxing factors, obesity, angiotensin II, NADPH oxidase, reactive oxygen species.

RÉSUMÉ

Le vieillissement vasculaire est caractérisé par une dysfonction de l'endothélium. De nombreux facteurs de risque cardiovasculaire tels que l'obésité et l'hypertension prédisposent l'endothélium à un stress oxydant élevé aboutissant à une dysfonction endothéliale, celle-ci étant communément accompagnée d'une diminution de la biodisponibilité du monoxyde d'azote. Bien que la fonction endothéliale soit un déterminant majeur de la prédiction du risque cardiovasculaire des patients, son évaluation individuelle reste très limitée. En conséquence, il existe un intérêt scientifique grandissant pour la recherche de meilleurs biomarqueurs.

L'Angiopoiétine like-2 (angptl2), une protéine identifiée récemment, joue un rôle pro-inflammatoire et pro-oxydant dans plusieurs désordres causés par une inflammation chronique allant de l'obésité à l'athérosclérose. L'inflammation et un stress oxydant accru ont été établis comme des mécanismes sous-jacents à l'apparition d'une dysfonction endothéliale, c'est pourquoi ce travail met l'accent sur le rôle de l'angptl2 dans la dysfonction endothéliale. Plus précisément, ce travail vise à: 1) déterminer les effets aigus de l'angptl2 sur la fonction endothéliale, 2) caractériser la fonction endothéliale et la contribution des différents facteurs relaxants dérivés de l'endothélium (EDRF) dans plusieurs lits vasculaires, et ce, dans un modèle de souris réprimant l'expression de l'angptl2 (knock-down, KD), et 3) examiner si l'absence d'expression angptl2 protège contre la dysfonction endothéliale induite par un régime riche en graisses (HFD) ou par perfusion d'angiotensine II (angII) chez la souris.

Dans la première étude, l'incubation aigue avec de l'angptl2 recombinante induit une dysfonction endothéliale dans les artères fémorales isolées de souris de type sauvage (WT), probablement en raison d'une production accrue d'espèces réactives oxygénées. Les artères fémorales de souris angptl2 KD présentent une meilleure fonction endothéliale en comparaison aux souris WT, vraisemblablement par une plus grande contribution de la prostacycline dans la vasodilatation. Après 3 mois d'une diète HFD, les principaux EDRF respectifs des artères fémorales et mésentériques sont conservés uniquement dans les souris angptl2 KD. Cette

préservation est associée à un meilleur profil métabolique, une moindre accumulation de triglycérides dans le foie et des adipocytes de plus petite taille. De plus, l'expression de gènes inflammatoires dans ces tissus adipeux n'est augmentée que chez les souris WT.

Dans la seconde étude, l'absence d'angptl2 résulte en une production accrue de monoxyde d'azote dans les artères cérébrales isolées par rapport à celles des souris WT. La perfusion chronique d'angII provoque, seulement chez les souris WT, une dysfonction endothéliale cérébrale probablement par le biais d'une augmentation de la production d'espèces réactives oxygénées, probablement dérivé des NADPH oxydase 1 et 2, ainsi que l'augmentation des facteurs constricteurs dérivés de l'endothélium issus de la cyclo-oxygénase. En revanche, l'apocynine réduit la dilatation cérébrale chez les souris KD traitées à l'angII, ce qui suggère le recrutement potentiel d'une voie de signalisation compensatoire impliquant les NADPH oxydases et qui aurait un effet vaso-dilatateur.

Ces études suggèrent fortement que l'angptl2 peut avoir un impact direct sur la fonction endothéliale par ses propriétés pro-inflammatoire et pro-oxydante. Dans une optique d'application à la pratique clinique, les niveaux sanguins d'angptl2 pourraient être un bon indicateur de la fonction endothéliale.

Mots-clés Angiopoietin like-2 (angptl2), facteurs relaxant dérivés de l'endothélium, obésité, angiotensine II, NADPH oxydase, espèces réactives de l'oxygène.

TABLE OF CONTENTS

ABSTRACT iii
RÉSUMÉ v
LIST OF TABLES xii
LIST OF FIGURES xiii
LIST OF ABBREVIATIONS xiv
ACKNOWLEDGEMENTS xix

1. Introduction 22

Chapter 1: The Endothelium 24

1.1. The endothelium – its function and dysfunction 24

1.1.1. Endothelium-derived relaxing factors (EDRFs) 25

1.1.1.1. Nitric oxide (NO) 26

1.1.1.1.1. Regulation of eNOS activity and its downstream pathways 26

1.1.1.1.2. Vascular protection and anti-oxidative effects of NO 28

1.1.1.2. Prostaglandins (PGs) 29

1.1.1.2.1. Other physiological roles of PGI_2 31

1.1.1.3. Endothelium-derived hyperpolarizing factor (EDHF) 32

1.1.1.3.1. Relevance of H_2O_2 in cerebral vasculature 33

1.1.1.3.2. The role of eNOS in generating H_2O_2 35

1.1.1.4. Heterogeneity in EDRF contribution to vasodilation 37

1.1.1.4.1. Interactions between different vasodilator pathways 39

1.1.2. Endothelium-derived contracting factors (EDCFs) 40

1.1.2.1. Prostanoids 40

1.1.2.2. Endothelin-1 (ET-1) 41

1.1.2.3. Angiotensin II (AngII) 42

1.1.2.4. Superoxide 43

1.1.3. Endothelial dysfunction 44
1.1.3.1. Alteration of the NO pathway contributing to endothelial dysfunction. 45
1.1.3.2. Oxidative stress: reactive oxygen species (ROS)....... 46
1.1.3.2.1.The uncoupled eNOS 47
1.1.3.2.2. Mitochondrial ROS 48
1.1.3.2.3. NADPH oxidases – focus on Nox1, Nox2, and Nox4 49
1.1.3.2.4. Generation of ROS by COX 52
1.1.4. Adaptation of EDRFs to endothelial dysfunction 53
1.1.5. Involvement of vasoconstrictors in endothelial dysfunction 54
1.1.5.1. Role of ET-1 in endothelial dysfunction....... 54
1.1.5.2. AngII and its signaling transduction pathways in endothelial dysfunction....... 54
1.1.5.3. COX pathway alteration....... 56
1.1.6. Pathologies associated with endothelial dysfunction....... 58
1.1.6.1. Obesity, insulin resistance and endothelial dysfunction 58
1.1.6.1.1. The adipocyte and its emerging importance in regulating endothelial function 60
1.1.6.1.2. Dyslipidemia in obesity and endothelial dysfunction 61
1.1.6.2. The renin-angiotensin system (RAS) and angII....... 61
1.1.6.2.1. Hypertension and its implications....... 62
1.1.6.2.2. AngII and its effects on the cerebrovasculature....... 64
Chapter 2: Angiopoietin-like proteins....... 67
1.2.1. The Angiopoietin-like protein family 68

1.2.2. History at a glance: identification and characterization of angiopoietin-like proteins 69
1.2.3. Physiological roles of angiopoietin-like proteins 72
1.2.3.1. Angiopoietin-like proteins in angiogenesis 72
1.2.3.2. Angiopoietin-like proteins in lipid metabolism 74
1.2.3.3. Angiopoietin-like proteins in glucose metabolism 79
1.2.4. Contribution of angptl proteins to cardiovascular risk factors 80
1.2.4.1 Angiopoietin-like proteins in dyslipidemia 80
1.2.4.2. Angiopoeitin-like proteins in obesity and insulin resistance 83
1.2.5. Angiopoietin-like proteins in endothelial dysfunction and atherosclerosis 86
Chapter 3: Angiopoietin-like-2 89
1.3.1. Angptl2 – a pro-inflammatory mediator 89
1.3.2. Angptl2 – a pro-oxidative mediator 91
1.3.3. Physiological roles of angptl2 92
1.3.3.1. Angptl2 in physiological angiogenesis 92
1.3.3.2. Angptl2 in tissue repair and remodeling 93
1.3.3.3. Angptl2 as a circadian gene 95
1.3.3.4. Angptl2 in AT1R recycling 95
1.3.4. Pathological roles of angptl2 96
1.3.4.1. Angptl2 in rheumatoid arthritis 96
1.3.4.2. Angptl2 in cancer 97
1.3.4.3. Angptl2 in obesity and insulin resistance 100

1.3.4.4. Angptl2 in vascular remodeling 103

1.3.4.5. Angptl2 in inflammatory tissue disorders 104

1.3.4.6. Angptl2 in abdominal aortic aneurysm (AAA) development 105

1.3.4.7. Angptl2 in atherogenesis 106

1.3.4.8. Angptl2 in endothelial dysfunction 107

1.3.5. Reported molecular pathways associated with angptl2 111

1.3.6. Reported mechanisms regulating angptl2 transcription and expression 112

2. Research Overview 116

2.1. Study #1: Does angptl2 knock-down protect against obesity-induced endothelial dysfunction? 117

2.1.1. Background 117

2.1.2. Hypothesis 117

2.1.3. Specific aims 117

2.2. Study #2: Does angptl2 knock-down protect against angII-induced endothelial dysfunction? 118

2.2.1. Background 118

2.2.2. Hypothesis 118

2.2.3. Specific aims 118

3. Articles 119

3.1. Article 1 119

3.1.1. Contribution of co-authors 119

3.2. Article 2 140

3.2.1. Contribution of co-authors 140

4. Discussion 153

4.1. Linking it all together: how does angptl2 mechanistically mediate endothelial dysfunction? 154

4.2. Can angptl2 lower endothelial cell stress resistance? 157

4.3. Could angptl2 also contribute to endothelial dysfunction *via* its role in AT1R recycling? 159

4.4. Could protection against high-fat diet-induced metabolic dysfunction in angptl2 KD mice be a consequence of blunted angiogenesis? 160

4.5. Is there a functional consequence of the cleaved angptl2 protein on adipocytes or ECs? 163

4.6. Lowering levels of angptl2: is that the solution? But how? 163

4.7. Limitations of the studies 166

5. Conclusion and Perspectives 170

REFERENCES 172

LIST OF TABLES

Table 1. Summary of angptl proteins and their current known functions.................67

LIST OF FIGURES

Figure 1. Proposed mechanisms of endothelium-derived relaxation and contraction in the smooth muscle cell by various known EDRFs and EDCFs........................25
Figure 2. Calcium-dependent and –independent activation of eNOS to produce NO for smooth muscle cell relaxation.. 28
Figure 3. A schematic representation of AA metabolism.......................................30
Figure 4. Proposed mechanisms of H_2O_2 production in the cerebral arteries and its downstream effects on the VSMC to cause vasorelaxation.................................35
Figure 5. EDRF heterogeneity in arteries of varying sizes......................................38
Figure 6. Activation of Nox isoforms..50
Figure 7. Proposed mechanisms by which angII stimulation of AT1R in the endothelium leads to endothelial dysfunction...56
Figure 8. ACh-induced and endothelium-dependent effects in normotensive and hypertensive rat aorta. 63
Figure 9. ROS is one of the links between cardiovascular risk factors and CVD....66
Figure 10. Schematic protein structure of an angptl protein....................................68
Figure 11. Alignment of the amino acid sequences and evolutionary relationships between angptl2 and its relatives, human angptl1, angiopoietin-1 and -2, demonstrating their homology...71
Figure 12. Summary of the different roles of angptls 3, 4, and 6 in maintaining metabolic homeostasis...78
Figure 13. The role of angptl2 in physiological and pathological adipose tissue remodeling..94
Figure 14. Proposed mechanism linking angptl2 and carcinogenesis......................99
Figure 15. A proposed model of adipocyte-derived angptl2 contribution to inflammation, insulin resistance, and vascular dysfunctions.............................109
Figure 16. Schematic representation of a selection of reported pathways that induce angptl2 expression and the pathways that are in turn induced by angptl2..........115
Figure 17. Exponentially growing scientific interests in angptl2 in physiology and pathophysiology..154

LIST OF ABBREVIATIONS

AA	Arachidonic acid
AAA	Abdominal aortic aneurysm
AC	Adenylate cyclase
ACE	Angiotensin converting enzyme
ACh	Acetylcholine
Akt	Protein kinase B
AngI	Angiotensin I
AngII	Angiotensin II
Angptl	Angiopoietin like protein
Angptl2	Angiopoietin like-2
ApoB	Apolipoprotein B
ApoE	Apolipoprotein E
ARAP1	Angiotensin II receptor-associated protein 1
ATF2	Activated transcription factor 2
AT1R	Angiotensin II receptor type 1
AT2R	Angiotensin II receptor type 2
BH_4	Tetrahydrobiopterin
BMAL1	Brain and muscle aryl hydrocarbon receptor nuclear translocator-l like protein 1
BMI	Body mass index
Ca^{2+}	Calcium
CAD	Coronary artery disease
CaM	Calmodulin
cAMP	Cyclic adenosine monophosphate
Cav-1	Caveolin-1
cGMP	Cyclic guanosine monophosphate
ChREBP	Carbohydrate responsive element binding protein
CLOCK	Circadian locomotor output cycles kaput
COX	Cyclo-oxygenase

Cu/ZnSOD	Copper- and zinc-containing superoxide dismutase
CVD	Cardiovascular disease
db/db	Diabetic (leptin receptor-deficient)
DETC	Diethyldithiocarbamate
DP	Prostaglandin D_2 receptor
EC	Endothelial cell
ECE	Endothelin-1 converting enzyme
ECM	Extracellular matrix
EDCF	Endothelium-derived contracting factor
EDHF	Endothelium-derived hyperpolarizing factor
EDRF	Endothelium-derived relaxing factor
EET	Epoxyeicosatrienoic acid
ERK	Extracellular signal regulated kinase
ETC	Electron transport chain
eNOS	Endothelial nitric oxide synthase
EP	Prostaglandin E_2 receptor
ER	Endoplasmic reticulum
ET-1	Endothelin-1
ET-2	Endothelin-2
ET-3	Endothelin-3
ET_A	Endothelin receptor A
ET_B	Endothelin receptor B
FA	Fatty acid
FAD	Flavin adenine dinucleotide
FFA	Free fatty acid
FMD	Flow-mediated dilation
FoxO1	Forkhead box protein O1
FP	Prostaglandin $F_{2\alpha}$ receptor
GC	Guanylate cyclase
GLUT4	Glucose transporter type 4
GPCR	G-protein coupled receptor

GPx	Glutathion peroxidase
GTP	Guanosine tri-phosphate
HDL	High-density lipoprotein
HFD	High-fat diet
H_2O_2	Hydrogen peroxide
HO-1	Heme-oxygenase 1
HSC	Hematopoietic stem cell
HSP90	Heat shock protein 90
HUVEC	Human umbilical vein endothelial cell
ICAM	Intracellular adhesion molecule
IFN γ	Interferon γ
IL	Interleukin
IκB	Inhibitor of kappa B
iNOS	Inducible nitric oxide synthase
IP	Prostaglandin I_2/prostacyclin receptor
IRS-1	Insulin receptor substrate 1
JAK	Janus kinase
K^+	Potassium
K_{Ca}	Calcium-activated potassium channel
KD	Knock-down
Keap1	Kelch-like erythroid cell-derived protein with cap ‘n’ collar homology-associated protein 1
KO	Knock-out
LCSFA	Long chain saturated fatty acid
LDL	Low-density lipoprotein
LDLr	Low-density lipoprotein receptor
LILRB2	Leukocyte immunoglobulin-like receptor B2
LNNA	Nω-nitro-L-arginine
LPL	Lipoprotein lipase
LXR	Liver X receptor
MAP	Mitogen-activated protein

MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractive protein 1
MMP	Metalloproteinase
MnSOD	Manganese superoxide dismutase
NAC	N-acetylcysteine
NADPH	Nicotinamide adenine dinucleotide phosphate
NFκB	Nuclear factor kappa B
NEFA	Non-esterified fatty acid
NFAT	Nuclear factor of activated T cells
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
NOX	Nicotinamide adenine dinucleotide phosphate oxidase
Nrf2	Nuclear-like factor 2
O_2	Oxygen
$O_2^{-\bullet}$	Superoxide
ob/ob	Obese (leptin-deficient)
$ONOO^-$	Peroxynitrite
PG	Prostaglandin
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGG_2	Prostaglandin G_2
PGH_2	Prostaglandin H_2
PGD_2S	Prostaglandin D_2 synthase
PGE_2S	Prostaglandin E_2 synthase
$PGF_{2\alpha}S$	Prostaglandin $F_{2\alpha}$ synthase
PGG_2S	Prostaglandin G_2 synthase
PGH_2S	Prostaglandin H_2 synthase
PGI_2	Prostaglandin I_2/Prostacyclin
PI3K	Phosphatidylinositol 3-kinase

PIRB	Paired immunoglobulin-like receptor
PKG	Protein kinase G
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PPAR	Peroxisome proliferator-activated receptor
RAS	Renin-angiotensin system
ROS	Reactive oxygen species
RXR	Retinoid X receptor
sGC	Soluble guanylate cyclase
SOD	Superoxide dismutase
STAT	Signal transducers and activators of transcription
TG	Triglyceride
TGF	Tumour growth factor
TLL1	Tolloid-like 1
TLR	Toll-like receptor
TNFα	Tumour necrosis factor α
TP	Thromboxane A_2 receptor
Trib3	Tribbles homolog 3
TXA_2	Thromboxane A_2
UCP-1	Uncoupling protein 1
UCP-2	Uncoupling protein 2
UCP-3	Uncoupling protein 3
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VLDL	Very low-density lipoprotein
VSMC	Vascular smooth muscle cell
WT	Wild-type

ACKNOWLEDGEMENTS

I have deliberately left this part of the thesis until the very end expecting myself to go through this section in no time. To my surprise, it is actually much more difficult than I imagined to express my gratitude towards each and everyone that has made this work possible. First and foremost, I would like to thank the members of my jury for this thesis. To Dr. Laher, Dr. Joyal, Dr. Girouard, and Dr. Lamontagne, thank you all for spending your time with this work and being on the jury panel, your comments are invaluable and I greatly appreciate them.

To Éric Thorin, my supervisor and mentor,
First of all, thank you for accepting me into your lab with open arms from the start. I remember I once joked that moving to Montreal was the best thing I have ever done, but in all honesty, it is one of the best decisions I have ever made. Not only has it been my honor working with you, it has been loads of fun. You are so down-to-earth, which makes work an enjoyable place to be at, and you always manage to have time for all your students. Of course it was not always easy and that “life is full of little challenges”, but you have proven time and again that things, inside or outside of the lab, do work out in the end. Thank you for being so patient, kind, generous, and overly optimistic with me throughout the past four years.

To Nathalie,
I cannot be more thankful for your teaching and help from the very beginning. It has been a great pleasure working with you. Thank you for the endless hours spent correcting this work and always giving me pointers for everything. I know you “suggest” them, but truly they are always so insightful. Although they are not always so peaceful, the discussions we have with Eric really open my mind. Working alongside the both of you, I was fortunate to be exposed to different kinds of thinking, and it has undoubtedly shaped me into a better thinker. And of course, thank you for all the delicious baked goods and food that help us last until the end of the day. Knowing that you are there, the lab is surely in good hands.

To Nada,

Thank you for your companionship throughout this time. This work would not have been completed as timely as it has without you. With you, there was never a moment of dullness, and you taught me what real multi-tasking is all about. Thank you for sharing the passion you have for your work, it is always inspiring working with you!

To Albert,

Where can I even start? Without that first-class training I had from you on the arteriograph, this work would not have been possible. It has been more than fun working with you. Thank you for always being so helpful, entertaining and witty.

To Xiaoyan,

Thank you for all your help with both of my projects. Without it, this would not have gone as smoothly as it did. You were always so patient and kind. Thank you for being so sweet to me. Working with you has been a great pleasure.

To Cécile,

It has been a great deal of awesomeness being with you both inside and outside of the lab. Thank you for all your help with all our little ones and with everything else, and always pushing me to keep going and improving.

To Adeline,

Merci for all the laughter we shared. It has been a blast working with you. You always manage to bring out my even more immature side and thanks to you, my French has “dramatically” improved, along with my exquisite taste in French cuisine.

To François,

You are one of the most organized people I know, and I greatly appreciate your help with my transitions into the lab from the start (in fact, you were the first person I met from the lab!). Thank you for everything!

To Virginie,

Working with you has been a great pleasure. You were always so determined, hard-working and dedicated to your work, so thank you for being such a great lab-mate.

To Youri,

Your arrival to the lab made me the second loudest person. I know we joke around all the time, but in all seriousness, it has been an honor working next to you. You are dedicated and always pay attention to details. Thank you for being a cool bro.

To Nour,

Although it was only a short few months that we worked together, those times were great times! Thank you for being so much fun inside and outside of the lab!

To Bruce,

Even though you always just make fun of me, I would still like to thank you for all the insightful work-related and –unrelated West coast-style conversations we've had over the last few years, and the coffee too!

I would like to thank all of our collaborators for their help in this work. I would also like to acknowledge Anie, Yolanda, Sirirat, and Eric, who I did not have a chance to directly work with, your friendship means the world to me. Thank you for being there, through the good times and the bad, and the (super loud) laughter we share. And to Maya Mamarbachi, Louis Villeneuve, Natacha Duquette, and Robert Parent, thank you for all the technical help you supported me with. To my family and friends, thank you for all the local and long-distance moral support throughout this whole time. Last but not least, I extend my great thanks to my funding agencies, the Natural Sciences and Engineering Research Council of Canada (NSERC), and the Univeristé de Montréal, for the financial support in this achievement.

1. Introduction

> "It has been said that one is as old as one's arteries. In view of the supreme importance of endothelium in arterial function, I should like to modify... this statement by saying that one is as old as one's endothelium."
>
> – *Rudolf Altschul, 1954*

Stated 6 decades ago, at a time when the endothelium was simply considered a passive cell-lining in the vasculature, this visionary statement clearly captures the relevance of endothelial health in the aging process. Indeed, endothelial cells (ECs) are known for their capabilities to secrete a wide spectrum of anti-atherosclerotic substances, as well as a balance of relaxing and contracting factors under physiological conditions (Sessa 1994; Moncada 1997; Feletou and Vanhoutte 2007; Feletou *et al.* 2011). However, under pathological conditions, where cardiovascular risk factors are present, persistent oxidative stress and endothelial dysfunction occur where ECs lose their protective role and become pro-atherosclerotic (Feletou and Vanhoutte 2006). Despite ample evidence demonstrating strong associations among cardiovascular risks, endothelial dysfunction and cardiovascular disease (CVD) (Feletou and Vanhoutte 2006), CVD remains one of the leading causes of death in Canada, and costs the Canadian economy more than $20.9 billion per year (Conference Board of Canada, 2010). The greatest and most established cardiovascular risk factor is metabolic syndrome (Katzmarzyk and Janssen 2004), which is characterized by obesity, often caused by sedentariness and excess energy intake, dyslipidemia, insulin resistance or glucose intolerance, a pro-inflammatory and pro-thrombotic state, as well as hypertension (Grundy *et al.* 2004).

Over the past years, it has become strikingly clear that endothelial dysfunction can occur in early childhood and silently progress through age (Deanfield *et al.* 2007), and recent findings have shown potentials in lowering later cardiovascular events with lifetime risk management and reduction in risk factors starting at an earlier stage (Ulrich *et al.* 2000; Cohen *et al.* 2006). Nonetheless, studies that focus on the clinical impact of endothelial dysfunction concentrate heavily on patient cohorts presented with established CVD (Deanfield *et al.* 2007). Even though endothelial function testing in patients have

shown enormous benefits in understanding the development of endothelial dysfunction and ultimately CVD, it is still not yet suitable for individual screening (Deanfield *et al.* 2007). Undoubtedly, the endothelium is a dynamic organ and its regulation is vascular district-specific with varying results in different vascular beds (Shimokawa *et al.* 1996; Urakami-Harasawa *et al.* 1997). Thus, a greater and more comprehensive understanding of its regulation is clearly warranted.

In this introductory section, the physiology and pathophysiology of the endothelium will first be discussed, followed by an introduction to a relatively new family of proteins, the "angiopoietin like-proteins", emphasizing on one in particular, angiopoietin like-2 (angptl2). How this protein is involved in regulating endothelial function, specifically in pathological settings, will be the focus of this work.

Chapter 1: The Endothelium

1.1. The endothelium – its function and dysfunction

The endothelium, consisting of a single layer of cells lining the interior surface of blood and lymphatic vessels, amount to roughly 10^{14} cells in the vasculature (Cines *et al.* 1998), and has been intensely studied since the discovery of nitric oxide (NO) as a vasodilating agent that is produced by ECs (Furchgott and Zawadzki 1980; Palmer *et al.* 1987). Indeed, vascular ECs play an extremely important role in maintaining cardiovascular homeostasis besides merely acting as a physical barrier between the lumen and vessel wall. It secretes a wide spectrum of mediators that can regulate cellular adhesion and permeability, vessel wall inflammation, smooth muscle cell proliferation, angiogenesis, and vascular tone (Sessa 1994; Moncada 1997). One of the most important features of the endothelium is its capacity to affect vascular tone by producing a balance of vasorelaxing and vasoconstricting factors (Luscher *et al.* 1989; Deanfield *et al.* 2007). Among them include NO (Furchgott and Zawadzki 1980), prostacyclin (PGI_2) (Moncada and Vane 1978), and endothelium-derived hyperpolarizing factor (EDHF) (Komori and Vanhoutte 1990; Garland *et al.* 1995), collectively called endothelium-derived-relaxing factors (EDRFs), which contribute to vasodilation, as well as endothelin-1 (ET-1) (Pernow *et al.* 2012), thromboxane (TXA_2) (Feletou and Vanhoutte 2006), superoxide anion ($O_2^{-\bullet}$) (Katusic and Vanhoutte 1989), collectively called endothelium-derived-contracting factors (EDCFs), which contribute to vasoconstriction, as depicted in Figure 1. Under the physiological state, endothelial nitric oxide synthase (eNOS)-generated NO mainly determines vascular tone (Sessa 1994; Moncada 1997), and is the major contributor of a quiescent state of the vascular wall by inhibition of inflammation and adhesion (Bath *et al.* 1991), cell proliferation (Yang *et al.* 1994), thrombosis (Ignarro 1989), and limits mitochondrial oxidative phosphorylation (Moncada and Erusalimsky 2002).

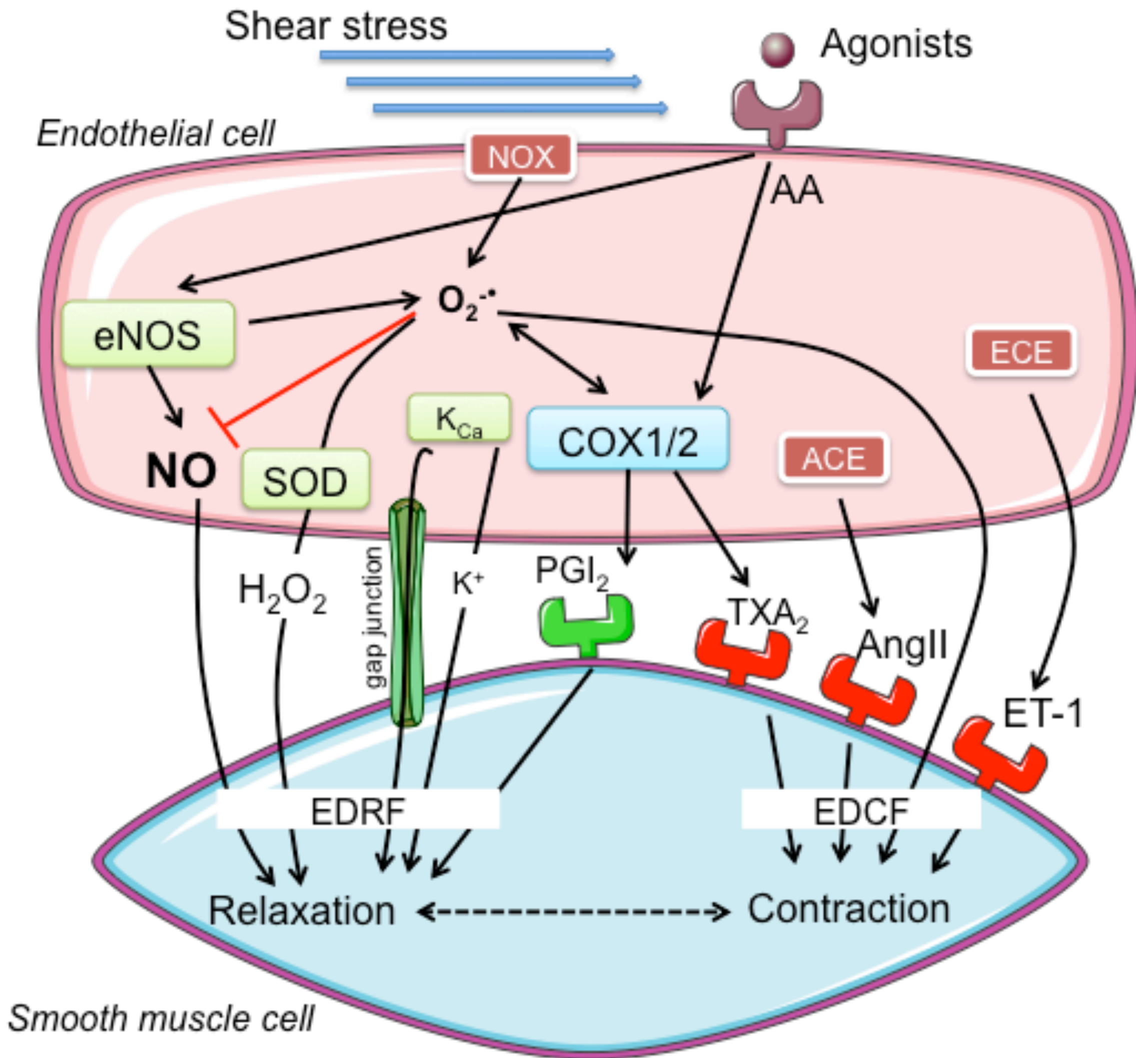

Figure 1. Proposed mechanisms of endothelium-derived relaxation and contraction in the smooth muscle cell by various known EDRFs and EDCFs. Abbreviations: EDRF: endothelium-derived relaxing factor; EDCF: endothelium-derived contracting factor; AA: arachidonic acid; eNOS: endothelial nitric oxide synthase; $O_2^{\cdot-}$: superoxide; SOD: superoxide dismutase; K_{Ca}: calcium-activated potassium channels; K^+: potassium ion; NOX: nicotinamide adenine dinucleotide phosphate oxidase; COX: cyclo-oxygenase; PGI_2: prostacyclin; TXA_2: thromboxane A_2; ACE: angiotensin converting enzyme; AngII: angiotensin II; ECE: endothelin-1 converting enzyme; ET-1: endothelin-1.

1.1.1. Endothelium-derived relaxing factors (EDRFs)

With the knowledge that the endothelium serves as a regulator of vascular tone, research has begun to focus on its mechanisms. The endothelium responds to both physical (such as shear stress and flow (Davies 1995)) and chemical (such as

acetylcholine (ACh) and bradykinin (Groves *et al.* 1995; Drexler 1999)) stimuli. The following sections will highlight the most studied EDRFs and their respective mechanisms leading to vasodilation.

1.1.1.1. Nitric oxide (NO)

First discovered by Furchgott and Zawadzki and an EDRF (Furchgott and Zawadzki 1980), this endothelium-dependent relaxing factor was identified as NO (Palmer *et al.* 1987), and was first documented to derive from vascular ECs from the conversion of L-arginine (Palmer *et al.* 1988). The contribution of NO to vasodilation is well recognized, largely in conduit arteries such as the aorta, but also extends to all types of blood vessels (Forstermann *et al.* 1994). The generation of NO, whether basal or stimulated, is dependent on the endothelial, constitutively-expressed enzyme eNOS, a dimeric, bi-domain enzyme with a C-terminal reductase domain and N-terminal oxidase domain. eNOS is responsible for the conversion of L-arginine into NO through a two-step, 5-electron-oxidation process in the presence of cofactors including tetrahydrobiopterin (BH_4) (Abu-Soud *et al.* 1994; Forstermann and Munzel 2006). Subsequent to its generation, NO diffuses from the endothelium to the vascular smooth muscle cells (VSMC), where it activates guanylate cyclase (GC), leading to cyclic guanosine monophosphate (cGMP)-mediated vasodilation (Deanfield *et al.* 2007), as shown in Figure 2. NO has an extremely short half-life ranging from 3 to 5 seconds (Ignarro 1989), and is readily degraded by $O_2^{-\bullet}$ (Gryglewski *et al.* 1986) and oxidized into nitrite and nitrate (Hibbs *et al.* 1988). Physiologically, a potent stimulator of NO generation is shear stress through a non-receptor-dependent mechanisms (Rubanyi *et al.* 1986; Corson *et al.* 1996), and pharmacologically, a number of agonists have been found able to stimulate NO generation including ACh and bradykinin, both of which bind to their respective receptor, which lead to subsequent downstream vasodilatory pathways (Doyle and Duling 1997).

1.1.1.1.1. Regulation of eNOS activity and its downstream pathways

There are 4 distinct isoforms of NOS – neuronal (nNOS), inducible (iNOS), endothelial (eNOS) (Forstermann *et al.* 1993), and red blood cell NOS (Jubelin and

Gierman 1996). As eNOS is the dominant NOS isoform expressed in the vasculature that produces NO under physiological conditions, it will be the primary focus of this chapter. The activity and expression of eNOS are tightly regulated both transcriptionally (Wang and Marsden 1995) and translationally (Sase and Michel 1997). Post-translationally and in its inactive form, eNOS is bound to inhibitory protein caveolin-1 and localized at caveolae in the cell membrane (Lisanti *et al.* 1994). Stimulation such as receptor-mediated rise in intracellular calcium has been shown to activate eNOS by disrupting the protein-protein interaction between eNOS and caveolin-1 (Brouet *et al.* 2001), thus increasing NO production.

There are two main branches of eNOS activation – calcium-dependent and calcium-independent activation, as shown in Figure 2. Classical agonists such as ACh act *via* G-protein-coupled receptors (GPCRs) to generate intracellular rise in calcium, which then binds to calmodulin and together bind to eNOS, resulting in its activation (Busse and Fleming 1995). In the other case, independently of calcium, stimuli such as shear stress (Ayajiki *et al.* 1996) and insulin (Zeng and Quon 1996) activate phosphatidylinositol 3-kinase (PI3K) and protein kinase B (Akt), which then phosphorylate and activate eNOS (Harris *et al.* 2001). This PI3K/Akt signaling pathway downstream of insulin stimulation is, interestingly, shared with insulin-dependent glucose transporter type 4 (GLUT-4)-mediated glucose uptake (Hsueh and Law 1999), which will gain importance in settings of insulin resistance and associated dysfunctions.

Subsequently, the activated eNOS generates NO, as described in section 1.1.1.1., which diffuses into VSMC and activates GC by binding to its heme group at the iron (Lowenstein *et al.* 1994). Activation of GC leads to its synthesis of cGMP from GTP, which in turn activates cGMP-dependent protein kinase G (Carvajal *et al.* 2000), initiating a cascade of phosphorylation reactions resulting in physiological effects such as lowering intracellular calcium in VSMC and ultimately leading to vasorelaxation (Francis and Corbin 1994; Lohmann *et al.* 1997), as shown in Figure 2.

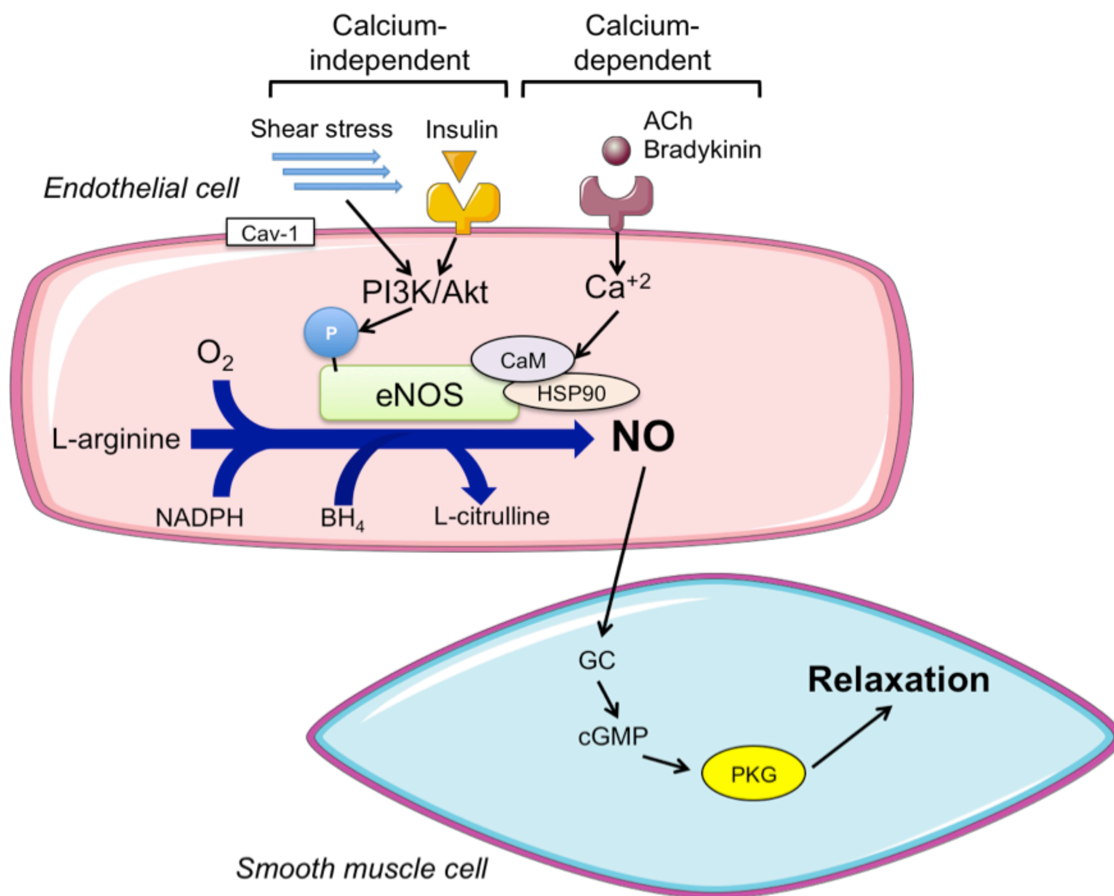

Figure 2. Calcium-dependent and –independent activation of eNOS to produce NO for smooth muscle cell relaxation. Abbreviations: ACh: acetylcholine; O_2: oxygen; NADPH: nicotinamide adenine dinucleotide phosphate; Cav-1: caveolin-1; eNOS: endothelial nitric oxide synthase; NO: nitric oxide; PI3K: phosphatidylinositol 3-kinase; Akt: protein kinase B; Ca^{2+}: calcium; CaM: calmodulin; HSP90: heat shock protein 90; BH_4: tetrahydrobiopterin; GC: guanylate cyclase; cGMP: cyclic guanosine monophospate: PKG: protein kinase G.

1.1.1.1.2. Vascular protection and anti-oxidative effects of NO

Besides its ability to act as a vasodilating agent, vascular NO possesses many other physiological properties. Importantly, NO has anti-platelet (Radomski and Moncada 1993), anti-adhesive (Bath *et al.* 1991), anti-proliferative (Yang *et al.* 1994) and anti-inflammatory (Bath *et al.* 1991) effects, and these properties become extremely important in settings of atherogenesis. For example, NO can potently inhibit aggregation of platelets and leukocytes onto the vessel wall. It does so by interfering with the

adhesive bonding between the leukocyte adhesion molecule and the endothelium surface, which is an early event of atherogenesis (Forstermann *et al.* 1994). In addition, it has been reported that NO could suppress DNA synthesis, mitogenesis, as well as proliferation of VSMC (Forstermann *et al.* 1994).

As mentioned, interactions between vascular signaling such as that of NO and oxidants such as superoxide are well documented. NO is best known for its ability to impair oxidation of free fatty acids (FFA), phosphatidylcholine and low-density lipoprotein (LDL) particles. NO induces endothelial ferritin formation (Recalcati *et al.* 1998), which then can reduce oxidative damage by preventing superoxide generations as ferritin binds free iron ions (Balla *et al.* 1992). Mechanistically, NO induces expression of heme oxygenase-1 (HO-1), which can then increase formation of bilirubin and carbon monoxide (Maines 1997), which in turn can scavenge superoxide and activate soluble GC (sGC), respectively (Stocker *et al.* 1987). NO has also been documented to induce extracellular superoxide dismutase both *in vitro* and *in vivo* in VSMC (Fukai *et al.* 2000). As a result, NO is able to decrease both superoxide and peroxynitrite levels in the vessel wall.

1.1.1.2. Prostaglandins (PGs)

Synthesized mainly from fatty acid (FA) arachidonic acid (AA) derived from the cell membrane released by phospholipase A_2, PGs were discovered before NO was identified as an endothelium-derived vasoactive substance (Moncada *et al.* 1976). AAs are then metabolized by different enzymes – prostaglandin H synthases (PGH synthases, or more commonly cyclo-oxygenases COX-1 or -2) (Vane *et al.* 1998), lipoxygenases, or cytochrome P450 (Morrow *et al.* 1990). COX-1 and -2 are the first and also rate-limiting enzymes to process PGs, and give rise to prostaglandin G_2 (PGG_2), which is reduced into short-lived prostaglandin H_2 (PGH_2). The fate of PGH_2, in turn, depends on the actions of PG synthases – prostaglandin D_2 synthase (PGDS), prostaglandin E_2 synthase (PGES), prostaglandin $F_{2\alpha}$ synthase (PGFS), prostaglandin I_2 synthase (PGIS) and thromboxane synthase (TXS), which form the five main PGs – prostaglandin D_2 (PGD_2), prostaglandin E_2 (PGE_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), prostaglandin I_2 (PGI_2), and thromboxane A_2

(TXA_2), respectively. A schematic representation of AA metabolism is shown in Figure 3.

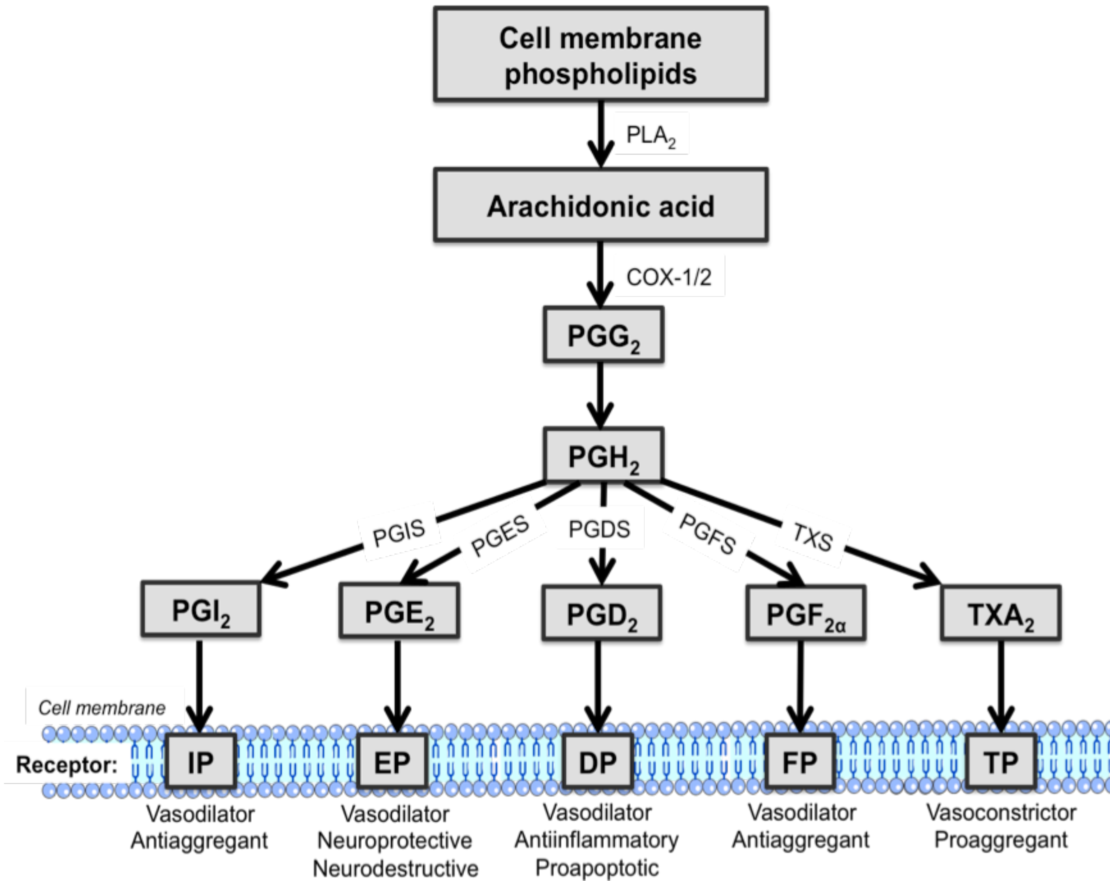

Figure 3. A schematic representation of AA metabolism. AA is first derived from cell membrane phospholipids by PLA_2, which is metabolized by COX-1 and COX-2 into PGG_2, and is then reduced into PGH_2. Subsequently, PGH_2 is further metabolized by different PG synthases into distinct prostanoids. Abbreviation: PLA_2: phospholipase A_2; COX-1/2: cyclo-oxygenase 1/2; PGG_2: prostaglandin G_2; PGH_2: prostaglandin H_2; PGI_2: prostaglandin I_2; PGE_2: prostaglandin E_2; PGD_2: prostaglandin D_2; $PGF_{2\alpha}$: prostaglandin $F_{2\alpha}$; TXA_2: thromboxane A_2; PGIS: prostaglandin I_2 synthase; PGES: prostaglandin E_2 synthase; PGDS: prostaglandin D_2 synthase; PGFS: prostaglandin $F_{2\alpha}$ synthase; TXS: thromboxane A_2 synthase; prostaglandin I_2 receptor: IP; prostaglandin E_2 receptor: EP; prostaglandin D_2 receptor: DP; prostaglandin $F_{2\alpha}$ receptor: FP; thromboxane A_2 receptor: TP.

PGI_2, derived from PGI_2 synthase (Hara *et al.* 1994), is typically described as an endothelium-derived vasodilator, as it can bind to and stimulate its receptor, the IP receptor, to activate adenylate cyclase and increase intracellular cAMP concentration and produce smooth muscle relaxation (Wise and Jones 1996). PGI_2 synthase is highly expressed in ECs (Tang and Vanhoutte 2008) and to a lesser degree also in VSMC (Wu and Liou 2005). Furthermore, vasodilation by PGI_2 has been accompanied by hyperpolarization of adjacent VSMC, which may involve potassium channels (Corriu *et al.* 2001). Of note, plasma concentrations of PGI_2 in human has been observed to peak at infancy and decrease throughout life (Kaapa *et al.* 1982). Hence, PGI_2 is usually not the main vasodilator in most vascular beds (Shimokawa *et al*. 1996); however, its vasodilatory role could become important in the face of decreased NO bioavailability, for instance, PGI_2 may compensate for the loss of NO in hypertensive patients (Bulut *et al.* 2003).

1.1.1.2.1. Other physiological roles of PGI_2

In most arterial beds, many believed that the synthesis of PGI_2 and downstream responses were not altered under pathological settings. A prime example was showcased by the intact PGI_2 vasodilating system in the coronary resistance arteries of atherosclerotic mice, despite damages in NO-dependent dilations (Godecke *et al.* 2002). However, this is not always the case, as shown in the abrogated PGI_2-dependent vasodilation in the aorta of aldosterone-treated normotensive and hypertensive rats (Blanco-Rivero *et al.* 2005). Importantly and in addition to its vasodilating features, PGI_2 is characterized by its powerful anti-coagulant and anti-adhesive properties (Moncada and Vane 1978). This was highlighted by the unexpected results coming from colorectal adenoma patients presented with adverse cardiovascular effects of rofecoxib, trademarked as Vioxx (Merck and Co.) (Bresalier *et al.* 2005), ultimately leading to its withdrawal from the market. This could be partly explained by its inhibition of protective PGI_2 synthesis, in spite of reduced TXA_2 synthesis (Griffoni *et al.* 2007). Indeed, PGI_2 is the most potent endogenous inhibitor of platelet aggregation (Bunting *et al.* 1976; Moncada *et al.* 1976). It can also act in a paracrine or endocrine manner to affect functions of other cells types *via* specific GPCRs (Negishi *et al.* 1995) or nuclear

receptors such as the peroxisomal proliferator-activated receptors (PPARs) (Forman *et al.* 1997), as well as regulate physiological events such as angiogenesis (Spisni *et al.* 1992) and apoptosis (Li *et al.* 2004).

1.1.1.3. Endothelium-derived hyperpolarizing factor (EDHF)

The most recently discovered group of EDRF has been broadly termed endothelium-derived hyperpolarizing factor (EDHF), with its first evidence reported in 1988 (Chen *et al.* 1988; Feletou and Vanhoutte 1988). By definition, EDHF is a substance or an electrical signal that is released from the endothelium and is able to hyperpolarize VSMC resulting in relaxation (Fleming 2000; Feletou and Vanhoutte 2007). Its contribution to vasodilation is found in the greatest levels in small arteries such as the resistance arteries (Shimokawa *et al.* 1996; Urakami-Harasawa *et al.* 1997; Luksha *et al.* 2009). Ongoing debates have accumulated over the recent years surrounding topics on the variable nature of EDHFs and their variable mechanisms of actions. Initially, EDHF referred to any relaxing factor that is neither NO nor PGI_2, but over the past decade, a number of possible candidates have been uncovered: epoxyeicosatrienoic acids (Rubanyi and Vanhoutte 1987), hydrogen peroxide (H_2O_2) (Beny and von der Weid 1991; Matoba *et al.* 2000), K^+ ions (Edwards *et al.* 1998), C-type natriuretic peptide (Chauhan *et al.* 2004), as well as contact-mediated mechanisms (Rummery and Hill 2004). Although the contribution by EDHF to vasodilation is limited in large conductance arteries under healthy conditions, it has been documented that EDHF can compensate for loss of NO bioavailability in disease states (Csanyi *et al.* 2012).

The basic mechanism of EDHF release and action can be separated into two stages. The first stage involves the pathways that take place in the endothelium: an increase in intracellular Ca^{2+}, K^+ efflux upon activation of Ca^{2+}-dependent K^+ channel, followed by hyperpolerization and generation of the EDHF that diffuses through myoendothelial gap junctions (McGuire *et al.* 2001; Busse *et al.* 2002). The second stage involves those taking place in the VSMC: EDHF activating K^+ channels and leading to endothelium-dependent hyperpolarization, followed by closure of voltage-gated Ca^{2+} channels and lastly, relaxation of the VSMC (Busse *et al*. 2002; McGuire *et al.* 2001).

1.1.1.3.1. Relevance of H_2O_2 in cerebral vasculature

While in most vascular beds eNOS-derived NO predominantly contributes to vasodilation including flow-mediated dilation (FMD) (Davies 1995), both NO (Iadecola 1992; White *et al.* 1998) and EDHF (Fujii *et al.* 1991) have been found involved in this mechanism. In particular, one of the EDHFs, namely H_2O_2, has been demonstrated to cause vasodilation in the cerebral vasculature from mice (Wei and Kontos 1990; Fraile *et al.* 1994; Wei *et al.* 1996; Iida and Katusic 2000; Drouin and Thorin 2009). Importantly, we previously demonstrated that H_2O_2-mediated cerebral vasodilation was inhibited by an sGC inhibitor, suggesting that H_2O_2 shared a similar downstream vasodilatory pathway with NO (Drouin *et al.* 2007). H_2O_2, although not a free radical itself, is the product of various superoxide dismutases (SOD), including copper- and zinc-containing SOD (Cu/ZnSOD) (Morikawa *et al.* 2003). In contrast to highly reactive reactive oxygen species (ROS) such as $O_2^{-\bullet}$, H_2O_2 is readily diffusible and is relatively stable. Most importantly, it does not react with NO to produce peroxynitrite (Pacher *et al.* 2007). Consistent with data from human coronary (Liu *et al.* 2011) and rat skeletal (Sindler *et al.* 2009) arterioles, H_2O_2 contributes to vasodilation of cerebral arteries *in vitro* (Fraile *et al*. 1994; Iida and Katusic 2000), with strong evidence pointing towards potassium channels mediating its effects (Iida and Katusic 2000; Paravicini *et al*. 2004; Sobey *et al.* 1997), which leads to hyperpolarization and vasodilation (Sobey *et al.* 1997). Indeed, in rat cerebral arterioles, H_2O_2 caused vasodilation *via* activation of calcium-dependent potassium channels (Sobey *et al.* 1997), and in cat cerebral arterioles, H_2O_2 activated ATP-sensitive potassium channels (Wei *et al.* 1996). Exogenous H_2O_2 and ACh-induced H_2O_2 production may also activate sGC in mouse cerebral arteries (Drouin *et al*. 2007), resulting in elevated cGMP levels that may cause hyperpolarization and subsequently relaxation (Nelson *et al.* 1990). Interestingly, cerebral arterial dilation to H_2O_2 can also stimulate AA release from VSMC *via* phospholipase A_2 activation (Rao *et al.* 1995), and this was confirmed by sensitivity of H_2O_2-dependent cerebral arteriolar dilation to indomethacin (Iida and Katusic 2000), a non-specific COX inhibitor, in addition to H_2O_2-mediated cAMP increase (Iida and Katusic 2000). Consistent with this, topical application of H_2O_2 onto piglet cerebral arterioles increased formation of 6-ketoprostaglandin F1α, thromboxane B_2, and PGE_2 (Leffler *et al.* 1990). Elevated cAMP

in the VSMC may cause relaxation by activating potassium channels to induce hyperpolarization and decrease calcium influx (Nelson *et al.* 1990). Collectively, these findings suggest that H_2O_2 may also cause dilation indirectly *via* the COX-mediated vasodilatory pathway. Proposed mechanisms of H_2O_2-mediated vasodilation are summarized in Figure 4. Moreover, vasodilatory effects of H_2O_2 have been documented in studies using different vasoactive stimuli – AA (Kontos *et al.* 1984), bradykinin (Kontos *et al.* 1984; Yang *et al.* 1991; Sobey *et al.* 1997), ACh (Drouin *et al*. 2007), as well as flow (Drouin and Thorin 2009).

The sources of H_2O_2 have been primarily reported from the dismutation of $O_2^{-\bullet}$, and also directly from various enzymes, such as lipooxygenases, xanthine oxidase and NADPH oxidase (Haas *et al.* 1994; Cai 2005). Importantly, the recent provocative proposal that eNOS may be involved as a source of H_2O_2 in cerebral arteries (Drouin and Thorin 2009; Drouin *et al*. 2007), associated with evidence showing eNOS-dependent H_2O_2-mediated dilation (Zembowicz *et al.* 1993; Bharadwaj and Prasad 1995; Cai *et al.* 2003) have shed light on the relationship between eNOS, the main generator of NO, and H_2O_2, in the cerebral vascular tone regulation.

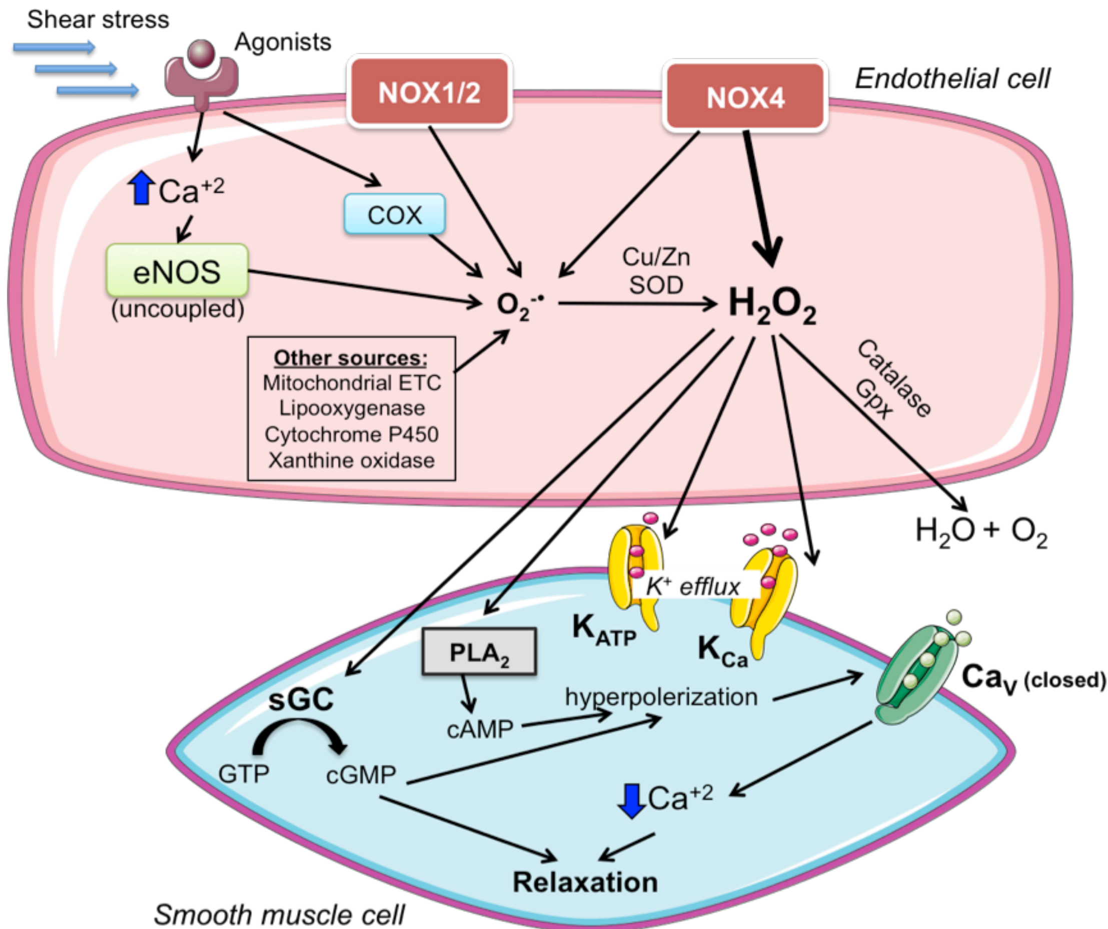

Figure 4. Proposed mechanisms of H_2O_2 production in the cerebral arteries and its downstream effects on the VSMC to cause vasorelaxation. Abbreviations: NOX: nicotinamide adenine dinucleotide phosphate oxidase; COX: cyclo-oxygenase; eNOS: endothelial nitric oxide synthase; ETC: electron transport chain; $O_2^{-\bullet}$: superoxide; Cu/Zn SOD: copper- and zinc-containing superoxide dismutase; H_2O_2: hydrogen peroxide; Gpx: glutathione peroxidase; H_2O: water; O_2: oxygen; sGC: soluble guanylate cyclase; GTP: guanosine tri-phosphate; cGMP: cyclic guanosine monophosphate; PLA_2: phospholipase A_2; cAMP: cyclic adenosine monophosphate; K_{ATP}: ATP-sensitive potassium channel; K_{Ca}: calcium-dependent potassium channel; Ca_V: voltage-gated calcium channel.

1.1.1.3.2. The role of eNOS in generating H_2O_2

The enzyme eNOS, under physiological conditions, is known for its ability to produce NO in its coupled state (Forstermann and Munzel 2006). However, in conditions where oxidative stress is abundant or cofactors are limited, eNOS is uncoupled resulting in lower NO generation but greater superoxide production (Forstermann and Munzel 2006; Vasquez-Vivar *et al.* 1998). In the presence of enzymes capable of reducing

superoxide such as SOD, dismutation reaction of superoxide can take place to result in H_2O_2 generation (Cai 2005).

The involvement of eNOS in H_2O_2 generation was confirmed in pressurized mouse cerebral arteries. In a seminal study by Drouin *et al*. using young and healthy C57Bl/6 mice, ACh-mediated cerebral vasodilation was abolished by eNOS inhibitor Nω-nitro-L-arginine (LNNA), catalase, SOD inhibitor diethyldithiocarbamate (DETC) but not by NO inhibitor pyruvate (Drouin *et al*. 2007). It was demonstrated that eNOS was, although uncoupled, physiologically producing H_2O_2 in these cerebral arteries in response to ACh (Drouin *et al*. 2007). The "uncoupled" state of eNOS to produce H_2O_2 was proposed to be physiological as H_2O_2 generation was not detected in eNOS KO mice and was not due to low BH_4 levels (Drouin *et al*. 2007), conditions where pathological eNOS uncoupling is commonly observed (Forstermann and Munzel 2006; Vasquez-Vivar *et al*. 1998). On the other hand, excess BH_4 induced both NO and H_2O_2 production (Drouin *et al*. 2007). In addition, eNOS-derived H_2O_2 activated sGC (Drouin *et al*. 2007), suggesting a shared vasodilatory pathway with NO, as previously proposed by others (Iesaki *et al.* 1999). Most likely, SOD was involved to dismutate superoxide into H_2O_2 as Also, cerebral arteriole ACh-mediated dilation impairment was previously reported in heterozygous manganese SOD $(MnSOD)^{+/-}$ mice (Faraci *et al.* 2006).

In addition to ACh, it was reported that FMD in mouse cerebral arteries also involved eNOS to produce H_2O_2 (Drouin and Thorin 2009). In this study where cerebral arterioles isolated from young and healthy C57Bl/6 mice were used, FMD activated eNOS in an Akt-dependent manner that was associated with H_2O_2 production (Drouin and Thorin 2009). Accordingly, the dilation driven by H_2O_2 was prevented by eNOS inhibition and H_2O_2 scavengers, but not by NO scavengers (Drouin and Thorin 2009), strongly implying eNOS involvement in FMD mediated by H_2O_2.

Although superoxide generated from eNOS has traditionally been viewed as a pathological state (Vasquez-Vivar *et al.* 1998; Yang *et al.* 2009), these recent studies, especially ones examining smaller cerebral arterioles (Drouin and Thorin 2009; Drouin *et al*. 2007), have provided examples that the unstable superoxide can be converted into H_2O_2 by SOD and that eNOS can potentially regulate vascular tone through H_2O_2 production.

1.1.1.4. Heterogeneity in EDRF contribution to vasodilation

Relaxation of blood vessels dependent on the endothelium is the results of various agents such as ACh and bradykinin, or physical stimulus such as shear stress. Not only does the nature of the stimulus determine the specific EDRF responsible for the resulting signaling events, contribution by various EDRF to dilation of arteries also heavily relies on the specific arterial bed. As a general rule, larger conductance arteries usually utilize NO to a greater degree than other EDRFs, while smaller resistance arteries depend on EDHF more preferentially (Shimokawa *et al*. 1996; Urakami-Harasawa *et al*. 1997). For instance, the aorta, a conductance artery, mainly utilizes NO for dilation (Shimokawa *et al*. 1996) whereas smaller resistance arteries typically rely on EDHF (Shimokawa *et al*. 1996; Urakami-Harasawa *et al*. 1997), as illustrated by the key role of H_2O_2 in small cerebral arteries (Drouin *et al.* 2007; Drouin and Thorin 2009). The contribution of PGI_2, on the other hand, usually does not play major roles regardless of vessel size (Shimokawa *et al*. 1996). This is summarized in Figure 5.

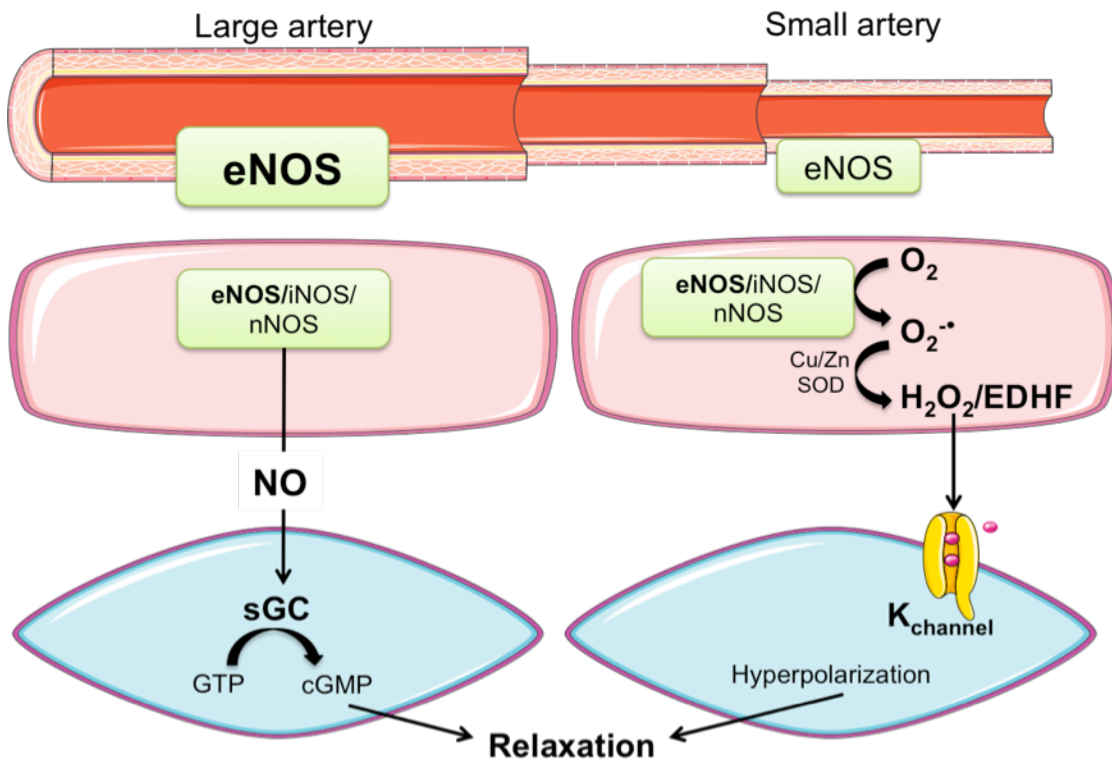

Figure 5. EDRF heterogeneity in arteries of varying sizes. NO is the main contributor to vasodilation in larger conductance artery whereas its role decreases as artery size decreases, and EDHF gains important roles in the small artery. Abbreviations: eNOS: endothelial nitric oxide synthase; iNOS: inducible nitric oxide synthase; nNOS: neuronal nitric oxide synthase; NO: nitric oxide; sGC: soluble guanylate cyclase; GTP: guanosine tri-phosphate; cGMP: cyclic guanosine monophosphate; Cu/ZnSOD: copper- and zinc-containing superoxide dismutase; $O_2^{-\bullet}$: superoxide; H_2O_2: hydrogen peroxide; EDHF: endothelium-derived hyperpolarizing factor.

Adapted with the permission from the Rockefeller University Press: J Exp Med. Takaki A, Morikawa K, Tsutsui M, Murayama Y, Tekes E, Yamagishi H, Ohashi J, Yada T, Yanagihara N, Shimokawa H. Crucial role of nitric oxide synthases system in endothelium-dependent hyperpolarization in mice. *J Exp Med*. **205**, 2053-2063. Copyright (2008).

However, the study of EDRF contribution to vasodilation is further complicated by their heterogeneity among different species and genders. Studies using different animal models have provided ample evidence demonstrating this rich diversity of vasodilatory pathways. For instance, FMD in porcine coronary arterioles exclusively depends on NO, demonstrated by complete abolishment of FMD by NOS inhibitors (Kuo

et al. 1991), while FMD is mainly mediated by PGI_2 in cremaster muscle arterioles in rats (Koller *et al.* 1995). In the guinea pig coronary arterioles, NO is the main vasodilator (Kelm and Schrader 1990; Kostic and Schrader 1992; Miura and Gutterman 1998), whereas in the same vascular bed in human, both NO and EDHF participate in vasodilation (Stork and Cocks 1994; Miura and Gutterman 1998; Miura *et al.* 1999), and in the rat, it is completely mediated by an EDHF (Fulton *et al.* 1995), whereas it is mediated by both NO and PGI_2 in the rabbit (Lamontagne *et al.* 1992). Furthermore, an evolution of vasodilators used in various stages of development has been observed in both animals and human. For example, in the vertebral artery, contribution of PGI_2 to ACh-mediated dilation is present at infancy but diminishes while NO takes over in adulthood (Charpie *et al.* 1994). As another example, endogenous H_2O_2 contributes to skeletal arteriole vasodilation in juvenile rats but not in weaning rats (Samora *et al.* 2008). Taken together, the study of endothelium-dependent dilator mechanisms is complex – usually not only one vasodilator is involved, and different EDRFs can interact and/or compensate for each other. Hence, the endothelium is a dynamic organ with heterogeneous responses depending on different factors.

1.1.1.4.1. Interactions between different vasodilator pathways

In addition to the diversity of endothelium-dependent vasodilatory mechanisms, there are complex interactions between EDRFs. It has been proposed that COX and NOS enzymes influence each another, such that inhibition of either system could enhance the role of the other (Ichihara *et al.* 1998), while COX-2-derived vasodilators were observed to compensate for NO in renal arteries when NOS was acutely inhibited (Beierwaltes 2002). A more recent study reported a direct binding interaction between iNOS and COX-2 that facilitates NO S-nitrosylation and activation of COX-2 (Kim *et al.* 2005). Interactions between NO and cAMP, a downstream mediator in the COX pathway to mediate vasodilation, have also been reported (Zhang and Hintze 2001). Indeed, the study by Zhang and Hintze suggests that cAMP can activate protein kinase A, which in turn, through a PI3K-dependent pathway, phosphorylates eNOS by Akt (Zhang and Hintze 2001). Once again, there are complicated interactions among different vasodilatory pathways even within the same arterial bed in the same species.

In conclusion, the endothelium is capable of secreting different EDRFs, namely NO, PGI_2, and EDHF in response to both mechanical and chemical stimuli in context-dependent manners which is also dependent on vascular bed and vessel size, with complex interactions among them. With that, we will now focus on the other main group of endothelial mediators – the EDCFs.

1.1.2. Endothelium-derived contracting factors (EDCFs)

The endothelium, besides mediating relaxation, also plays a vital role to mediate vasocontraction to maintain vascular tone. Endothelium-dependent contractions are induced by both physical (such as pressure, stretch, and flow) and chemical (such as cytokines) stimuli (Luscher *et al.* 1992) and in response, the endothelium releases EDCFs. Endothelium-dependent contractions have been demonstrated in different vascular beds – aorta (Luscher and Vanhoutte 1986), carotid arteries (Traupe *et al.* 2002), cerebral arterioles (Mayhan 1992), femoral arteries (Shi *et al.* 2007) and many more. Particularly, in disease states such as hypertension, effects of EDCFs become more prominent as they may counteract actions of EDRFs to increase vascular tone. Many EDCFs have been identified and characterized, and will be briefly outlined below.

1.1.2.1. Prostanoids

Derived from COX-1 and -2 enzymes, prostanoids including endoperoxides (Auch-Schwelk *et al.* 1990), PGI_2 (Rapoport and Williams 1996) and thromboxane A_2 (Shirahase *et al.* 1988), have all been proposed to be EDCFs. Endoperoxides are immediate products of COX enzymes, which are then spontaneously or enzymatically converted into downstream prostanoids such as PGI_2, prostaglandin D_2 and E_2 (Bos *et al.* 2004), but are also vasoconstrictors themselves (Ito *et al.* 1991).

PGI_2, which was previously mentioned as one of the EDRFs, can also act as a vasoconstrictor at high doses (Levy 1980; Williams *et al.* 1994) through activation of the TP receptors. This usually occurs in pathological states such as hypertension, where there are dysfunctions in the main IP receptor (Rapoport and Williams 1996).

Lastly, TXA_2 is the major COX-derived vasoconstrictor resulting from the thromboxane synthase-catalyzed reaction from endoperoxides (Moncada and Vane 1978)

and acts mainly on TP receptors (Figure 3), resulting in elevated calcium levels through calcium entry in VSMC, leading to contraction (Berridge and Irvine 1984; Shenker *et al.* 1991). The role of TXA_2 becomes increasingly important both in aging (Drouin *et al*. 2011) and dyslipidemia (Gendron and Thorin 2007) in mouse cerebral arteries, as well as during atherosclerosis in mouse renal arteries (Gendron and Thorin 2007). In mouse cerebral arteries, increased TXA_2 production was reported to limit eNOS activity (Drouin *et al*. 2011), which was partly restored when the mice were given preventive polyphenol catechin treatment (Drouin *et al*. 2011). In mouse renal arteries, TXA_2 production was augmented in the presence of dyslipidemia, and was associated with a change in the redox environment (Gendron and Thorin 2007).

1.1.2.2. Endothelin-1 (ET-1)

Isolation of the 21-amino acid protein endothelin was first reported in 1988 (Yanagisawa *et al.* 1988) and include 3 main isoforms – ET-1, ET-2, and ET-3 (Inoue *et al.* 1989). ET-1, which is the predominant isoform expressed in the vasculature and released by ECs, potently vasoconstricts both *in vitro* and *in vivo* (Hickey *et al.* 1985; Yanagisawa *et al.* 1988; Miller *et al.* 1989), and will be the main focus herein. Through activation of two receptor subtypes – ET_A (localized in VSMC only) and ET_B (localized in EC and VSMC), ET-1 exerts its vascular actions to result in phospholipase C (PLC) activation, increased cytosolic calcium and myosin kinase phosphorylation for smooth muscle contraction and ultimately vasoconstriction (Seo *et al.* 1994). In ECs, ET-1 activation of ET_B would increase intracellular calcium leading to eNOS activation and dilation (Tsukahara *et al.* 1994), whereas stimulation of ET_B receptors in VSMC would cause vasoconstriction (Haynes *et al.* 1995). Therefore, receptor localization and the balance between ET_A and ET_B receptors would ultimately determine the net effects of ET-1. ET-1 can, in healthy human beings, cause increases in mean arterial blood pressure, reduction in heart rate, cardiac output and stroke volume (Weitzberg *et al.* 1993), suggesting involvement of this peptide to regulate vascular homeostasis.

1.1.2.3. Angiotensin II (AngII)

AngII is a peptide hormone well known to cause vasoconstriction, and in the long run, to cause increased blood pressure. AngII is considered the main final mediator of the renin-angiotensin system (RAS), where renin is a protease released by the juxtaglomerular cells of the kidney. Renin functions to cleave angiotensinogen, which is a glycoprotein released in the blood mainly from the liver, converting it into angiotensin I (angI) (Skeggs *et al.* 1954), which is then subsequently converted into angII (Skeggs *et al.* 1956b) by angiotensin converting enzyme (ACE) (Skeggs *et al.* 1956a). AngII then acts as the effector molecule by stimulating two possible receptor subtypes – the angII receptor type 1 (AT1R) (Murphy *et al.* 1991; Sasaki *et al.* 1991) and angII receptor type 2 (AT2R) (Chiu *et al.* 1989; Whitebread *et al.* 1989; Mukoyama *et al.* 1993). Besides merely acting as a vasoconstrictor, angII plays a pivotal role in the maintenance of cardiovascular homeostasis, while it is also implicated in many CVDs (Higuchi *et al.* 2007), which will be discussed later.

It is well established that the AT1R and AT2R subtypes, although sharing 34% sequence homology, are distinct in their expression patterns and functions (Lemarie and Schiffrin 2010). While the AT1R is ubiquitously expressed in the cardiovascular system, the AT2R is found highly expressed during fetal development and its expression is reduced rapidly after birth (Henrion *et al.* 2001). Furthermore, binding of angII to these receptors results in distinct signaling pathways and cellular responses (Lemarie and Schiffrin 2010). For instance, downstream signaling pathways of AT1 include vasoconstriction, superoxide production, cell proliferation and hypertrophy, whereas those of AT2 include vasodilation, growth inhibition and NO production (Lemarie and Schiffrin 2010). Importantly, pathological effects of angII have mainly been attributed to angII binding to the AT1R (Mehta and Griendling 2007; Lemarie and Schiffrin 2010).

The AT1R can be further categorized into the A and B subtype (Inagami *et al.* 1994), which share 96% homology as well as ligand binding and downstream signaling mechanisms. It is now clear that angII binding to AT1R leads to downstream effects that are also time-dependent. Within seconds, angII can activate PLC (Griendling *et al.* 1986), generating inositol phosphate and calcium mobilization (Brock *et al.* 1985), leading to

vasoconstriction. In the scale of minutes, angII can stimulate mitogen-activated protein kinase (MAPK) activity (Morrell *et al.* 1999) and in hours, the Janus kinase (JAK) signal transducers and activators of transcription (STAT) pathway (Marrero *et al.* 1995). Downstream transduction pathway of the AT1R is also cell type- and tissue-specific, such as hypertrophy and vasoconstriction in the VSMC, and sodium re-absorption in the kidneys (Lemarie and Schiffrin 2010).

The signaling transduction pathway of angII through the AT1R is tightly regulated. Besides the classic regulating mechanisms such as receptor transcription (Lassegue *et al.* 1995), phosphorylation (Kai *et al.* 1994) and receptor desensitization after angII binding (Sasamura *et al.* 1994), and heterodimerization with other receptors (AbdAlla *et al.* 2000), additional proteins have been identified to regulate the local sensitivity of the receptor to angII by modifying receptor surface expression *via* its recycling (Daviet *et al.* 1999; Lopez-Ilasaca *et al.* 2003; Tsurumi *et al.* 2006). In particular, the cytoplasmic C-terminal of the AT1R may interact with specific AT1R-associated proteins, such as angptl2 (Guo *et al*. 2003, Guo *et al*. 2001), which can ultimately determine receptor efficacy and function, and will be discussed in detail later (section 1.3.2.4.).

1.1.2.4. Superoxide

Superoxide is generated from molecular oxygen by various sources, such as the COX enzymes, xanthine oxidases, and NOX enzymes (Cai and Harrison 2000), can give rise to multiple ROS and reactive nitrogen species (Faraci 2006), and can act as an EDCF (Katusic and Vanhoutte 1989). Superoxide is most reactive with NO, with NO reacting three times as quickly with superoxide to form peroxynitrite than with SOD (Faraci 2005). Loss of NO function to vasodilate is the main consequence of its inactivation by superoxide. Interestingly, however, effects of superoxide have been suggested to be biphasic, as demonstrated in basilar cerebral arterioles from rabbits, with low concentration resulting in relaxation but high concentration resulting in contraction (Didion and Faraci 2002). Furthermore, superoxide is capable of directly contracting vascular smooth muscle in cerebral arteries independent of the endothelium (Tosaka *et al.* 2002).

Taken together, the single-cell-layer endothelium plays a vital role in regulating vascular tone by secreting both EDRFs and EDCFs in response to multiple stimuli. To achieve vascular homeostasis, the endothelium maintains the balance between vasodilation and vasoconstriction, prevention and stimulation of VSMC proliferation and migration, inhibition and promotion of platelet adhesion and aggregation, as well as between thrombogenesis and fibrinolysis (Davignon and Ganz 2004). When this balance is tipped, endothelial dysfunction occurs.

1.1.3. Endothelial dysfunction

Endothelial dysfunction has long been regarded as the primary first step in the cascade leading up to coronary events. In the past, the term "endothelial dysfunction" has been used to refer to altered anti-coagulant and anti-inflammatory features of the endothelium and vascular remodeling (Feletou and Vanhoutte 2006). The term "endothelial dysfunction", in recent literature, broadly describes impaired endothelium-dependent relaxation (Winquist *et al.* 1984; Lockette *et al.* 1986), and represents a switch from the quiescent phenotype of the endothelium to activation of a molecular machinery that produces chemokines, cytokines, as well as adhesion molecules, which ultimately leads to an inflammatory state (Deanfield *et al.* 2007). Under this pathological state, ROS can uncouple eNOS and lead to superoxide generation (Forstermann and Munzel 2006). Loss of NO bioavailability is the hallmark of endothelial dysfunction, which ultimately contributes to CVD pathogenesis. Indeed, all major cardiovascular risk factors in human including hypercholesterolemia, hypertension, and smoking, have been found associated with endothelial dysfunction (Cai and Harrison 2000). Also in this state, there is typically imbalance of EDRFs and EDCFs, in favor of the EDCFs, as it is commonly observed in patients with CVD.

The term "endothelial dysfunction" is rather a broad terminology, and in fact is quite heterogeneous in nature depending on multiple factors. In the following sections, endothelial dysfunction and its mechanisms in different pathological states will be introduced and compared.

1.1.3.1. Alteration of the NO pathway contributing to endothelial dysfunction

The balance between the synthesis and degradation of NO determines its bioavailability. While the biosynthesis of NO is mostly due to activity of eNOS, its inactivation can be caused by reactions with various molecules. Most importantly, NO is inactivated by superoxide at an extremely rapid rate, 6.7×10^9 mol/L^{-1} • s^{-1} (Thomson *et al.* 1995), which is approximately three times faster than the dismutation of superoxide by SOD. Endothelial dysfunction generally describes the loss of NO bioavailability, which can be caused by a myriad of factors and pathological states.

Obvious factors that could result in decreased NO bioavailability include decreased eNOS expression (Wilcox *et al.* 1997), limited substrates or cofactors for eNOS (Pou *et al.* 1992), possible alterations in the signaling pathways that activate eNOS (Shimokawa *et al.* 1991), as well as decreased VSMC sensitivity to NO (Drexler 1997). Most importantly, and for the scope of the current work, is the degradation or deactivation of NO by ROS (Cai and Harrison 2000).

Although it may seem paradoxical, one of the first studies using hypercholesterolemic rabbit aorta, which displayed impaired endothelium-dependent vasorelaxation, showed augmented production of NO upon stimulation by ACh, implicating functional eNOS activation and the pathways leading up to it (Minor *et al.* 1990). These results generated the speculation that although NO was normally produced in the hypercholesterolemic state, it was being oxidized to become nitrogen oxides, namely nitrites and nitrates. Indeed, when cholesterol-fed rabbits were treated with polyethylene-glycolated-SOD, which was able to reduce superoxide, endothelium-dependent vasorelaxation was improved (Mugge *et al.* 1991), supporting the notion that decreased NO bioavailability was a direct consequence of increased superoxide. In line with this, *in vitro* studies using purified eNOS have also demonstrated that the enzyme, besides mainly participating in NO production, can potentially produce superoxide (Xia *et al*. 1998). Besides decreased eNOS function, up-regulation and increased activity of another isoform of NOS, namely the iNOS, has been reported to be associated with endothelial dysfunction in diabetic rats (Nagareddy *et al.* 2005) and mice (Gunnett *et al.* 2003), hypothyroid rats (Virdis *et al.* 2009), and lipopolysaccharide-treated mice

(Chauhan *et al.* 2003). Related to this, iNOS was involved in age-related ROS production in the rat aorta (Oudot *et al.* 2006), and was found induced by low-grade vascular inflammation (Busse and Mulsch 1990; Kessler *et al.* 1997). Indeed, it has been demonstrated that iNOS generates superoxide at its reductase domain (Xia *et al.* 1998), which may simultaneously react with NO to form peroxynitrite (Beckman *et al.* 1990).

Decreased NO bioavailability is also associated with other pathologies, with majority of which involving chronic inflammation, such as insulin resistance and diabetes, obesity, hypertension, atherosclerosis and many more (Lockette *et al.* 1986; Durante *et al.* 1988). Before further detailing these pathological states, ROS and their sources will first be described.

1.1.3.2. Oxidative stress: reactive oxygen species (ROS)

For over half a century, many have focused on the topic of ROS and scientists have long suspected that ROS are key players in the aging process and the pathogenesis of age-related diseases. ROS are oxygen-derived small molecules produced by all aerobic cells, including all vascular cell types – endothelial, vascular smooth muscle, and adventitial cells. They are typically generated by a cascade of reactions starting with superoxide, and this occurs within the mitochondria (Balaban *et al.* 2005), peroxisomes *via* cytochrome P-450 (Gonzalez 2005), and through other cellular activities (Mueller *et al.* 2005). Under pathological vascular settings, the most important players in the generation of ROS include mitochondria, COX, xanthine oxidase, uncoupled eNOS, and NADPH oxidase (Paravicini and Touyz 2008). ROS include oxygen radicals such as superoxide, hydroxyl, and peroxyl as well as non-radicals such as H_2O_2. The observation of ROS involvement in the aging process was first documented in 1956 by Denham Harman (Harman 1956), which gave rise to the "free radical theory of aging". It was observed that there was a relationship between life cycle length and metabolic rates and that irradiation of living organisms caused aging, mutations, and cancer by production of superoxide and hydroxyl molecules (Harman 1956). Harman proposed that the free oxygen radicals produced are the cause of damage and aging of a cell over time (Harman 1956). Despite early wide acceptance of ROS being involved in cellular aging, ROS was

also recognized to have beneficial effects, in particular its role in innate immunity (Matsuzawa *et al.* 2005), and as important signaling molecules (Ray *et al.* 2012).

Under conditions of oxidative stress, there is an imbalance between pro- and anti-oxidative activities, resulting in high levels of ROS, which typically activates pathways of inflammation, proliferation, angiogenesis, apoptosis, and more (Alfadda and Sallam 2012; Ray *et al.* 2012). Ultimately, these pathways pre-set the stage for vascular remodeling and endothelial dysfunction, as mentioned earlier, which then prime the system into pathological settings of a multitude of diseases including heart failure, atherosclerosis, hypertension, obesity, diabetes, and so on (Virdis *et al.* 2004; Harrison *et al.* 2006; Nistala *et al.* 2009).

Of the aforementioned enzymes responsible in ROS generation, xanthine oxidase has mostly been implicated in the context of cardiac diseases and only a few studies have proposed its role in endothelial dysfunction (Suzuki *et al.* 1998; Mervaala *et al.* 2001), hence only the uncoupled eNOS, mitochondrial enzymes, COX and Nox enzymes will be further discussed.

1.1.3.2.1.The uncoupled eNOS

Numerous studies have reported that the function of eNOS is altered in disease states such as diabetes, and transforms from a NO-producing enzyme to a superoxide-producing enzyme (Yang *et al*. 2009). While all four isoforms of NOS - eNOS, nNOS, iNOS and red blood cell NOS, can be uncoupled, eNOS is a particularly important generator of ROS, when uncoupled, within the vasculature since eNOS is the most abundant form present and is responsible for the majority of vascular NO produced (Forstermann *et al.* 1994). eNOS is a cytochrome p450 reductase-like enzyme facilitating and catalyzing electron transport from NADPH to a heme group, and requires two molecules of BH_4 to transfer electrons to L-arginine to form NO (Abu-Soud *et al*. 1994; Forstermann and Munzel 2006). This flow of electrons within eNOS is tightly regulated. When it is disturbed, however, the ferrous dioxygen complex becomes dissociated, resulting in superoxide generation instead of NO, which is also referred to "uncoupling" (Forstermann and Munzel 2006) – that is, uncoupling of NADPH oxidation and NO synthesis resulting in oxygen being the terminal electron acceptor instead of L-arginine

(Wever *et al.* 1997). Under normal circumstances, the functional eNOS is present as a dimer and BH_4 can preserve its dimerization (Cai *et al.* 2005). The eNOS is uncoupled when there is limited amount of co-factors such as L-arginine and BH_4 (Vasquez-Vivar *et al.* 1998). Failure of eNOS to dimerize results in greater monomer-to-dimer ratio and superoxide formation (Landmesser *et al.* 2003).

Uncoupling of eNOS has been observed in both *in vitro* (Pritchard *et al.* 1995) and *in vivo* (Cosentino and Luscher 1998) models of CVDs, as well as in patients with cardiovascular risk factors such as smoking (Heitzer *et al.* 2000) and hypercholesterolemia (Stroes *et al.* 1997). Supplementation with vitamin C has been shown to stabilize (Heller *et al.* 2001) and increase BH_4 production (Huang *et al.* 2000), thus correcting eNOS uncoupling and minimizing its superoxide production (Schmidt and Alp 2007).

1.1.3.2.2. Mitochondrial ROS

The mitochondria generate a significant amount of ROS by nature of its respiratory function in order to metabolize nutrients. Through ATP production, ROS formation is a byproduct (Murphy 2009). The mitochondrial transport chain consists of complexes I through IV, which are all capable of producing superoxide that is rapidly converted into H_2O_2, with only complex III being able to generate ROS in the intermembrane space (Turrens 2003). In addition, dysfunction of complexes II and IV was shown to result in electron leak leading to ROS generation (Shen 2010). Physiologically, there exists a tight regulation of the mitochondrial redox balance through mechanisms such as the dismutation of superoxide by MnSOD and conversion into H_2O_2 (Balaban *et al.* 2005; Murphy 2009), and endogenous uncoupling by uncoupling proteins (Zamzami *et al.* 1995), which involves limitation of free radicals by mitochondrial membrane transporters, the uncoupling protein 1, 2, and 3 (UCP1, UCP2, UCP3) (Rousset *et al.* 2004). On the other hand, factors such as hyperpolarization of the mitochondria can trigger superoxide formation by complex III (Zamzami *et al*. 1995). There is evidence of association between mitochondrial dysfunction and insulin resistance (Stump *et al.* 2003). Moreover, endothelial dysfunction induced by hyperglycemia could be reversed when ROS production from mitochondria was blocked

(Nishikawa *et al.* 2000), altogether suggesting involvement of ROS generated by the mitochondria in the development of endothelial dysfunction.

1.1.3.2.3. NADPH oxidases – focus on Nox1, Nox2, and Nox4

NADPH oxidases, or Nox, are ROS generators that transfer electrons across biological membranes with oxygen being the electron acceptor and superoxide being the product of this reaction (Bedard and Krause 2007). The main function of NADPH oxidases is ROS generation (Lambeth 2004; Wingler *et al.* 2011). Nox 1, 2, 4 and 5 are expressed in the endothelium but Nox5 is absent in rodents (Drummond and Sobey 2014). Therefore, the focus of this section will be Nox isoforms 1, 2, and 4.

Historically, before the identification of the Nox enzymes, early observations of a respiratory burst in phagocytes were first made (MacLeod 1943), reporting that the phagocyte respiratory burst depended on glucose metabolism and required energy (Sbarra and Karnovsky 1959), and giving superoxide as an initial product (Babior *et al.* 1973). In the Nox terminology, $gp91^{phox}$, a catalytic subunit of the phagocyte NADPH oxidase first cloned by Royer-Pokora *et al*. (Royer-Pokora *et al.* 1986) and Teahan *et al*. (Teahan *et al.* 1987), is called Nox2. Besides this catalytic subunit, the phagocyte enzyme also consists of a transmembrane stabilizer protein $p22^{phox}$ (Dinauer *et al.* 1987; Parkos *et al.* 1988), cytosolic subunits $p47^{phox}$, $p67^{phox}$ (Nunoi *et al.* 1988; Volpp *et al.* 1988), and $p40^{phox}$ (Wientjes *et al.* 1993). Altogether in the Nox family, there are, so far, seven described Nox isoforms, two organizer subunits ($p47^{phox}$ and NOXO1), two activator subunits ($p67^{phox}$ and NOXO1), DUOX1 and DUOX2 (Bedard and Krause 2007), as shown in Figure 6 below.

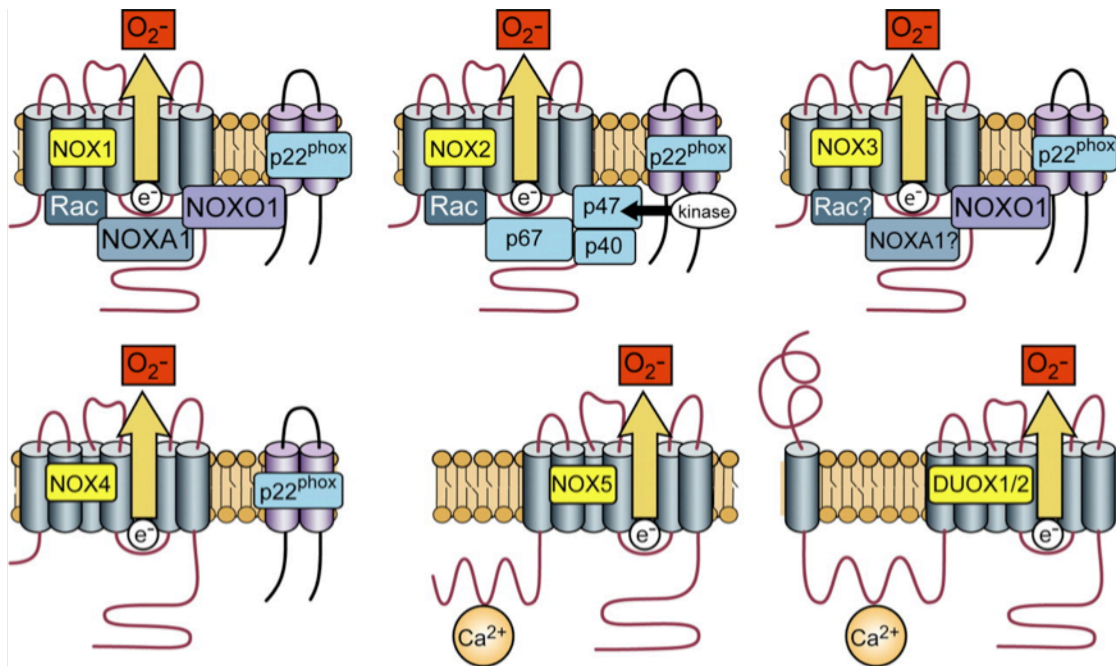

Figure 6. Activation of Nox isoforms. Although they have similar structures and function, different Nox isoforms require different subunits and have different mechanisms of actions. From top left to bottom right: 1) Nox1 requires $p22^{phox}$, NOXO1 and Rac. 2) Nox2 requires $p22^{phox}$, $p47^{phox}$, $p67^{phox}$, Rac, and possibly $p40^{phox}$. 3) Nox3 requires $p22^{phox}$ and NOXO1. 4) Nox4 requires $p22^{phox}$ and possibly Rac. 5 and 6) Nox5 and DUOX1/2 do not require any subunits, but are activated by Ca^{2+}.

Reprinted with the permission of The American Physiological Society: Physiol Rev. Bedard K, Krause KH. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiol Rev*. **87**, 245-313. Copyright (2007).

All members of the Nox family are transmembrane proteins responsible for the reduction of oxygen to superoxide *via* electron transport across cell membranes. Conserved structural properties common to all Nox enzymes include an NADPH-binding site, a flavin adenine dinucleotide (FAD)-binding region, 6 transmembrane domains, and 4 heme-binding histidines (Bedard and Krause 2007).

Exact mechanisms of superoxide generation by the Nox enzymes are not clearly established. Extensive research has focused on the role of Nox2, which is the prototype NADPH oxidase. Structurally, it has been predicted to have between 4 to 6 transmembrane domains (Segal *et al.* 1992; Henderson *et al.* 1995), with both the $-NH_2$

and -COOH termini facing the cytoplasm except for DUOX1/2 (Burritt *et al.* 2003; Paclet *et al.* 2004), as shown in Figure 6. Nox2 is constitutively associated with $p22^{phox}$ (Groemping and Rittinger 2005), and all Nox enzymes but Nox5 and DUOX1/2 seem to require $p22^{phox}$ (Ambasta *et al.* 2004; Kawahara *et al.* 2005; Bedard and Krause 2007). Presently, it is believed that phosphorylation of $p47^{phox}$ leads to a conformational change, which allows for its interaction with $p22^{phox}$ (Groemping *et al.* 2003; Capone *et al.* 2010), and that its phosphorylation is the key rate-limiting step. Indeed, the kinetics of $p47^{phox}$ parallel that of NADPH oxidase activation (Rotrosen and Leto 1990). Nox enzymes respond to a number of stimuli, including various growth factors, cytokines, mechanical forces, metabolic factors, as well as GPCR-coupled agonists including bradykinin, ET-1, and angII (Lassegue and Clempus 2003).

Another important isoform, Nox1, has recently gained attention in the field of hypertension as improvement of vasodilation in response to ACh and blunted vasopressor effects of angII in Nox1-deficient mice were reported (Matsuno *et al.* 2005; Gavazzi *et al.* 2006). Consistent with this, overexpression of Nox1 in VSMC of mice showed more pronounced aortic vascular hypertrophy and hypertension when infused with angII (Dikalova *et al.* 2005). The cloning and ROS-generating properties of Nox1 were first described in 1999 (Suh *et al.* 1999), and it has been shown that Nox1 constitutively produces low levels of superoxide anion (Dutta and Rittinger 2010).

Up until now, Nox1 and Nox2 have been the only isoforms consistently found associated with endothelial dysfunction (Jung *et al.* 2004; Matsuno *et al.* 2005; Gavazzi *et al.* 2006) and it is generally believed that while the Nox1 and Nox2 homologue generate superoxide anions, which then lead to vascular dysfunction, Nox4 mainly produces H_2O_2 (Montezano *et al.* 2011). Notably, patients who had a rare genetic loss of Nox2 were presented with greater NO-, flow- and endothelium-dependent vasodilation, as well as increased NO levels in platelets (Violi *et al.* 2009). Furthermore, these patients did not develop endothelial dysfunction after ischemia-reperfusion (Loukogeorgakis *et al.* 2010). These two compelling studies in human subjects strongly support studies in animals, and suggest a link between Nox2 and its superoxide producing function with endothelial dysfunction. Copious work has shown the consequences of increased superoxide anions, which is diminishing levels of NO, *via* interaction to produce

peroxynitrites (Beckman and Koppenol 1996; Takac *et al.* 2011). As such, these highly reactive peroxynitrites are able to oxidize cysteines and BH_4, which is an essential cofactor for eNOS. Since Nox4 predominately produces H_2O_2, which is not capable to react with NO and scavenge it, this particular isoform has been recently proposed to be beneficial (Schroder *et al.* 2012; Drummond and Sobey 2014).

1.1.3.2.4. Generation of ROS by COX

In earlier sections, the roles of COX-1 and -2 in ECs were discussed in the context of vasodilator and vasoconstrictor production, where they catalyze the synthesis of PGI_2 or TXA_2 *via* PGG_2 (Figure 3). Along with prostanoids, these enzymes are also capable of generating ROS (Rosenblum 1987; Katusic 1996; Niwa *et al.* 2001; Yang *et al.* 2002), which can be inhibited by anti-inflammatory drugs (Simon 1996). Whereas COX-1 has been suggested to provide homeostatic functions (Dubois *et al.* 1998), COX-2 has been primarily associated with pathophysiological states such as inflammation (Seibert and Masferrer 1994). Increased COX-2 expression was found in inflamed joint tissues (Anderson *et al.* 1996) and its induction was detected in human osteoarthritis-affected cartilage (Amin *et al.* 1997) and synovial tissues of rheumatoid arthritis patients (Kang *et al.* 1996). Selective inhibition of COX-2, on the other hand, has been shown to reduce endothelial dysfunction in patients with peripheral artery disease and hypertension (Florez *et al.* 2009) and reduce atherosclerotic lesions in atherosclerotic mice (Burleigh *et al.* 2005). In line with this, our laboratory observed increased TXA_2-dependent free radical production *via* COX before the onset of endothelial dysfunction in mouse renal arteries, which led to decreased EDHF-dependent dilation (Gendron and Thorin 2007).

Mechanistically, there is evidence that COX-2 produces ROS (Katusic 1996) as it uses molecular oxygen to generate ROS as an intermediate (Marnett 2000). In human ECs subjected to high glucose, COX-2 expression was up-regulated *via* increased PKC pathway activation, which was associated with increased ROS production (Cosentino *et al.* 2003).

In summary, the possible sources of oxidative stress leading to endothelial dysfunction are the uncoupled eNOS, mitochondria, the Nox, and the COX enzymes.

1.1.4. Adaptation of EDRFs to endothelial dysfunction

Whereas there seems to be dynamic sources of oxidative stress that contribute to endothelial dysfunction, the diversity of EDRFs in blood vessels allows the endothelium to compensate for the loss of NO (Durand and Gutterman 2013), which is the hallmark of endothelial dysfunction. It is clear that EDRFs are capable of interacting with one another (Beierwaltes 2002; Ichihara *et al*. 1998; Zhang and Hintze 2001), as presented earlier. Various reports have shown compensatory as well as redundant mechanisms in mediating endothelial responses (Lamping *et al.* 2000; Bulut *et al.* 2003; Bagi 2009; Goto *et al.* 2012), and strongly suggest that complex interactions among various vasodilating pathways in fact act to preserve endothelial function when challenged with risk factors. For instance, when the normal mechanisms are down-regulated in face of a disease state, another overlapping mechanism could be up-regulated to maintain function near normal, at least up to a certain point in time. A classic example is seen in the eNOS knock-out (KO) mice, where eNOS-derived NO is absent, there is compensation by other vasodilators such as PGI_2 in coronary arteries (Lamping *et al*. 2000). Different NOS can also compensate for the loss of eNOS function. Indeed, another study by Kelly *et al*. showed that in small coronary arteries isolated from eNOS KO mice, NO production is shifted to nNOS (Kelly *et al.* 1996). Likewise, in atherosclerotic patients (Wilcox *et al*. 1997) and hypertensive rats (Boulanger *et al.* 1998), up-regulation of nNOS may represent a compensatory mechanism in the face of decreased NO production from eNOS. In essential hypertensive patients, COX-2-derived prostaglandins partly compensated for the decreased NO bioavailability to vasodilate (Bulut *et al*. 2003). In healthy settings, where NO is the predominant vasodilator, NO inhibits production of EDHF but during pathological settings, such as in hypertension, diminished NO up-regulates EDHF contribution to vasodilation (Goto *et al*. 2012). On the other hand, severe hypercholesterolemic mice exhibited preserved EDHF-mediated vasodilation to ACh in the cremaster muscle resistance arteriole even though NO-mediated vasodilation was attenuated (Wolfle and de Wit 2005). Innate endothelial function of human subjects may also pre-determine compensations in vasodilatory pathways. For example, FMD was impaired in sedentary subjects following acute exercise-induced hypertension whereas

FMD was preserved in athletic subjects, but was shifted from NO to H_2O_2 (Phillips *et al.* 2009), revealing complex response systems to compensate for NO loss.

1.1.5. Involvement of vasoconstrictors in endothelial dysfunction

Although endothelial dysfunction is primarily concerned with impaired production and bioavailability of the main vasodilators, an additional important alteration associated with endothelial dysfunction is increased production of vasoconstrictors, such as ET-1, angII, and COX-derived prostanoids.

1.1.5.1. Role of ET-1 in endothelial dysfunction

As a potent vasoconstrictor, ET-1 has been implicated in endothelial dysfunction associated with CVDs. For instance, ET-1 levels were found elevated in patients with pulmonary arterial hypertension (Giaid *et al.* 1993), while there was a more pronounced forearm vasoconstriction in response to ET-1 in patients with atherosclerosis compared to control (Bohm *et al.* 2002). Increased contractile responsiveness to ET-1 (Donato *et al.* 2005) as well as elevated plasma ET-1 levels (Maeda *et al.* 2003) associated with aging and endothelial dysfunction were also reported, suggesting involvement of this vasoactive peptide in pathological settings. Indirectly, ET-1 can further augment vasoconstriction by also inducing generation of TXA_2 (Taddei and Vanhoutte 1993). With the knowledge that the ET_B receptor activation in VSMC leads to vasoconstriction (Haynes *et al.* 1995), the evidence of increased ET_B receptor expression in atherosclerotic arteries in human (Iwasa *et al.* 1999) provides one of the explanations of altered ET-1 effects on vascular reactivity, which ultimately leads to endothelial dysfunction.

1.1.5.2. AngII and its signaling transduction pathways in endothelial dysfunction

With more than a century of research on the RAS, involvement of angII in the pathophysiology of CVDs is now widely accepted. Much of what is known about angII effects on the vasculature comes from studies on VSMC. AngII-stimulated AT1Rs in VSMC induced rapid protein tyrosine phosphorylation, leading to growth promoting

signal including MAPK/extracellular signal-regulated kinase (ERK) action. AngII is able to activate a number of tyrosine kinase receptors, such as platelet-derived growth factor (Heeneman *et al.* 2000) and epidermal growth factor receptors (Eguchi *et al.* 2003; Lin and Freeman 2003), *via* cross-talk with the AT1R. AngII-stimulated AT1Rs, through nicotinamide adenine dinucleotide (NADH)/NADPH oxidase-dependent pathway, have also been shown to produce ROS as second messengers (Griendling *et al.* 1997). Knowledge on angII/AT1R signaling mechanisms in ECs is limited, but there is evidence suggesting that endothelial dysfunction is induced through inhibition of NO function (Millatt *et al.* 1999; Yan *et al.* 2003), and will be extensively discussed in the context of the cerebrovasculature in a later section (1.1.6.2.2.). AngII also induced ET-1 mRNA expression in ECs *via* activating ROS-sensitive ERK (Hsu *et al.* 2004), suggesting synergistic interactions between the two vasoconstrictors. In human umbilical vein endothelial cells (HUVECs), it has been shown that angII signaling *via* AT1R inhibited insulin-induced NO production by increasing phosphorylation of insulin receptor substrate 1 (IRS-1) (Andreozzi *et al.* 2004), while another study reported that in HUVECs, angII induced nuclear factor kappa B (NFκB)-dependent transcriptional up-regulation of adhesion molecules intracellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1, which involved ROS and p38 MAPK activation (Costanzo *et al.* 2003). Taken together, angII is strongly implicated in CVD settings, and exerts its deleterious actions through other receptors besides the AT1R (summarized in Figure 7).

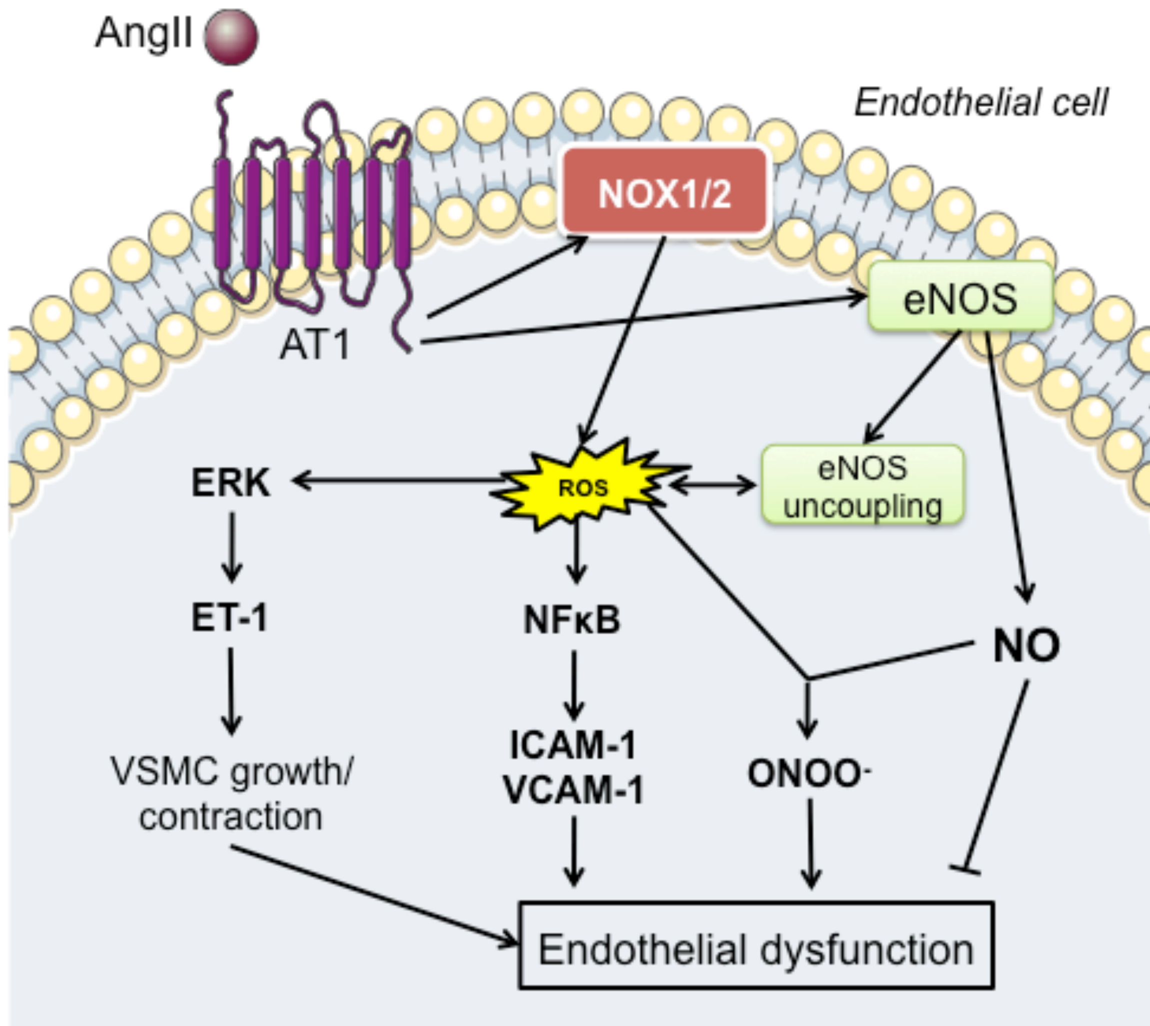

Figure 7. Proposed mechanisms by which angII stimulation of AT1R in the endothelium leads to endothelial dysfunction. Abbreviations: AngII: angiotensin II; AT1: angiotensin II receptor type 1; ERK: extracellular signal-regulated kinase; ET-1: endothelin-1; VSMC: vascular smooth muscle cell; NOX: nicotinamide adenine dinucleotide phosphate oxidase; ROS: reactive oxygen species; NFκB: nuclear factor kappa B; ICAM-1: intracellular adhesion molecule-1; VCAM-1: vascular cell adhesion molecule-1; eNOS: endothelial nitric oxide synthase; NO: nitric oxide; $ONOO^-$: peroxynitrite.

1.1.5.3. COX pathway alteration

Another aspect of endothelial dysfunction involves the COX-mediated pathways. Under physiological circumstances, COX enzymes participate in regulating vascular tone by producing both vasorelaxant PGI_2 and vasoconstrictor TXA_2, which are in tight balance, and bind to their respective receptors to initiate downstream reactions.

However, during aging or pathological states, this tight balance is shifted from PGI_2- to TXA_2-production. The enzyme responsible for PGI_2 synthesis, PGI_2 synthase (Hara *et al*. 1994), is one of the most sensitive enzymes inactivated by even extremely low concentrations of peroxynitrites (Zou *et al.* 2002). In agreement with this, in ECs exposed to high glucose, PGI_2 synthase was inactivated by tyrosine nitration (Cosentino *et al.* 2003), which has been a proposed mechanism of its inactivation by peroxynitrite (Zou *et al.* 2002). It has also been reported that the vasodilatory function of PGI_2 is reduced during aging in human (Schrage *et al.* 2007) as well as in rodents (Woodman *et al.* 2003; Liu *et al.* 2012), likely due to impaired function of its receptor, the IP receptor (Rapoport and Williams 1996). On the other hand, production of COX-derived vasoconstrictors was associated with aging (Drouin *et al.* 2011), dyslipidemia (Gendron and Thorin 2007), and hypertension (Taddei *et al.* 1997). Impaired endothelial function could, indeed, be improved by TP-receptor antagonists (Rodriguez-Manas *et al.* 2009). TP receptors, as mentioned earlier, are normally the main receptors for TXA_2 but can also be stimulated by high concentration of PGI_2 (Levy 1980; Williams *et al.* 1994). Conversely, paradoxical vasoconstriction resulting from PGI_2 has been reported, likely due to an up-regulation of TP receptor and its activation, compromising PGI_2 binding to IP receptor and their downstream vasodilatory pathways (Liu *et al.* 2012). In line with this, ROS was also reported to enhance TP receptor stability post-transcriptionally (Valentin *et al.* 2004), supporting the notion that PGI_2 can act as a vasoconstrictor under pathological circumstances.

Additionally, there have been reported interactions between NO and peroxynitrite activities with that of COX (Upmacis *et al.* 2006). For example, while peroxynitrite resulting from NO could interact with the heme in the catalytic domain of COX, forming an unstable FeIII-ONOO intermediate, which in turn could activate COX (Tsai *et al.* 1992), it was also shown that nitration of specific tyrosine residues of COX could result in its inactivation (Goodwin *et al.* 1998). Furthermore, specific interactions between iNOS and COX-2 have been reported (Kim *et al.* 2005). In a macrophage cell line, iNOS was co-immunoprecipitated with COX-2, and iNOS-derived NO, by proximity, could nitrosylate COX-2, resulting in its activation (Kim *et al.* 2005). This could be important in settings of atherosclerosis, where there is increased expression of iNOS and COX-2 in

atherosclerotic lesions, and that production of respective NO and prostanoids lead to peroxynitrite production and acceleration of the inflammatory cascade (Baker *et al.* 1999). Altogether, these interactions add complexity and synergism between the two different pathways.

1.1.6. Pathologies associated with endothelial dysfunction

Endothelial dysfunction is a multifaceted vascular disorder that has been associated with a wide spectrum of diseases such as obesity and diabetes (Beckman *et al.* 2002; Xu and Zou 2009), hypertension (Puddu *et al.* 2000), heart failure (Landmesser *et al.* 2002), atherosclerosis (Beckman *et al.* 2002; Davignon and Ganz 2004), and coronary syndrome (Fichtlscherer *et al.* 2004). In the following section, endothelial dysfunction in the context of obesity and insulin resistance, as well as hypertension, will be discussed, as these are the main pathologies studied in this thesis.

1.1.6.1. Obesity, insulin resistance and endothelial dysfunction

Obesity is one of the main forces that accelerate vascular aging, and is a major risk factor of premature cardiovascular, peripheral vascular, as well as cerebrovascular diseases (Chudek and Wiecek 2006). Endothelial dysfunction is a prominent feature of obesity. Over the past decades, the prevalence of obesity in both adults and children in developed countries has dramatically risen. Obesity is currently classified as having a body mass index (BMI) of greater than 30 kg/m^2 in human. There is an emerging concept of obesity being a state of chronic inflammation (Lumeng and Saltiel 2011). Inflammation is the combination of responses to harmful stimuli, whereby the system tries to return itself to the normal baseline. In obesity, the inflammatory response involves the classic recruitment of leukocytes to inflamed tissues, in this case the adipose tissues, increasing systemic circulatory inflammatory cytokines (Hotamisligil *et al.* 1995) as well as acute phase proteins such as C-reactive protein, and fibrosis of tissues (Spencer *et al.* 2010).

This chronic low-grade inflammatory state in adipose tissues can also directly promote systemic insulin resistance (Neels and Olefsky 2006). Furthermore, obesity does not only involve dysfunction in adipose tissues, but also affects other organs. In obesity

and insulin resistance, endothelial dysfunction is a prominent feature (Xu and Zou 2009). Accumulating evidence shows that insulin resistance leads to endothelial dysfunction, that endothelial dysfunction can in turn contribute to insulin resistance, and that the combination of the two act synergistically to accelerate the aging process (Kim *et al.* 2006; Hadi and Suwaidi 2007; Avogaro *et al.* 2013). Metabolic abnormalities typically arise when energy intake outbalances energy expenditure, resulting in increasing accumulation of body fat compared to lean mass, which has important consequences – resistance of insulin action to stimulate glucose transport in skeletal muscle is among one of the primary major condition and precedes the development of type 2 diabetes (Cavaghan *et al.* 2000; Kim *et al.* 2008).

Insulin mainly targets its metabolic effects on the liver, adipose tissues, and skeletal muscle (Potenza *et al.* 2009). In the liver and skeletal muscle, insulin increases glycogen synthesis (Saltiel and Kahn 2001). In adipocytes, insulin promotes triglyceride (TG) synthesis and its deposition (Saltiel and Kahn 2001). Insulin can promote glucose uptake and oxidation (Klip *et al.* 1993), which in turn is required for the generation of ATP. Other than its metabolic functions, insulin can also contribute to NO-dependent vasodilation in the endothelium (Dimmeler *et al*. 1999; Laakso *et al*. 1990), thereby increasing blood blow. Indeed, the presence of insulin receptors have been documented in ECs (Zeng and Quon 1996) and the effect of insulin on NO release has been a proposed pathway to increase glucose uptake in the skeletal muscle (Steinberg *et al.* 1996). As previously mentioned, stimulation of glucose uptake by insulin and activation of eNOS for NO generation share the PI3K/Akt signaling pathway (Hsueh and Law 1999). In healthy mice with normal insulin signaling, insulin-mediated vasodilation appeared to be dependent on both the endothelium and NO (Wheatcroft *et al.* 2004). Moreover, hyperinsulinemia actually increased eNOS expression and NO production in mice with intact insulin signaling, suggesting that insulin, in the presence of intact and normal signaling, has beneficial effects on endothelial function (Wheatcroft *et al.* 2003). This vasodilatory effect, however, was blunted in insulin resistance states (Laakso *et al.* 1992) and indeed, ACh in the coronary circulation of diabetic patients paradoxically caused vasoconstriction instead of vasodilation (Nitenberg *et al.* 1993). One of the possible mechanisms could be due to low levels of BH_4 and defects of the antioxidant defenses,

leading to excessive superoxide production, deactivating NO (Shinozaki *et al.* 2001). Accordingly, accumulating evidence has demonstrated that insulin resistance can influence endothelial function by disturbing vasorelaxing effects from one or more of the 3 main EDRFs (Du *et al.* 2001; Bolego *et al.* 2006; Murphy *et al.* 2007), thereby disrupting the balance between vasodilation and vasoconstriction.

Insulin resistance and endothelial dysfunction are directly linked. Multiple reports have repeatedly shown reduced NO bioavailability that results from insulin resistance (Kim *et al.* 2006), most likely due to, but not limited to, damages caused by hyperglycemia and increased FFA (Tripathy *et al.* 2003). The PGI_2 synthase was found inactivated in obese Zucker rats and in insulin resistant mice induced by a high-fat diet (HFD) (Du *et al*. 2001). ECs isolated from diabetic patients have decreased expressions of COX-2 and eNOS, as well as lower PGI_2 production (Bolego *et al*. 2006). Notably, elevated ROS levels are the initial source of endothelial dysfunction in this pro-inflammatory setting, which disrupts the function of all 3 main EDRFs (Shi and Vanhoutte 2009). A crucial and recognized producer of ROS in obesity and insulin resistance is the chronically inflamed adipocyte (Xu *et al.* 2003).

1.1.6.1.1. The adipocyte and its emerging importance in regulating endothelial function

Historically, adipose tissues have been considered merely a site for excess energy storage. Work in the past decade has revealed that adipocytes are capable of controlling glucose and lipid homeostasis under both physiological and pathological conditions (Guilherme *et al.* 2008). They have high capacities for expansion and increase in cell number. Insulin stimulates glucose uptake in the fed states, to yield glycerol-3-phosphate for FA esterificaiton and produce TG and *de novo* FA, as well as direct esterification of incoming FA by adipocyte lipoprotein lipase (LPL) (Saltiel and Kahn 2001; Goldberg *et al.* 2009).

Besides their classic role to sequester lipids as adipose TG stores, adipocytes can act as endocrine cells and are able to secrete adipokines. They include leptin, adiponectin, monocyte chemotactic protein (MCP)-1, and tumour necrosis factor α (TNFα) that are able to regulate insulin sensitivity in the periphery (Guilherme *et al*. 2008), as well as

metabolic, endocrine and immune functions (Walker *et al.* 2007). In recent years, angptl2 has also emerged as an adipokine that regulates insulin sensitivity (Oike and Tabata 2009; Tabata *et al.* 2009), which will be discussed in a later section (1.3.3.3.). It is clear now that many of these adipokines act on ECs and influence their function, and that many of these adipokines have been linked to pathogenesis of obesity-related diseases including atherosclerosis (Chudek and Wiecek 2006; Horio *et al.* 2014).

1.1.6.1.2. Dyslipidemia in obesity and endothelial dysfunction

One aspect of obesity is the disruption of the plasma lipoprotein system, which gives the concept of the term “dyslipidemia”. To a significant extent, the changes in lipid profiles seen in obesity give rise to the increase in cardiovascular risks (Castelli 1998). In human, the dyslipidemia phenotype is typically characterized by increased TG, decreased high-density lipoprotein (HDL), increased total cholesterol and LDL shifting into small dense LDL (Franssen *et al.* 2008). In patients with or without coronary artery disease (CAD), hypercholesterolemia was an independent predictor for endothelial dysfunction (Creager *et al.* 1990; Drexler and Zeiher 1991), while it has been shown that serum total- and LDL-cholesterol were inversely correlated with endothelial function (Seiler *et al.* 1993). A main consequence of high LDL levels, due to its capacities to become oxidized, is the initial process of atherosclerosis (Sobenin *et al.* 1996).

1.1.6.2. The renin-angiotensin system (RAS) and angII

Closely related to obesity is the activation of the sympathetic nervous system and there is a well-established relationship between obesity and hypertension, or high blood pressure, where the regulation of vascular tone is affected (Kotsis *et al.* 2010). Apart from NO being the major regulator of vascular tone, the autocrine and paracrine systems shared by ECs and VSMCs, one of which is the RAS, are also involved in maintaining this homeostasis. Physiologically, the main product of RAS, angII, co-ordinates the signaling cascade to regulate renal function, fluid and electrolyte balance, as well as blood pressure (Ferrario 2006). Along with evolutionary development, the RAS has also become involved with a continuum of pathologies, one of which is vascular diseases such as hypertension, where RAS is over-stimulated resulting in increased angII production. In

turn, the over-production of angII can exert deleterious effects by causing vascular injury, increasing oxidative stress, stimulating VSMC proliferation and growth (Ibrahim 2006), and promoting thrombosis (Brown *et al.* 1998). Importantly, elevated angII raises blood pressure by increased vasoconstriction and sympathetic nervous stimulation and thus, angII is heavily involved in the pathophysiology of hypertension.

1.1.6.2.1. Hypertension and its implications

Hypertension is currently defined as having ≥140 mmHg and/or ≥90 mmHg for systolic and diastolic blood pressure, respectively, or being on medication that controls blood pressure (Go *et al.* 2013). In chronic hypertension, both functional and structural changes occur in the vasculature, leading to overall increased peripheral resistance (Touyz 2003). Functional changes include enhanced vasoconstriction as well as decreased vasodilation, whereas structural changes mainly involves increased resistance arteries lumen narrowing (Touyz 2003), reduced diameter and increased media thickness, also known as vascular remodeling. Indeed, in the aorta of hypertensive rats, ACh-induced and endothelium-dependent relaxations were impaired, which have been found associated with increased COX-derived contractile prostanoid generation (Luscher and Vanhoutte 1986) and the subsequent activation of TP receptors (Gluais *et al.* 2005), as well as elevated ROS production and subsequent NO scavenging (Grunfeld *et al.* 1995; Beswick *et al.* 2001; Wind *et al.* 2010) (summarized in Figure 8).

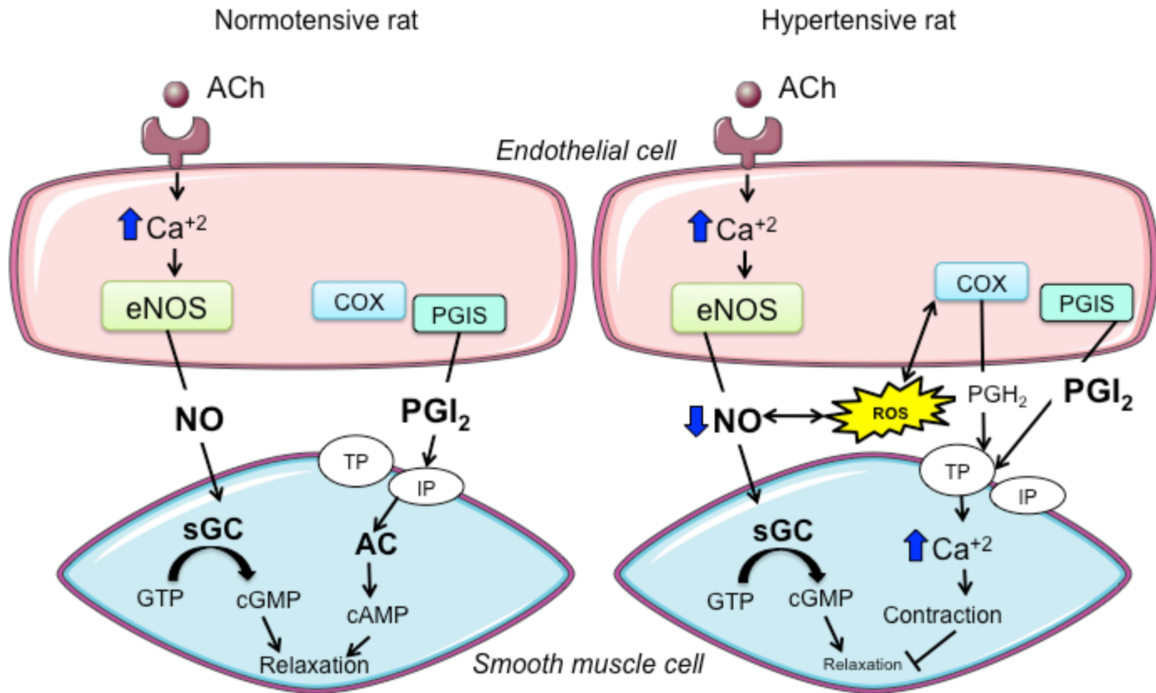

Figure 8. ACh-induced and endothelium-dependent effects in normotensive and hypertensive rat aorta. *Left*: endothelium-dependent relaxation in the aorta of the normotensive rat. *Right*: endothelium-dependent relaxation in the aorta are impaired in the hypertensive rat aorta. Abbreviations: ACh: acetylcholine; eNOS: endothelial nitric oxide synthase; NO: nitric oxde; sGC: soluble guanylate cyclase; GTP: guanosine tri-phosphate; cGMP: cyclic guanosine monophosphate; AC: adenylyl cyclase; cAMP: cyclic adenosine monophosphate; COX: cyclo-oxygenase; PGH_2: prostaglandin H_2; PGIS: prostacyclin synthase; PGI_2: prostacyclin; TP: thromboxane A_2 receptor; IP: prostacyclin receptor; Ca^{2+}: calcium; ROS: reactive oxygen species.

Another major consequence of hypertension is altered cerebrovascular regulation leading to increased risks of dementia and stroke (Lawes *et al.* 2004), with hypertension responsible for 62% of cerebrovascular diseases (Lawes *et al.* 2006). Several agents are well-studied in the state of hypertension, including ET-1 (Schiffrin 1999), aldosterone (El-Gharbawy *et al.* 2001) and norepinephrine (Goldstein 1981), but for the purpose of this work, only angII impact on the cerebrovasculature is majorly discussed.

1.1.6.2.2. AngII and its effects on the cerebrovasculature

The cerebral circulation is responsible for an adequate blood supply for the brain, an organ that has minimal storage of energy sources. Hence, it is a supremely specialized vascular bed that responds to a range of external and internal conditions, yet is able to maintain constant blood flow (Faraci and Heistad 1998). The brain, which weighs relatively little compared to the whole body, utilizes 20% of total body oxygen consumption (Floyd and Carney 1992), yet it is not enriched in anti-oxidant defense systems evidenced by its low catalase activities (Marklund *et al.* 1982). Furthermore, as cerebral blood vessels have greater capacities to generate NOX-derived ROS compared to systemic vessels (Miller *et al.* 2005), they are highly susceptible to inflammation and oxidative stress (Faraci and Lentz 2004; Chrissobolis *et al.* 2011). Hypertension induces inflammation within the cerebral vasculature and in this context, angII seems to play an integral role (De Silva and Faraci 2013). In spontaneously hypertensive rats, there is increased expression of ICAM-1 (Ando *et al.* 2004), which could be partly reversed with AT1R antagonist treatment (Ando *et al*. 2004), suggesting involvement of angII in ICAM-1 up-regulation.

Another major consequence of angII exposure to the vasculature is increased ROS generation. In cerebral arteries of mice and rats, acute exposure to angII could increase ROS production (Kazama *et al.* 2004; Miller *et al.* 2005). Chronic exposure to angII also elevated cerebrovascular ROS levels (Chrissobolis *et al.* 2012). To date, most evidence points towards Nox2 as the primary source of ROS when stimulated by angII in the cerebral vasculature (Girouard *et al.* 2006; Girouard *et al.* 2007). In turn, ROS can modulate vascular tone by multiple mechanisms including scavenging of NO by $O_2^{-\bullet}$, reducing its bioavailability and resulting in peroxynitrite formation, which leads to oxidation of protein and thiol, in addition to tyrosine nitration (Gavazzi *et al.* 2007). Interestingly, both exogenous and endogenous H_2O_2 were reported to dilate cerebral arteries (Faraci and Sobey 1998), and the generation of H_2O_2 in chronic hypertension was associated with elevated Nox4 expression (Paravicini *et al.* 2004), which may be beneficial in the face of decreased NO bioavailability, as in the context of hypertension.

AngII is also suggested to be an important player in inward remodeling, a reduction in vessel size, in cerebral arterioles (Faraci 2011) as angII-independent

hypertension does not cause inward remodeling (Baumbach *et al.* 2003). Moreover, Nox2 may be involved in mediating this process as Nox2-deficient mice were protected against cerebral arteriole inward remodeling induced by angII (Chan and Baumbach 2013).

In summary, obesity and elevated plasma angII levels are among some of the well-established cardiovascular risk factors that contribute to increased vasculature ROS production and inflammation, the two main drivers for endothelial dysfunction, which is the obligatory first step towards cardiovascular events, as depicted in Figure 9. Therefore, targeting endothelial dysfunction seems like a potential therapeutic strategy in patients with CVD. However, despite considerable evidence supporting the notion that the inflammatory cascade is the main culprit behind endothelial dysfunction, the exact mechanisms causing this dysfunction remain elusive and CVD is still the leading cause of death in North America. Thus, greater therapeutic targets are clearly warranted. In the upcoming chapters, the angptl proteins, a recently identified family of proteins, will be introduced, followed by an in-depth introduction of one particular family member, angptl2, which is known for its pro-inflammatory properties in general (Kadomatsu *et al.* 2014), and its pro-inflammatory capacities in the adipocytes (Tabata *et al.* 2009) and in the ECs (Horio *et al.* 2014; Farhat *et al.* 2013).

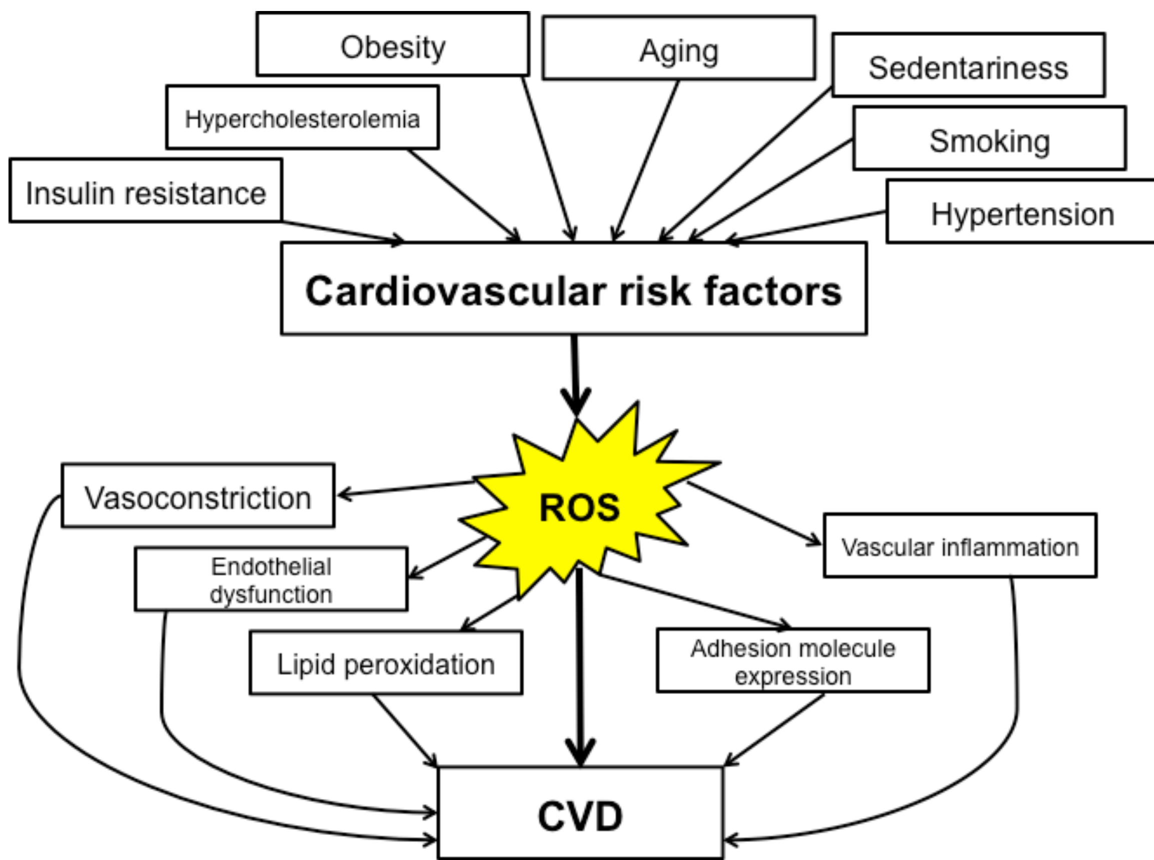

Figure 9. ROS is one of the links between cardiovascular risk factors and CVD. Obesity and elevated angII are among some of the greatest known cardiovascular risk factors, which lead to increased ROS production, which is a common feature in CVDs.

Chapter 2: Angiopoietin-like proteins

The protein of interest in this thesis, i.e. angptl2, belongs to a greater family of proteins, called angiopoietin-like protein (angptl), was first cloned in 1999 (Kim *et al.* 1999a), and are structurally similar to the angiopoietins. There are, to date, a total of 8 angptls in this family of proteins (Table 1), and although they share certain degrees of homology among them, their roles seem to differ in biology. This chapter will briefly highlight the different angptls in both physiology and pathophysiology. Greater detail of angptl2 will be provided in the following chapter.

Table 1. Summary of angptl proteins and their current known functions

Gene	Other names	Tissue expression	Angiogenesis	Effect on lipoproteins	Expansion of HSC
Angptl1	ARP1, ANG3, AngY, ANGPT3, angioarrestin	Adrenal gland, placenta, thyroid gland, heart, small intestine, liver, muscle	Pro/anti	No report	No report
Angptl2	ARP2, HARP ARAP-1	Heart, vessels, small intestine, spleen, stomach, adipose tissue, liver	Pro	No report	Yes
Angptl3	ANGPT5	Liver (exclusively)	Pro	HDL↓; TG↑	Yes
Angptl4	ARP4, NL2, pp1158, HFARP, FIAF, PGAR, HARP	Liver (highest), pericardium, adipose tissue, others (adrenal glands, lung, pancreas, placenta)	Pro/anti	HDL↓; TG↑	No report
Angptl5	None	Adipose tissue (highest), bronchus, epididymis, vena cava, heart	No report	No report	Yes
Angptl6	ARP3, ARP5, AGF	Liver	Pro	No report	No report
Angptl7	CDT6, AngX	Cornea (exclusively)	No report	No report	Yes
Angptl8	Lipasin, betatrophin, TD26	Liver, adipose tissue	No report	TG↓	No report

1.2.1. The Angiopoietin-like protein family

A family of secreted and circulating protein, collectively called the angptl, was initially cloned in 1999 by Kim *et al*. (Kim *et al*. 1999a). They are called "angiopoietin-like" due to their structural similarities shared with angiopoietins, as they possess a similar N-terminal helical coiled-coil domain, a short linker region, and a C-terminal globular fibrionogen-like domain (Kim *et al*. 1999a), as illustrated in Figure 10. Despite that, however, angptls do not bind to tie-1 or tie-2 receptors like angiopoietins-1 and -2 (Oike and Tabata 2009). Whereas angiopoietin-1 and -2 are involved mainly in mediating angiogenic and hematopoietic effects, some angptls mediate angiogenesis and hematopoietic stem cell expansion (Table 1), while others also play various roles in a wide spectrum of physiological and pathological mechanisms. To date, 8 members of this protein family have been discovered and all of them, except for angptl8 (Quagliarini *et al.* 2012), possess the coiled-coil N-terminus and a fibrinogen-like C-terminus (Kim *et al*. 1999a). While the coiled-coil domain may serve as its protein secretion purpose, the fibrinogen-like domain suggests its ability to bind a receptor. Since the cloning of the first angptl protein, much work has been completed to understand their roles and underlying molecular mechanisms. Although they share similarities in their structures, each angptl protein is distinct in their physiological and pathological roles. A brief history of the discovery of the angptl protein will first be introduced, followed by involvement and biological roles played by each angptl protein member in physiological settings and different diseases.

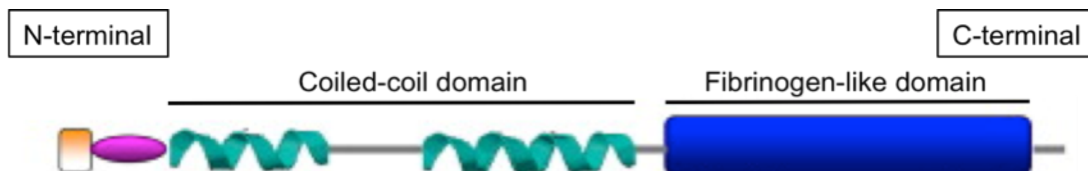

Figure 10. Schematic protein structure of an angptl protein. Orange region depicts a hydrophobic signaling peptide sequence; green coils depict the two coiled-coil domains; blue region depicts the predicted fibrinogen-like domain.

Adapted and reprinted with the permission from Elsevier: Biochem Biophys Res Commun. Dhanabal M, Jeffers M, LaRochelle WJ, Lichenstein HS. Angioarrestin: a

unique angiopoietin-related protein with anti-angiogenic properties. *Biochem Biophys Res Commun*. **333**, 308-315. Copyright (2005). License number: 3453380425828

1.2.2. History at a glance: identification and characterization of angiopoietin-like proteins

The discovery of the first angptl protein was made in 1999 (Kim *et al*. 1999a) by homology-based PCR to isolate a cDNA, which encoded a novel 491-amino acid protein from human adult heart, as shown in Figure 11. Owing to the structural homologies of 29% and 26% with angiopoiein-1 and -2, respectively, this new protein was initially named angiopoietin-3 (Kim *et al*. 1999a). Concurrently, the group of Yancopoulos was also successful in cloning two members in this family with opposing functions, which also shared homologies with angiopoietin-1 and -2, and called them angiopoietin-3, and -4 (Valenzuela *et al.* 1999). Subsequently, the name for angiopoietin-3 was replaced by Dr. Koh and his team with angiopoietin-related protein (ARP)1, or angiopoietin-like 1 (angptl1). Not long after, another protein that shared 59% of homology with angptl1, was identified and was named angptl2 (Kim *et al*. 1999b). Expression of another angptl, angptl3, was first found in the human liver (Conklin *et al*. 1999), while the closely-rated angptl4 was identified in the year of 2000 by three separate groups (Kersten *et al.* 2000; Kim *et al.* 2000; Yoon *et al.* 2000), in the liver and adipose tissues. Angptl5 was then identified in the adult human heart in 2003 (Zeng *et al.* 2003), angptl6 in the liver, platelets and mast cells (Oike *et al.* 2003), angptl7 by using the technique of comparative integromics in 2007 (Katoh and Katoh 2006), and finally angptl8 in the liver and adipose tissues in 2012 (Quagliarini *et al*. 2012).

In terms of conformation, angptl proteins conserve 4 out of 6 cysteines found in angiopoietins (Oike *et al.* 2004a), which are meant for intermolecular linkages by disulfide bonds as in angiopoietins, and may explain inability of angptl proteins to bind to tie-1 or tie-2 (Oike *et al.* 2004b). Similar to angiopoietins, at their C-terminus is a fibrinogen-like domain, which suggests their potential to bind to specific receptors (Hato *et al.* 2008). The N-terminus is a highly hydrophobic region in angptl proteins, suggestive of their ability for secretion (Hato *et al.* 2008). Indeed, angptls 2,3,4, and 6 have been detected in the circulation (Farhat *et al*. 2013; Ge *et al*. 2004; Kim *et al*. 2000; Kim *et al.*

1999b; Oike *et al*. 2005; Ono *et al*. 2003; Shimizugawa *et al*. 2002; Tabata *et al*. 2009), suggesting their possible endocrinic roles. In accordance to this, angptl vector transfection in cells resulted in secretion of the protein in the supernatants (Kim *et al.* 1999b; Ito *et al.* 2003). Angptl proteins also possess glycosylation consensus sites (Kim *et al.* 1999b), which are likely required for the biological activities (Farhat *et al.* 2014). In the next section, diverse roles of angptl proteins will be discussed including both their physiological and pathological roles.

```
hARP2   MRPLCVTCW- WLGLLAAMGA VAGQEDG--F EGTEEGSPRE FIYLNRYKRA   47
hARP1   M---KTFTW- TLGVLFFLLV DTGHCRGGQF K-IKKINQR- --RYPRATDG   42
hAng2   M---WQIVFF TL--SCDLVL AAAYNNFRKS M-DSIG--K- --KQYQVQHG   39
hAng1   M---TVFLS- -FAFLAAILT HIGCSNQRRS P-ENSG--R- --RYNRIQHG   39
              •                                →
hARP2   GESQDKCTYT FIVPQ----- -QRVTGAICV NSK-EPEVLL ENRVHKQELE   90
hARP1   KEEAKKCAYT FLVPE----- -QRITGPICV NTKGQDASTI KDMITRMDLE   86
hAng2   S-----CSYT FLLPEMD-NC R-SSSSPYVS NAVQRDAP-L EYDDSVQRLQ   81
hAng1   Q-----CAYT FILPEHDGNC RESTTDQYNT NALQRDAPHV EPDFSSQKLQ   84
                                                         ⇒
                           Coiled-coil domain
hARP2   LLNNELLKQK RQIETLQQL- V--------E VDGGIV-S-- ----E--VKL  122
hARP1   NLKDVLSRQK REIDVLQLV- V--------D VDGNIV-N-- ----E--VKL  118
hAng2   VLENIMENNT QWLMKLENYI QDNMKKEMVE IQQNAVQNQT AVMIEIGTNL  131
hAng1   HLEHVMENYT QWLQKLENYI VENMKSEMAQ IQQNAVQNHT ATMLEIGTSL  134
                           Coiled-coil domain
hARP2   L--RKE-SRN MNSRVTQLYM QLLHEIIRKR DNALELSQLE NRILNQTADM  169
hARP1   L--RKE-SRN MNSRVTQLYM QLLHEIIRKR DNSLELSQLE NKILNVTTEM  165
hAng2   LNQTAEQTRK LTDVEAQVLN QTTRLELQLL EHSLSTNKLE KQILDQTSEI  181
hAng1   LSQTAEQTRK LTDVETQVLN QTSRLEIQLL ENSLSTYKLE KQLLQQTNEI  184
                           Coiled-coil domain
hARP2   LQLASKYKDL EHKYQHLATL AHNQSEIIAQ LEE-HCQRVP SARPVPQPPP  218
hARP1   LKMATRYREL EVKYASLTDL VNNQSVMITL LEE-QCLRIF SRQDTHVSPP  214
hAng2   NKLQDKNSFL EKKVLAMED- -KHIIQLQSI KEEKDQLQVL VSKQNSIIEE  229
hAng1   LKIHEKNSLL EHKILEMEG- -KHKEELDTL KEEKENLQGL VTRQTYIIQE  232
                           Coiled-coil domain  ←
hARP2   ---AAPPRVY QPPTYNRIIN QISTNEIQSD QNLK--VLPP P-LPT-MPTL  261
hARP1   LVQVVPQHIP NSQQYTPGL- -LGGNEIQRD PGYPRDLMPP PDLAT-SPTK  261
hAng2   L--------- -EKKIVTAT- -VNNSVLQKQ QHDLMETVN- -NLLTMMSTS  265
hAng1   L--------- -EKQLNRAT- -TNNSVLQKQ QLELMDTVH- -NLVN-LCTK  267
                                                     ⇐
                   →•                                         •
hARP2   T--SLPSSTD KPSGPWRDCL QALEDGHDTS SIYLVKPENT NRLMQVWCDQ  309
hARP1   SPFKIPPVTF INEGPFKDCQ QAKEAGHSVS GIYMIKPENS NGPMQLWCEN  311
hAng2   NSAKDPTVAK EEQISFRDCA EVFKSGHTTN GIYTLTFPNS TEEIKAYCDM  315
hAng1   EGVLLKGGKR EEEKPFRDCA DVYQAGFNKS GIYTIYINNM PEPKKVFCNM  317
                        Fibrinogen-like domain
hARP2   RHDPGGWTVI QRRLDGSVNF FRNWETYKQG FGNIDGEYWL GLENIYWLTN  359
hARP1   SLDPGGWTVI QKRTDGSVNF FRNWENYKKG FGNIDGEYWL GLENIYMLSN  361
hAng2   EAGGGGWTII QRREDGSVDF QRTWKEYKVG FGNPSGEYWL GNEFVSQLTN  365
hAng1   DVNGGGWTVI QHREDGSLDF QRGWKEYKMG FGNPSGEYWL GNEFIFAITS  367
                        Fibrinogen-like domain
hARP2   QGNYKLLVTM EDWSGRKVFA EYASFRLEPE SEYYKLRLGR YHGNAGD-SF  408
hARP1   QDNYKLLIEL EDWSDKKVYA EYSSFRLEPE SEFYRLRLGT YQGNAGD-SM  410
hAng2   QQRYVLKIHL KDWEGNEAYS LYEHFYLSSE ELNYRIHLKG LTGTAGKISS  415
hAng1   QRQYMLRIEL MDWEGNRAYS QYDRFHIGNE KQNYRLYLKG HTGTAGKQSS  417
                                   •          •
hARP2   TWHNGKQFTT LDRDHDVYTG NCAHYQKGGW WYNACAHSNL NGVWYRGGHY  458
hARP1   MWHNGKQFTT LDRDKDMYAG NCAHFHKGGW WYNACAHSNL NGVWYRGGHY  460
hAng2   ISQPGNDFST KDGDNDKCIC KCSQMLTGGW WFDACGPSNL NGMYYPQRQN  465
hAng1   LILHGADFST KDADNDNCMC KCALMLTGGW WFDACGPSNL NGMFYTAGQN  467
        ←
hARP2   RSRYQDGVYW AEFRGGSYSL KKVVMMIRPN PNTFH      493
hARP1   RSKHQDGIFW AEYRGGSYSL RAVQMMIKPI D          491
hAng2   TNK-FNGIKW YYWKGSGYSL KATTMMIRPA DF         496
hAng1   HGK-LNGIKW HYFKGPSYSL RSTTMMIRPL DF         498
```

Figure 11. Alignment of the amino acid sequences and evolutionary relationships between angptl2 and its relatives, human angptl1, angiopoietin-1 and -2, demonstrating their homology. The conserved cysteines among them are shaded.

Reprinted with the permission from the Journal of Biological Chemistry: It is the policy of the American Society for Biochemistry and Molecular Biology to allow reuse of any

material published in its journals (the Journal of Biological Chemistry, Molecular and Cellular Proteomics and the Journal of Lipid Research) in a thesis or dissertation at no cost and with no explicit permission needed.

Kim I, Moon SO, Koh KN, Kim H, Uhm CS, Kwak HJ, Kim NG, Koh GY. Molecular cloning, expression, and characterization of angiopoietin-related protein. angiopoietin-related protein induces endothelial cell sprouting. *J Biol Chem*. **274**, 26523-26528. Copyright (1999).

1.2.3. Physiological roles of angiopoietin-like proteins

1.2.3.1. Angiopoietin-like proteins in angiogenesis

Due to similarities in their structural properties shared between angptl proteins and the angiopoietins, the first effort was made to investigate whether angptl proteins were involved in the process of angiogenesis, or in other words the formation of new blood vessels from pre-existing ones (Folkman 1971). It has been reported that angptls 1, 2, 3, 4 and 6 participate in regulating angiogenesis (Hato *et al.* 2008) (Table 1).

In 1999, at the time of discovery of the angptl proteins, Kim *et al*. made the first observations that angptl1 and angptl2 weakly stimulated *in vitro* sprouting of ECs (Kim *et al*. 1999a; Kim *et al*. 1999b). Subsequently, with the generation of transgenic mice that express angptl1 or angptl2 under the control of a K14 keratinocyte-specific promoter, Tabata *et al*. showed that the K14-angptl2 mice displayed significantly greater number of blood vessels in the dermis (Tabata *et al*. 2009). K14-angptl1 mice, on the other hand, did not significantly differ from wild-type (WT) mice, suggesting minimal role of angptl1 in promoting angiogenesis (Table 1). This finding was in contrast with another study, where treatment with recombinant angptl1 in ECs inhibited their angiogenic properties, including tube formation and adhesion (Dhanabal *et al.* 2002), suggesting an anti-angiogenic effect of angptl1 (Table 1). Dhanabal *et al*. proposed that angptl1 and angptl2 could act in context-dependent manners, depending on interactions with unknown receptors (Dhanabal *et al*. 2002).

Angptl3 was reported to be a pro-angiogenic factor (Table 1), as it induced blood vessel formation in a rat corneal assay (Camenisch *et al.* 2002), and that its fibrinogen-like domain is sufficient to induce angiogenesis on its own (Camenisch *et al*. 2002). In

this report, investigators also reported that recombinant angptl3 bound to α5β1 integrin but not to tie-1 or tie-2, and induced Akt phosphorylation, MAPK activation, and focal adhesion kinase (Camenisch *et al*. 2002).

Interestingly, angptl4 has been reported as both pro- and anti-angiogenic (Ito *et al.* 2003; Le Jan *et al.* 2003) (Table 1). As transcription of angptl4 was induced by hypoxia (Le Jan *et al*. 2003), it may have a biological role in angiogenesis. Indeed, independent laboratories showed that angptl4 could act as an anti-angiogenic factor (Kim *et al.* 2000; Cazes *et al.* 2006; Wang *et al.* 2013a). For instance, recombinant angptl4 exerted protective effects on ECs against apoptosis in an endocrinic manner (Kim *et al*. 2000), and by using a hind-limb ischemic mouse model, Cases *et al*. showed that angptl4 mRNA was upregulated (Cazes *et al*. 2006). Overexpression of angptl4 also protected ECs against lipopolysaccharide-induced vascular permeability and thus increasing cell viability (Wang *et al.* 2013a). It was shown that in a graft of Chinese hamster ovary cells that expressed angptl4, there was a strong pro-angiogenic response that was insensitive to inhibitors of vascular endothelial growth factor (VEGF) (Le Jan *et al*. 2003). Angptl4 mRNA was also found in hypoxic environments such as that in ischemic tissues in renal carcinoma tumor cells (Le Jan *et al*. 2003). In contrast, recombinant angptl4 in both corneal neovascularization and Miles permeability assays inhibited VEGF-induced neovascularization and vascular leakiness, suggesting anti-angiogenic properties (Ito *et al*. 2003). In the same study, K14-angptl4 transgenic mice did not show increased neovascularization in their skin tissues (Ito *et al*. 2003). Finally, in accordance with the anti-angiogenic effects of angptl4, tumor angiogenesis was blunted in the skin of K14-angptl4 mice with tumor transplantation (Ito *et al*. 2003).

Another angptl protein, angptl6, was also reported to have pro-angiogenic effects (Oike *et al.* 2003; Oike *et al.* 2004a; Urano *et al.* 2008; Okazaki *et al.* 2012) (Table 1). In mouse skin, targeted overexpression of angptl6 resulted in induction of angiogenesis and epidermal hyperplasia, and also showed significantly enhanced cutaneous wound healing in the tissue repair process compared to WT mice (Oike *et al*. 2003). Using the same mouse model, the same group further reported that angptl6 promoted angiogenesis independently of other angiogenic factors, such as VEGF and angiopoietin-1 (Oike *et al.* 2004a). In the same study, recombinant angptl6 also exerted chemo-attractive effects on

ECs in the Matrigel plug and corneal pocket assays (Oike *et al.* 2004a). It was also reported by Dr. Oike's group that an intramuscular injection of an adenovirus expressing angptl6 into the ischemic limb could enhance blood flow in a mouse hind-limb ischemic model through induction of angiogenesis and arteriogenesis (Urano *et al*. 2008). This was likely due to increased eNOS signaling and NO production as angptl6 did not have any effects in eNOS KO mice (Urano *et al*. 2008). Furthermore, exposing HUVECs to angptl6 led to activation of the ERK1/2-eNOS signaling pathway (Urano *et al*. 2008). Very recently, Okazaki *et al*. reported that angptl6 also accelerated the tissue repair process induced by acute or chronic UV-B irradiation by promoting angiogenesis (Okazaki *et al*. 2012), again confirming the pro-angiogenic role of angptl6.

In summary, out of the angptl protein family, angptls 1, 2, 3, 4 and 6 are likely important players in regulating angiogenesis, with angptls 1 and 4 having both pro- and anti-angiogenic effects, while angptls 2, 3, and 6 most likely are pro-angiogenic.

1.2.3.2. Angiopoietin-like proteins in lipid metabolism

In the literature, the roles of angptls 3, 4 and 6 have been mainly focused around maintaining metabolic homeostasis, independent of their angiogenic effects (Hato *et al.* 2008). In addition, the recently identified angptl8 has also been implicated in lipid metabolism by regulating angptl3 (Wang *et al.* 2013b).

Both angptl3 and angptl4 can be cleaved by proprotein convertases (Chomel *et al.* 2009; Essalmani *et al.* 2013), although their activities do not require cleavages. Expression of angptl3 is limited to the liver (Koishi *et al.* 2002) and is active mainly in the fed state (Ge *et al.* 2005; Mattijssen and Kersten 2012). It has been reported that a truncated angptl3 containing only the coiled-coil domain was sufficient to regulate metabolism (Ono *et al.* 2003). On the other hand, angptl4 expression is found in liver, adipose tissues (Kim *et al*. 2000), and skeletal muscle (Staiger *et al.* 2009), and is regulated by feeding and fasting (Kersten *et al.* 2000; Yoon *et al.* 2000). It was reported that adipose tissue secretes the full-length angptl4, whereas the liver secretes the truncated form (Mandard *et al.* 2004).

The link between angptl3 and lipid metabolism was first reported by Koishi *et al*. by positional cloning to map a genetic defect in the angptl3 gene in the KK/San mice

(Koishi *et al*. 2002), which is a mutant strain of KK obese mice with lower plasma non-esterified fatty acid (NEFA) and TG levels. Furthermore, angptl3 has been established as a hepatocyte-derived circulating factor in regulation of lipid metabolism (Shimamura *et al.* 2003). It directly targets adipocytes, activates lipolysis (Kersten *et al*. 2000; Shimamura *et al*. 2003), binds to and inhibits LPL activity thereby decreasing clearance of very low density lipoprotein (VLDL) TG and thus increasing FFA and glycerol release from adipocytes (Ono *et al.* 2003; Jin *et al.* 2007).

LPL is a key enzyme that is responsible for the metabolism of TG-enriched lipoproteins (Eisenberg 1984), and is anchored to endothelial surfaces of peripheral tissues (Beigneux *et al.* 2007). In turn, its function to uptake TG is governed by nutritional status (Wang and Eckel 2009). For instance, LPL activity is reduced in adipose tissue but increased in heart and skeletal muscle during fasting, which directs TG to oxidation in muscle cells. On the other hand, LPL activity is enhanced in adipose tissues but reduced in muscle during the fed state, which directs TG for storage in adipose tissues (Kuwajima *et al.* 1988).

Accordingly, angptl3-deficient mice had lower levels of plasma lipids (Koishi *et al.* 2002; Fujimoto *et al.* 2006), TG, cholesterol and FFA (Shimamura *et al*. 2003), which was found associated with higher heparin-releasable LPL activity (Koster *et al.* 2005). In these angptl3-deficient mice, Shimamura *et al*. also observed lower plasma levels of HDL and HDL phospholipid, which were increased by adenoviral treatment with angptl3 (Shimamura *et al.* 2007), suggesting a metabolic role of angptl3 in regulating HDL cholesterol (Table 1). Mechanistically, angptl3 could inhibit also the activity of endothelial lipase (Shimamura *et al*. 2007), which hydrolyzes HDL phospholipid, thereby decreasing HDL levels (McCoy *et al.* 2002). It was also shown that angptl3 is a direct target of liver X receptor (Inaba *et al.* 2003), a nuclear receptor that activates transcription of several lipid metabolic genes (Lu *et al.* 2001). In human, loss-of-function of angptl3 was associated with familial combined hypolipidemia (Musunuru *et al.* 2010), characterized by much lower cholesterol and TG levels than normal, but these patients may also suffer from symptoms such as malnutrition. Nonetheless, the findings that an angptl3 mutation is linked to hypolipidemia underscores a potential strategy, such as decreasing angptl3 activities, for lowering cholesterol in human (Musunuru *et al.* 2010).

The evidence that agonists of lipid-sensing PPARs upregulated angptl4 expression provided initial clues that angptl4 was involved in lipid metabolism (Mandard *et al*. 2004). Indeed, overexpression of angptl4 in mice resulted in a 50% reduction in adipose tissue weight, increased TG, FFA, glycerol, total cholesterol, and HDL in the plasma (Mandard *et al.* 2006) (Table 1). Angptl4, similar to angptl3, has been shown to be a potent inhibitor of LPL (Yau *et al.* 2009; Zhu *et al.* 2012). Structurally, angptl4 could bind to LPL with its coiled-coil domain, thereby converting the active dimers into inactive monomers (Sukonina *et al.* 2006; Yau *et al.* 2009). By this mechanism, angptl4 has been reported to suppress NEFA release and their uptake by adjacent tissues such as adipose tissue and skeletal muscle (Yoshida *et al.* 2002), and circulating TG clearance (Yau *et al*. 2009) (Table 1). More recently, there is data suggesting that plasma NEFA could raise plasma angptl4 in human (Jonker *et al.* 2013). Moreover, Jonker *et al*. reported that in human, there was a positive correlation between angptl4 and NEFA concentrations but a negative correlation in TG concentrations (Jonker *et al*. 2013). Interestingly, insulin was demonstrated to decrease circulating angptl4 and its expression in adipose tissues only in young and healthy subjects, an effect that was blunted in older and diabetic subjects (Ruge *et al.* 2012). Regulation of angptl4 by insulin was further shown to be both dependent (Jonker *et al*. 2013) and independent (Mizutani *et al.* 2012; van Raalte *et al.* 2012) on NEFA in mouse and human, respectively. Taken together, there is evidence suggesting that angptl4 acts as a signal from adipocytes, skeletal muscles and other tissues to govern adiposity by decreasing lipid storage and increasing fat mobilization.

There is also evidence indicating that angptl4 levels could be up-regulated by fasting (Kersten *et al.* 2009) and recent data also suggested that angptl4 can regulate food intake and energy expenditure (Kim *et al.* 2010), as deletion of angptl4 in mice resulted in increased body weight and reduced energy expenditure, while central administration of angptl4 increased energy expenditure and suppressed food intake and weight gain (Kim *et al*. 2010).

Angptl6 was detected in the systemic circulation and was predominantly secreted from the liver (Oike *et al.* 2005). In this study by Oike *et al*., more than 80% of mice deficient of angptl6 died around embryonic day 13 seemingly due to cardiovascular

defects, while the remaining surviving mice showed marked obesity characterized by increased fat mass and adipocyte size, insulin resistance, lipid accumulation in both the skeletal muscle and liver, with reduced energy expenditure compared to WT littermates (Oike *et al*. 2005). The increased fat mass was linked to decreased energy expenditure that was unrelated to food intake (Oike *et al*. 2005). On the other hand, mice with targeted angptl6 activation were resistant to the detrimental effects of a HFD including obesity and insulin resistance, which was associated with increased energy expenditure and decreased fat mass (Oike *et al*. 2005). In addition, overexpression of angptl6 in the liver resulted in higher circulating angptl6 and a significantly lower body weight (Oike *et al*. 2005). As plasma TG levels were not influenced by angptl6 overexpression or depletion, angptl6 may not be involved in inhibiting LPL activity, unlike angptls 3 and 4 (Oike *et al*. 2005). It remains to be determined whether angptl6 has a direct effect on insulin, cholesterol, and FA levels (Oike *et al*. 2005). Taken together, this study suggests that angptl6 may regulate energy metabolism, at least in mice, and could play a role in antagonizing the effects of a HFD by increasing energy expenditure (Oike *et al*. 2005).

The putative roles of angptls 3, 4, and 6 in regulating metabolism are summarized in Figure 12.

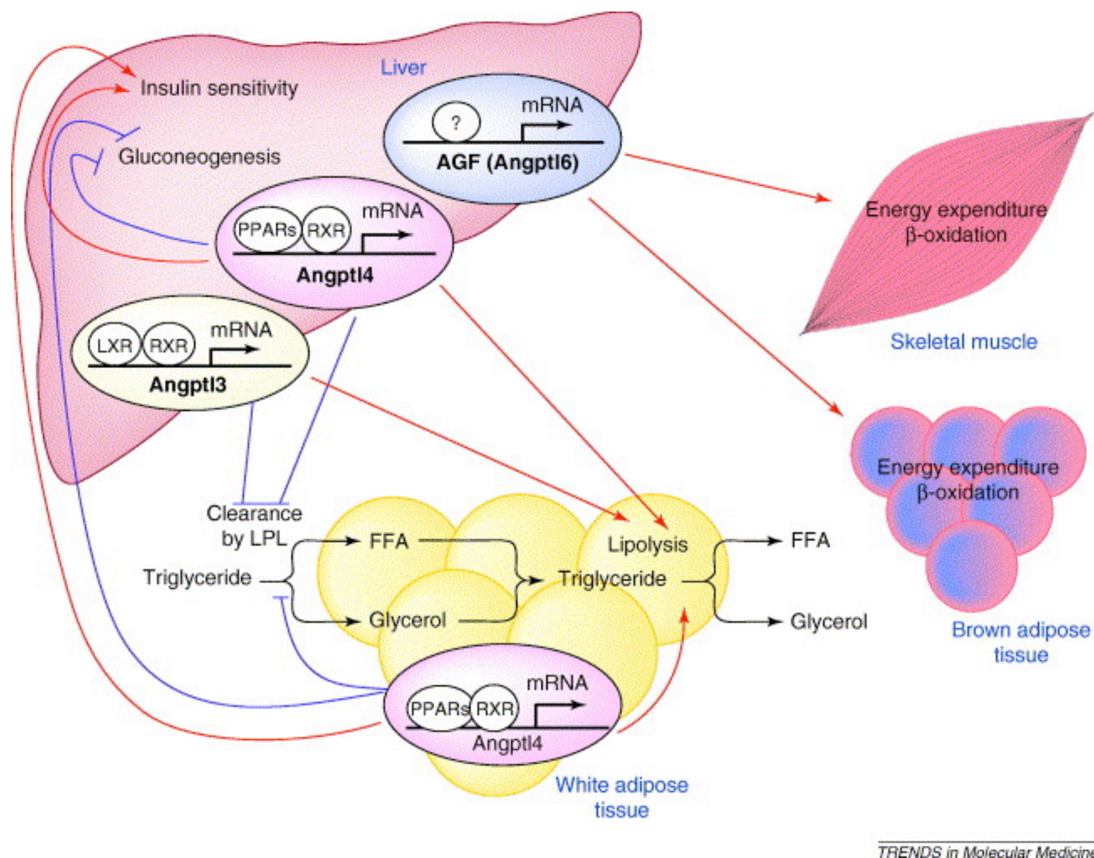

Figure 12. Summary of the different roles of angptls 3, 4, and 6 in maintaining metabolic homeostasis. Angptl3 is mainly derived from the liver and targets adipocytes in an endocrinic manner to inhibit LPL activity, thus promoting lipolysis. Angptl4 is derived from both the liver and adipocytes and targets both hepatocytes and adipocytes as an endocrine or paracrine to inhibit gluconeogenesis and stimulate lipolysis, respectively. Angptl4 also enhances insulin sensitivity. Angptl6 is derived from the liver and promotes fat burning and energy expenditure in peripheral tissues. Abbreviations: LXR: liver X receptor; RXR: retinoid X receptor; PPAR: peroxisome proliferator-activated receptor; LPL: lipoprotein lipase; FFA: free fatty acid.

Reprinted with the permission from Elsevier: Trends Mol Med. Oike Y, Akao M, Kubota Y, Suda T. Angiopoietin-like proteins: potential new targets for metabolic syndrome therapy. *Trends Mol Med*. **11**, 473-479. Copyright (2005).

Lastly, the newly identified angptl protein, angptl8, was also reported to play a major role in TG trafficking (Wang *et al*. 2013b), while its expression was found highly induced during adipocyte differentiation (Ren *et al.* 2012). Expression of angptl8 was found highly abundant in the liver and adipose tissues, and was up-regulated by feeding but suppressed by fasting (Quagliarini *et al*. 2012; Ren *et al.* 2012; Zhang 2012). The N-

terminal of angptl8 possesses a domain that is homologous to angptl3's N-terminal domain that is responsible for blood lipid regulation, as well as a domain that is homologous to angptl4's N-terminal domain that is responsible for LPL binding (Zhang 2012), suggesting the potential role of angptl8 to regulate lipid metabolism. Indeed, it has been shown that an adenovirus-mediated overexpression of angptl8 increased plasma TG levels in mice and recombinant angptl8 could inhibit LPL activity (Zhang 2012). Interestingly, co-expression of angptl8 and angptl3 resulted in more than a 10-fold increase in plasma TG levels (Quagliarini *et al*. 2012), and that the two proteins can be co-immunoprecipitated in mouse plasma and cell media (Quagliarini *et al*. 2012), strongly suggesting interactions and synergistic activities between the two proteins. Furthermore, the study by Wang *et al*. reported angptl8 as a key mediator of TG-FA trafficking in the fed state (Wang *et al*. 2013b), as disruption of angptl8 resulted in pronounced reduction in plasma TG levels in fed, but not fasted animals (Wang *et al*. 2013b). This was reflected by the significantly reduced VLDL secretion, increased intravascular LPL activity and chylomicron-TG clearance in these angptl8 KO mice (Wang *et al*. 2013b), as well as abolished VLDL-FA uptake into adipose tissues in fed KO mice, suggesting a role of angptl8 in replenishment of TG stores in adipose tissue in the fed state.

Collectively, of all the angptl proteins, angptls 3, 4, 6 and 8 seem to be involved in lipid metabolism.

1.2.3.3. Angiopoietin-like proteins in glucose metabolism

In addition to being involved in lipid metabolism, angptls 4, 6, and 8 have also been reported to regulate glucose metabolism. Regarding angptl4, there have been controversial findings – while supplementing recombinant angptl4 in hepatocyte supernatants suppressed glucose production (Xu *et al.* 2005) and reduced hyperglycemia in diabetic mice (Xu *et al*. 2005), overexpression of angptl4 in the liver did not result in any effects on glucose metabolism (Koster *et al.* 2005), but even worse glucose tolerance when angptl4 was overexpressed in the adipose tissue (Mandard *et al*. 2006). This deleterious effect of angptl4 on glucose metabolism was further supported by data

demonstrating a decrease in insulin-mediated glucose clearance (Lichtenstein *et al.* 2007).

Angptl6, on the other hand, has been reported to exert a beneficial role on glucose homeostasis in the liver (Kitazawa *et al.* 2007). In this study, Kitazawa *et al*. showed that angptl6 suppressed glucose production in a dose-dependent manner *in vitro*, which was associated with decreased transcriptional and translational expression of glucose-6-phosphatase (Kitazawa *et al*. 2007), a key gluconeogenic enzyme. Mechanistically, results suggested that angptl6 reduced transcriptional activity of forkhead box class O1 (FoxO1), a key transcription factor for glucose-6-phosphate, which led to activation of the PI3K/Akt signaling pathway in hepatocytes (Kitazawa *et al*. 2007).

The newly recognized angptl8 has recently been suggested to contribute to glucose homeostasis (Yi *et al.* 2013). In this study, hepatic overexpression of angptl8 promoted pancreatic β-cell proliferation and increased insulin secretion (Yi *et al.* 2013). However, disruption of angptl8 in a mouse model did not lead to changes in glucose homeostasis or insulin levels (Wang *et al*. 2013b).

Taken together, angptls 4, 6 and 8 may be involved in glucose metabolism, which may partly explain their roles in the pathogenesis of insulin resistance. Owing to their global metabolic properties, angptl proteins have been implicated in a number of pathologies that contribute to cardiovascular risk factors, namely dyslipidemia, obesity, and insulin resistance.

1.2.4. Contribution of angptl proteins to cardiovascular risk factors

1.2.4.1 Angiopoietin-like proteins in dyslipidemia

The findings that angptl proteins function to modulate lipid metabolism suggest that they are potentially involved in dyslipidemia, a state where blood lipids are abnormally elevated. Since both angptls 3 and 4 are involved in regulation of lipid storage and breakdown (Hato *et al.* 2008), it is without surprise to find studies showing angptls 3 and 4 involvement in dyslipidemia both in mice (Koishi *et al.* 2002; Inukai *et al.* 2004; Desai *et al.* 2007; Adachi *et al.* 2011) and in human (Shoji *et al.* 2009; Pisciotta

et al. 2012). On the other hand, two studies on human genetic variants concerning the role of angptl6 in dyslipidemia have also been published (Legry *et al.* 2009; Romeo *et al.* 2009). This section will focus on angptl proteins in dyslipidemia, except for angptl2, which will be covered in the next chapter.

The very first evidence that shed light on the roles of angptl proteins in dyslipidemia came from the study in mice by Shimizugawa *et al*. in 2002 (Shimizugawa *et al.* 2002). In this study, an adenoviral injection of angptl3 or a recombinant angptl3 protein into a mutant mouse strain characterized by its low plasma lipid levels resulted in marked increase in plasma lipid levels, including plasma total cholesterol, NEFAs, and TG levels (Shimizugawa *et al*. 2002). Overexpression of angptl3 in these mutant mice further elicited a significant increase in TG-enriched VLDL (Shimizugawa *et al*. 2002), a typical profile in dyslipidemia. The authors of this study concluded that the effects of angptl3 on VLDL were due to its ability to inhibit LPL activity, and thus inhibiting VLDL TG clearance (Shimizugawa *et al*. 2002). Consistent with this, the study by Ando *et al*. showed that mice with a mutant recessive gene encoding for angptl3 exhibited significant reductions in VLDL levels (Ando *et al.* 2003). Furthermore, in hyperlipidemic apolipoprotien E (ApoE) KO mice with this recessive mutation in the angptl3 gene showed a marked reduction of VLDL TG, VLDL, as well as plasma apolipoprotein (ApoB) levels compared to ApoE KO mice (Ando *et al*. 2003), which was attributed to enhanced lipid metabolism by increased LPL and hepatic lipase activities (Ando *et al*. 2003). Interestingly, in ApoE KO mice, the beneficial effects on dyslipidemia of an 8-week treatment with a xanthone compound, which has been found to have anti-oxidative effects (Jiang *et al.* 2003), were associated with down-regulated hepatic expressions of angptl3 mRNA and protein (Xiao *et al.* 2008), along with increased LPL mRNA expression (Xiao *et al*. 2008). Together, these studies suggest that low levels of angptl3 expression would be protective against dyslipidemia *via* increased lipid metabolism.

In human uremic dyslipidemia that is characterized by elevated TG-rich lipoproteins and low HDL levels, Shoji *et al*. found that high plasma angptl3 concentrations were associated with the disease (Shoji *et al.* 2009). Furthermore, in these uremic dyslipidemic subjects, angptl3 levels were inversely correlated with TG or

cholesterol ratios to both LDL and HDL, strongly suggesting angptl3 involvement in the pathogenesis of uremic dyslipidemia (Shoji *et al*. 2009).

The closely related angptl protein, angptl4, has also been implicated in dyslipidemia. The ability of angpt4 to suppress LPL activity *in vitro* first suggested the mechanism by which it could induce hyperlipidemia *in vivo* (Yoshida *et al.* 2002). Other studies using the adenoviral- (Ge *et al.* 2004; Xu *et al.* 2005) or transgene- (Koster *et al*. 2005) mediated overexpression of angptl4 in the liver reported marked TG increase. Moreover, transgenic mice mildly overexpressing angptl4 in peripheral tissues showed a 50% reduction in adipose tissue weight, which was associated with increased FA oxidation, increased uncoupling in fat, and greater plasma levels of TG, FFA, glycerol, total cholesterol and HDL (Mandard *et al*. 2006). Consistent with this, angptl4 KO mice had a 65 to 90% lower fasting TG as well as total cholesterol levels, accompanied by lower plasma VLDL and increased LPL activity (Backhed *et al.* 2004; Koster *et al.* 2005). The study by Desai *et al*. reported lipid-lowering effects of an anti-angptl4 antibody in C57Bl6/J, dyslipidemic ApoE KO, low-density lipoprotein receptor (LDLr) KO, and diabetic (db/db) mice (Desai *et al*. 2007). This was associated with increased VLDL clearance and decreased VLDL production (Desai *et al.* 2007). In line with this, in LDLr and angptl4 double KO mice, fasting total cholesterol, LDL, HDL, and TG levels were lower compared to LDLr single KO mice (Adachi *et al.* 2011). In addition, diabetic angptl4 KO mice showed improved fasting and post-prandial hypertriglyceridemia (Adachi *et al.* 2011). Notably, population-based sequencing uncovered associations between a variant (E40K) in the angptl4 gene and markedly lower plasma levels of TG and higher HDL levels (Romeo *et al.* 2007). These results were confirmed by other studies in human (Talmud *et al.* 2008; Nettleton *et al.* 2009). Also in human, angptl4 has been implicated in dyslipidemia, where a change in angptl4 in the plasma positively correlated with the change in NEFA (Jonker *et al.* 2013). Altogether, these studies indicate that disturbances in angptl4 signaling are involved in dyslipidemia, and highly suggest angptl4 as a potential therapeutic target. Similarly to angptl3, low levels of angptl4 expression would be protective against dyslipidemia.

Angptl6, a more recently identified hepatokine (Hato *et al.* 2008), also showed therapeutic implications in dyslipidemia. Serum angptl6 has been found significantly

greater in human with metabolic syndrome when compared with healthy controls (Namkung *et al.* 2011). Furthermore, subjects with high waist circumference or low HDL levels were also reported to have significantly increased serum angptl6 levels (Namkung *et al.* 2011). On the contrary, angptl6 null mice were presented with marked obesity that was associated with lower energy expenditure and insulin resistance (Oike *et al*. 2005), which will be discussed further in the next section.

Lastly, mice deficient of angptl8 revealed slower weight gain than WT mice, which was due to reduced adipose tissue accretion (Wang *et al*. 2013b). Specifically, post-prandial TG delivery was blunted in these angptl8 KO mice, which failed to replenish TG stores in their adipose tissues (Wang *et al*. 2013b). This study suggests that partial inhibition of angptl8 may be beneficial in combating dyslipidemia.

In summary, angptls 3, 4, 6 and 8 may be potential therapeutic targets in the treatment of dyslipidemia, a common feature of atherosclerosis.

1.2.4.2. Angiopoeitin-like proteins in obesity and insulin resistance

As angptl proteins play major roles in dyslipidemia, they are undoubtedly key players in obesity and insulin resistance. Most recent studies have focused on angptls 2, 3, 4, 6 and 8 in these pathological states.

Several studies have shown increased hepatic angptl3 expression as well as its circulating levels in leptin resistance and insulin resistance (Inukai *et al.* 2004; Shimamura *et al.* 2004). Levels of angptl3 mRNA and protein were found increased by about 2.2-fold in the liver of streptozotocin diabetic mice, which was reversed by insulin administration (Inukai *et al*. 2004). Similarly, hepatic angptl3 protein mRNA expressions were also greater than 3.0-fold in db/db mice compared to age-matched littermates (Inukai *et al*. 2004). In diabetic leptin-resistant and -deficent mice, angptl3 expression and its plasma levels were increased in comparison to control mice (Shimamura *et al*. 2004), which was associated with changes in plasma TG and FFA (Shimamura *et al*. 2004). Treatment with leptin or insulin in hepatocytes, on the other hand, decreased angptl3 expression (Shimamura *et al*. 2004). Together, these results suggest that in diabetes and insulin resistance, high expression of angptl3 in the leptin- or insulin-resistant state most likely involves the induction of hypertriglyceridemia and

hyperfattyacidemia. Therefore, abnormalities in angptl3 function may be involved in insulin resistance and related obesity.

Unlike angptl3, the role of angptl4 on obesity and insulin resistance has not been consistent. With the knowledge that agonists of PPARα and PPARγ also induce angptl4 expression levels (Mandard *et al*. 2004), overexpression of angptl4 in mice that led to hyperlipidemia and liver steatosis (Xu *et al*. 2005) seemed paradoxical. Moreover, mice that mildly overexpress angptl4, as mentioned earlier, had elevated plasma TG, FFA, glycerol, total cholesterol and HDL levels (Mandard *et al*. 2006). Thus, angptl4 is likely not an effector of PPAR agonists' beneficial effects on lipid metabolism. Nonetheless, the study by Xu *et al*. implied a beneficial role of angptl4 on glucose metabolism (Xu *et al*. 2005): indeed, treatment with angptl4 in db/db mice led to improved glucose tolerance and hyperinsulinemia, and patients with type 2 diabetes have substantially lower plasma angptl4 levels compared to obese subjects without diabetes (Xu *et al*. 2005). This was further strengthened by Koster *et al*., demonstrating significantly lower plasma angptl4 levels in patients with type 2 diabetes compared to healthy volunteers (Koster *et al*. 2005). Taken together, angptl4 may exert different effects on glucose and lipid metabolism, but its beneficial role in maintaining glucose homeostasis could be a potential target for treatment of diabetes. Contrarily, another study showed that microbial suppression of intestinal angptl4 in mice led to increased adiposity, suggesting that high angptl4 levels may promote leanness (Backhed *et al.* 2004). In summary, angptl4 has multiple metabolic effects, and further investigations are necessary to decipher out its role in obesity-related metabolic disorders.

Similar to angptl4, the role of angptl6 in obesity and insulin resistance has been inconsistent in recent literature. Whereas angptl6 was initially found to be able to "antagonize" obesity (Oike *et al.* 2005), which was also supported by recently published data showing its positive effects on resting metabolic rate in human (Mirzaei *et al.* 2011), another recent study reported elevated circulating levels of angptl6 in obese or diabetic human subjects (Ebert *et al.* 2009). In the study by Oike *et al*., angptl6-deficient mice showed marked obesity through decreased energy expenditure and insulin resistance (Oike *et al*. 2005). Contrarily, transgenic mice with overexpressed angptl6 driven by the CAG promoter were lean and had greater energy expenditure (Oike *et al*. 2005). In

parallel, adenoviral overexpression of angptl6 in the liver of diet-induced obese mice caused amelioration in obesity and insulin resistance (Oike *et al*. 2005), suggesting a counteracting effect of angptl6 on obesity. However, the study by Ebert *et al*. raised the possible phenomenon of "angptl6 resistance", which occurs in the state of obesity or diabetes (Kadomatsu *et al.* 2011). It was proposed that in physiological settings, where angptl6 levels are optimal, normal hepatic production of angptl6 may be effective in counteracting weight gain as well as promoting insulin sensitivity (Kadomatsu *et al.* 2011). However, this effect of angptl6 may be attenuated in obesity (Kadomatsu *et al.* 2011), leading to "angptl6 resistance". Highly relevant to obesity and insulin resistance, a potential role of angptl6 in endothelial dysfunction has recently been proposed by a Finnish group (Tuuri *et al.* 2013). Preliminary results from this group demonstrated that during the second trimester in relatively overweight pregnant women, angptl6 levels were higher in those who developed subsequent pregnancy-induced hypertension, although endothelial function was not tested (Tuuri *et al*. 2013). Collectively, angptl6 may have an anti-obesity effect, but additional studies are definitely needed to clarify how its expression is regulated, and to identify its receptor, so that its underlying signaling mechanisms can be further defined.

As mentioned earlier, since the recently identified angptl8 plays a significant role in TG trafficking (Wang *et al*. 2013b), disruption in its expression or signaling is expected to result in metabolic abnormalities. Serum levels of angptl8 were found higher in overweight and obese human subjects in comparison to lean subjects, and were positively correlated to BMI and glucose levels (Fu *et al.* 2014). This may be partially explained by the function of angptl8 to inhibit LPL and suppress TG clearance and increase serum TG (Zhang 2012), yet there was no correlation between fasting angptl8 levels and TG (Fu *et al.* 2014). Nonetheless, other studies have consistently shown evidence regarding the role of angptl8 in obesity: angptl8 transcript levels were around 8-fold higher in adipose tissue in obese (ob/ob) compared to WT mice (Ren *et al.* 2012), angptl8 KO mice displayed lower serum TG levels (Wang *et al*. 2013b), and overexpression of angptl8 resulted in dramatically elevated TG levels (Quagliarini *et al*. 2012; Zhang 2012), a hallmark of obesity (Miller *et al.* 2011). Interestingly, it was proposed that glucose could regulate angptl8 expression (Fu *et al.* 2014), as the human

angptl8 promoter has an almost perfect carbohydrate responsive element binding protein (ChREBP) binding site (Fu *et al.* 2014), which is consistent with findings that ChREBP indeed bound to the angptl8 promoter in hepatic Hep G2 cells (Jeong *et al.* 2011). Taken together, the recently identified angptl8 is likely a hepatokine that is involved in the pathogenesis of obesity and diabetes.

1.2.5. Angiopoietin-like proteins in endothelial dysfunction and atherosclerosis

With the knowledge that angptl proteins are key players in regulating angiogenesis, lipid and glucose metabolism, and that they are heavily implicated in dyslipidemia, obesity, and insulin resistance, one may expect that this protein family profoundly contributes to endothelial dysfunction and atherogenesis. Surprisingly, only a few studies have focused on angptl proteins in atherogenesis, while almost next to none have mentioned the role of angptls in endothelial dysfunction.

Due to angptl3's function in regulating lipid metabolism, ApoE KO mice with a mutant recessive angptl3 also exhibited less severe plaque development (Ando *et al.* 2003), which showed the first evidence of the link between angptl3 and atherosclerosis. In addition, Camenisch *et al*. demonstrated direct effects of angptl3 on vascular ECs by binding with integrin α5β3, thus modulating cell adhesion and migration (Camenisch *et al.* 2002). The study by Korstanje *et al*. in 2004 further linked murine atherosclerosis susceptibility to the angptl3 gene (Korstanje *et al.* 2004) and also found a strong association between single nucleotide polymorphisms in the angptl3 gene and atherosclerotic lesions in human (Korstanje *et al.* 2004). In parallel, plasma angptl3 levels were also positively correlated with carotid artery and femoral artery intima-media thickness in healthy human subjects, which was independent of other classical risk factors such as age, blood pressure, blood glucose and lipid levels (Hatsuda *et al.* 2007). Thus, angptl3 may be associated with EC adhesion, arterial wall thickness, as well as the development of atherosclerosis.

With the development of a new angptl4 ELISA assay (Stejskal *et al.* 2008), the study by Stejskal et al. reported correlations between plasma angptl4 levels and characteristics of metabolic syndrome, and provided the initial hypothesis that angptl4

could be a predictor of accelerated atherosclerosis (Stejskal *et al*. 2008). By a microarray analysis, Katano and Yamada demonstrated that angptl4 expression was significantly elevated in severely calcified carotid plaques (Katano and Yamada 2013). The recent generation of the LDLr/angptl4 double KO mouse showed decreased cholesterol and TG levels in comparison to LDLr single KO mice (Adachi *et al.* 2011), which typically develop premature spontaneous atherosclerosis characterized by increased plasma TG-rich lipoprotein remnants (Kolovou *et al.* 2011). In another similar study, ApoE/angptl4 double KO mice also displayed improved lipid profiles compared to ApoE single KO mice (Adachi *et al*. 2009). Notably, macrophages isolated form angptl4 KO mice showed suppressed ability for foam cell formation (Adachi *et al.* 2009), and may partly explain the effects of angptl4 absence on reducing atherosclerotic lesion size (Adachi *et al*. 2009). However, another recent study using the atherosclerosis-prone E3L mouse model contrarily showed that angptl4 suppressed foam cell formation, hence reducing atherosclerosis development, which was independent of changes in plasma cholesterol and TG (Georgiadi *et al*. 2013). In this study, authors found decreased uptake of oxidized LDL in macrophages by angptl4 (Georgiadi *et al.* 2013), most likely through its ability to inhibit LPL (Zhu *et al.* 2012). The discrepancies among these studies in mice could be attributed to the different atherosclerotic mouse model used, as the LDLr and ApoE KO mice develop significantly more severe atherosclerotic lesions compared to the E3L mouse model (van Vlijmen *et al.* 1994).

Prospective studies in human population also generated conflicting results. Whereas the prospective, population-based Atherosclerosis Risk in Communities Study reported associations between the common E40K loss-of-function variant in the angptl4 gene and lower genetic risks for developing coronary heart disease (Folsom *et al.* 2008), another study showed associations between the same common E40K variant and increased coronary heart disease risks (Talmud *et al.* 2008), and a more recent study reported no association between the E40K variant and coronary heart disease risks (Smart-Halajko *et al*. 2010).

Angptl6 may also be involved in atherosclerosis (Zhang *et al.* 2006b). In porcine ECs, *in vitro* studies showed that a recombinant angptl6 was able to potently support cell adhesion and migration mediated through integrin binding (Zhang *et al*. 2006a), which is

an important process in the inflammatory signaling cascade in ECs (Ley *et al.* 2007). Related to this, angptl6 may also be involved in endothelial dysfunction, as a study in Caucasian pregnant women reported for the first time that serum levels of angptl6 were higher in women with preeclampsia compared to healthy controls (Stepan *et al.* 2009), which suggested a link between angptl6 and endothelial dysfunction since preeclampsia is a complication in pregnancy characterized by hypertension and endothelial dysfunction (Sibai *et al.* 2005). Another recent Finnish study extended this potential link between angptl6 and endothelial dysfunction (Tuuri *et al*. 2013), as mentioned earlier. In this study, serum angptl6 levels were found higher in pregnant women who later developed pregnancy-induced hypertension (Tuuri *et al*. 2013). Although endothelial function was not directly measured (Tuuri *et al*. 2013), hypertension is implicated in endothelial dysfunction, as introduced in section 1.1.6.2. Interestingly, angptl6 has been shown to promote angiogenesis (Oike *et al.* 2004a) and blood flow *via* activation of the ERK1/2-eNOS-NO pathway (Urano *et al.* 2008), which may suggest its beneficial effects on endothelial function. Thus, further investigations are clearly warranted to understand the role of angptl6 in regulating endothelial function.

In summary, most studies have focused on the role of angptls 3 and 4 in the development of atherosclerosis due to their important functions in regulating lipoprotein metabolism, but findings were not always consistent. Whether angptls 3, 4, and 6 can regulate endothelial function, however, is still largely unknown. Therefore, whether inhibiting angptl proteins could become useful in preventing CVD including atherosclerosis remains to be elucidated.

Chapter 3: Angiopoietin-like-2

In the final introductory chapter, I will focus on angptl2, which is well known for its function in regulating metabolism as well as its pro-inflammatory role. Most of the literature regarding angptl2, interestingly, has come from Dr. Oike's group from Japan, with only a few other studies originating from other laboratories including our own. I will discuss both the physiological and pathological roles of angptl2, which will then lead me to the main question – does angptl2 play a role in regulating endothelial function?

1.3.1. Angptl2 – a pro-inflammatory mediator

Cloned 15 years ago by Kim *et al*. (Kim *et al*. 1999b), angptl2 is now known for its pro-inflammatory properties (Aoi *et al*. 2011; Aoi *et al*. 2014; Farhat *et al*. 2013; Horio *et al*. 2014; Ogata *et al*. 2012; Tazume *et al*. 2012). Accordingly, angptl2 has been studied in a number of pathologies, ranging from insulin resistance (Tabata *et al*. 2009) to tumour growth (Aoi *et al*. 2011; Aoi *et al*. 2014; Endo *et al*. 2014). Angptl2 is a protein of 493 amino acids with a molecular weight of 57 kDa. It can also be glycosylated at 64 kDa (Kim *et al*., 1999b), and is a circulating protein (Kadomatsu, Tabata, and Oike 2011; Kim *et al*. 1999b; Tabata *et al*. 2009).

Similar to the other angptl proteins, angptl2 possesses an N-terminal coiled-coil domain and a C-terminal fibrinogen-like domain (Kim *et al*. 1999b; Tabata *et al*. 2009), as depicted in Figure 10. However, there is limited information about the specific roles of these domains, other than the fact that the helical domain at the N-terminus may be required for oligomerization and maximal activity (Broxmeyer *et al.* 2011), and that the fibrinogen-like domain may be necessary for receptor binding (Kubota *et al*. 2005). Of note, the recent findings that the coiled-coil domain of angptl2 significantly enhanced hematopoietic stem cell expansion and survival (Broxmeyer *et al*. 2011) may begin to unfold the specific role of the coiled-coil domain. Very recently, it was proposed that angptl2 could be cleaved into fragments by the tolloid-like 1 (TLL1) protease *in vitro* at the coiled-coil domain and fibrinogen-like domain linker region (Odagiri *et al.* 2014). In this study using a cancer cell line, the cleaved form of angptl2 was unable to enhance tumour progression, unlike the full form of angptl2 (Odagiri *et al.* 2014).

As mentioned, angptl2 has been found in the circulation and expression of angptl2 has been abundantly found in skeletal muscle, heart, intestine, stomach, uterus, and adipose tissues (Kim *et al*. 1999b). In human, the physiological circulating level of angptl2 ranges from approximately 1.0 to 3.0 ng/ml (Kim *et al*. 1999b; Tabata *et al*. 2009; Kadomatsu *et al*. 2011), as measured in normal-weight, healthy volunteers, which was also confirmed with data from our own laboratory (Farhat *et al.* 2013), and is similar between male and female (Tabata *et al*. 2009; Usui *et al*. 2013). Work from our laboratory showed that gene expression of angptl2 in isolated ECs from atherosclerotic chronic smokers, compared to age-matched non-smokers was 4-fold higher (Farhat *et al*. 2008), which prompted us to study the role of angptl2 in the pathogenesis of atherosclerosis. To this end, our laboratory has since generated a recombinant form of human angptl2 (Farhat *et al.* 2013; Farhat *et al.* 2014), as well as a knock-down (KD) mouse model (Yu *et al*. 2014).

During the last decade, a number of other studies from different laboratories have been published regarding the pro-inflammatory role played by angptl2 in contexts of various inflammatory diseases. Expression of angptl2 has been found induced by obesity-associated pathological conditions including hypoxia, endoplasmic reticulum (ER) stress (Tabata *et al*. 2009), and a pro-inflammatory environment (Aoi *et al*. 2014; Farhat *et al*. 2013). Unfortunately, a clear known receptor for angptl2 has not been found, making it the greatest hinder in the field of angptl2 research. Until recently, a few potential candidate receptors that could bind to angptl2 have been proposed – the first one was the integrin α5β1 in adipocytes and ECs (Horio *et al*. 2014; Tabata *et al*. 2009). In the study published in 2009, Tabata *et al*. reasoned that the highly homologous fibrinogen-like domain in the angptl2 protein may, similar to fibrinogen (Herrick *et al.* 1999), act as a ligand for integrins (Tabata *et al*. 2009). Indeed, a neutralizing antibody for integrin α5β1 blocked effects of angptl2 (Tabata *et al*. 2009), suggesting angptl2 interacting with integrin α5β1. Additionally, the Toll-like receptor (TLR4) in ECs and monocytes (Oike and Tabata 2009) was also a proposed receptor, as the fibrinogen-like domain in angptl2 and fibrinogen can act as intrinsic TLR4 ligands (Oike and Tabata 2009). However, no direct evidence, such as binding assays, has ever shown TLR4 or integrin directly binding

to angptl2. Moreover, we were unable to detect integrin α5β1 mRNA expression in native aortic ECs treated with or without a recombinant angptl2 (Farhat *et al* 2013).

Recent reports demonstrated that the immune inhibitory receptor leukocyte immunoglobulin-like receptor B2 (LILRB2) in human (Zheng *et al.* 2012; Deng *et al.* 2014) and the paired immunoglobulin-like receptor (PIRB) in mouse can bind to angptl2, in addition to angptls1, 5, and 7, in hematopoietic cells (Zheng *et al*. 2012), as shown by co-immunoprecipitation and surface plasmon resonance studies (Zheng *et al*. 2012). Moreover, angptl2 was shown by flow cytometry to bind to LILRB2 with high affinity (Zheng *et al*. 2012). However, they still are not receptors that exclusively bind to angptl proteins, since LILRB2 and PIRB also bind other ligands (Shiroishi *et al.* 2003; Atwal *et al.* 2008). Furthermore, minimal LILRB2 mRNA expression in human coronary artery ECs (Horio *et al*. 2014) and mesenchymal cells (Odagiri *et al.* 2014) was reported, although both cells are able to secrete angptl2 (Farhat *et al.* 2013; Odagiri *et al.* 2014). As a result, a specific antagonist of the angptl2 receptor is still not yet available. Despite this shortcoming, studies on angptl2 have made tremendous progress using techniques to knock-down the protein with antisense (Kubota *et al.* 2005), siRNA or miRNA (Toyono *et al.* 2013; Odagiri *et al.* 2014; Richardson *et al.* 2014), the genetically modified angptl2 KO mice (Tabata *et al*. 2009), the angptl2 KD mice generated by our laboratory (Yu *et al.* 2014), as well as cell-specific KO or knock-in mice (Horio *et al*. 2014).

1.3.2. Angptl2 – a pro-oxidative mediator

With the different tools used to study angptl2, a recent study documented the role of angptl2 as a pro-oxidative mediator (Aoi *et al.* 2014). In a chemically-induced skin squamous cell carcinoma mouse model, Aoi et al. reported that mice overexpressing angptl2 in skin epithelial cells showed greater oxidative stress by quantifying lipid peroxidation product in mouse skin tissues (Aoi *et al.* 2014). Moreover, treatment with N-acetyl cysteine in drinking water reduced ROS levels in the skin of these mice, which was also associated with significantly attenuated incidence of papillomas (Aoi *et al.* 2014). In addition to this, preliminary data from our laboratory showed that in ECs, acute treatment with a recombinant angptl2 protein induced massive ROS production (Farhat *et al.* unpublished data), suggesting a pro-oxidative role of angptl2.

In the following few sections, physiological roles of angptl2 are first described, followed by evidence of angptl2 participation in inflammation and oxidative stress outlined as categorized by different pathologies.

1.3.3. Physiological roles of angptl2

Most recent studies have primarily focused on angptl2 participation in disease settings where its levels are in excess and where angptl2 acts as a pro-inflammatory mediator (Kadomatsu *et al.* 2014). Nonetheless, the inflammatory cascade is not involved strictly in disease settings but also in tissue homeostasis as protection against tissue damage (Medzhitov 2008). Thus, pro-inflammatory angptl2 can also take part in physiological settings.

1.3.3.1. Angptl2 in physiological angiogenesis

As the name implies, angptl2, being part of the angptl family, has properties similar to that of angiopoietins, proteins known for their angiogenic features (Tsigkos *et al.* 2003). Indeed, at the time of its initial cloning, angptl2 was shown to be pro-angiogenic by inducing EC sprouting (Kim *et al*. 1999b) (Table 1), which was further confirmed by our laboratory (Farhat *et al.* 2014), and also when the transgenic mice over-expressing angptl2 controlled by the keratinocyte-specific promoter K14 showed increased blood vessel growth in the skin (Hato *et al.* 2008). In line with this, knocking down angptl2 in zebrafish with antisense resulted in defective sprouting in ECs due to increased apoptosis (Kubota *et al*. 2005), suggesting a pro-angiogenic role of angptl2. However, globally knocking out angptl2, as in the angptl2 KO mouse model (Tabata *et al*. 2009), did not result in abnormal vascular development (Tabata *et al*. 2009). Interestingly, it was also shown that angptl2 cooperates with highly homologous angptl1 to exert anti-apoptotic effects, and that the two may even share complementary functions in vascular development (Kubota *et al*. 2005). Besides angiogenesis, another recent study demonstrated a potential role of angptl2 in the regulation of vasculogenesis (Richardson *et al.* 2014), a process of blood vessel formation *via de novo* production of ECs (Pardanaud *et al.* 1989). Indeed, knock-down of angptl2 by siRNA treatment in endothelial colony forming cell attenuated 36% of cell migration, but without much

effects on cell proliferation or apoptosis (Richardson *et al.* 2014). Moreover, the angiogenic role of angptl2 has been reported in bovine oestrous cycle maintenance (Mitko *et al.* 2008) and chick embryonic development (Niki *et al.* 2009), whereas its hematopoietic role has been reported in hematopoietic stem cell expansion *ex vivo* (Zhang *et al.* 2006a; Zheng *et al.* 2012; Akhter *et al.* 2013). Of course, angiogenesis is not strictly physiological, as pathological angiogenesis is an integral player in tumour growth (Folkman 1971), but the role of angptl2 in patholgocial angiogenesis will be discussed in a later section. Taken together, there is substantial evidence supporting the angiogenic involvement of angptl2 in physiological settings.

1.3.3.2. Angptl2 in tissue repair and remodeling

In aging, tissue repair and remodeling is important for the repair of damage caused by external and internal stresses and maintenance of tissue homeostasis (Medzhitov 2008). Normal angptl2 signaling has been demonstrated in tissue repair (Kim *et al*. 1999b; Kubota *et al*. 2005; Tabata *et al*. 2009). The role of angptl2 in tissue repair mechanisms has been demonstrated in zebrafish, which are characteristic for their tissue regeneration capacities (Akimenko *et al.* 2003). In studying embryogenesis in this model, angptl2 expression was first detected in yolk sac extension, spinal cord and branchial arches, and then subsequently expressed in liver primordium and pectoral fin buds (Kubota *et al*. 2005). In addition, angptl2 expression was induced in adult fin regeneration (Kubota *et al*. 2005). Notably, metalloproteinases (MMPs) were also expressed during blastema in fin regeneration of zebrafish (Bai *et al*. 2005), and angptl2 has recently been shown to induce MMP expressions and activities (Odagiri *et al*. 2014; Tazume *et al*. 2012). The recently published work linking angptl2 and MMPs reported that angptl2 signaling led to extracellular matrix (ECM) remodeling by increasing expressions and activities of MMPs *via* activation of p38 MAPK mediated by integrin $\alpha5\beta1$ (Odagiri *et al.* 2014). Relevant to this, angptl2 has been implicated in adipose tissue modeling (Tabata *et al*. 2009). Modest tissue remodeling is important in obesity, as least in its first stages when adipose tissues remodel to cope with excess lipids (Sun *et al.* 2011), which is a physiological response. Adipose tissue remodeling includes adipocyte hypertrophy, adipogenesis, angiogenesis, as well as ECM remodeling (Sun *et al.* 2011).

In obese mice, Tabata *et al*. reported abundant circulating and visceral adipose tissue angptl2 mRNA (Tabata *et al*. 2009). Transgenic mice overexpressing angptl2 in adipose tissue, although not obese, showed vascular inflammation and inflammatory macrophage infiltration (Tabata *et al*. 2009). Conversely, angptl2-deficient mice fed a HFD displayed fewer macrophage infiltration (Tabata *et al*. 2009). Altogether, this study suggests involvement of angptl2 in promoting adipose tissue remodeling in order to store excess energy, at least in early phases of obesity. This is summarized in Figure 13.

Collectively, there is evidence showing angptl2 contribution to tissue repair and remodeling, but more work is necessary to decipher out the exact mechanisms by which angptl2 promotes them, especially in human.

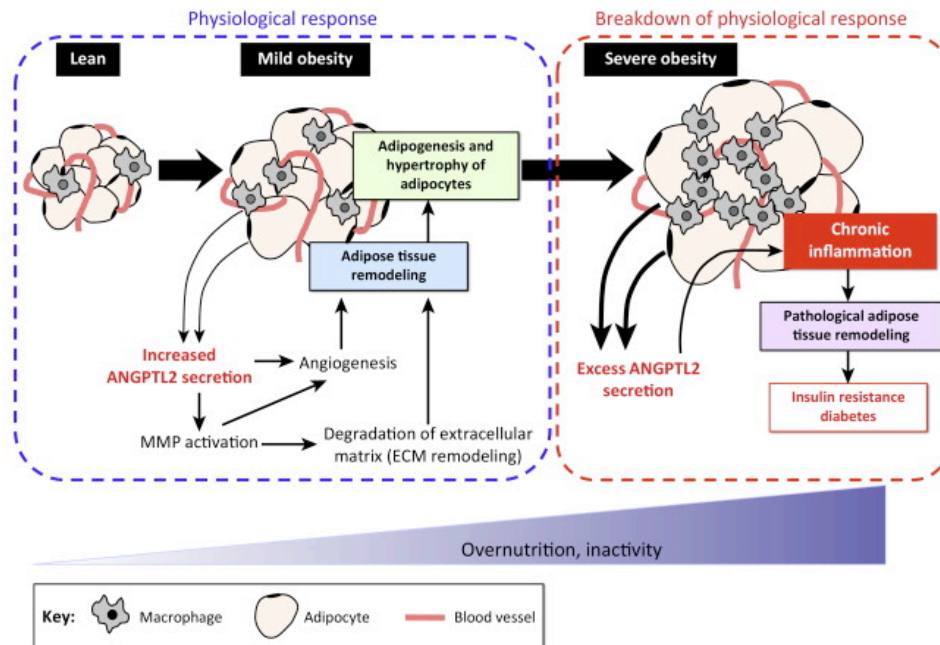

Figure 13. The role of angptl2 in physiological and pathological adipose tissue remodeling. In mild obesity, physiological adipose tissue remodeling involves increased angptl2 production from adipocytes promoting MMP activation, thus inducing adipose tissue remodeling, which leads to adipogenesis and adipocyte hypertrophy to store excess energy. In severe obesity, pathological adipose tissue remodeling involves excess angptl2 production, which leads to vascular inflammation and macrophage infiltration into adipocytes.

Reprinted with the permission from Elsevier: Trends Endocrinol Metab. Kadomatsu T, Endo M, Miyata K, Oike Y. Diverse roles of ANGPTL2 in physiology and pathophysiology. *Trends Endocrinol Metab*. **25**, 245-254. Copyright (2014).

1.3.3.3. Angptl2 as a circadian gene

Circadian rhythmicity is exhibited by a number of physiological processes such as metabolism (Bass and Takahashi 2010) and is tightly regulated in most cells by a set of important genes, including the circadian locomotor output cycles kaput (CLOCK) and the brain and muscle aryl hydrocarbon receptor nuclear translocator-like protein 1 (BMAL1) genes (Ukai and Ueda 2010). Disruption of the circadian clock, on the other hand, has been linked to the pathophysiogenesis of a number of diseases, such as sleep disorders, metabolic syndrome, and CVDs (Takahashi *et al.* 2008). Interesting insights on angptl2 acting as a circadian gene were reported in one study in 2011, where Kitazawa *et al*. first showed differential mRNA expression of angptl2 in epididymal adipose tissue dependent on the circadian rhythm in mice, with peak expression levels at times of active feeding, with induction of the angptl2 promoter activity by CLOCK and BMAL1 (Kitazawa *et al.* 2011), which was also confirmed by Kadomatsu *et al*. in various mouse tissues and synchronized human osteosarcoma cells (Kadomatsu *et al.* 2013). Rhythmic expression of angptl2 may therefore imply that the disruption of its circadian regulation can lead to lifestyle-related diseases (Kadomatsu *et al*. 2013). For example, chronic constitutive expression of angptl2, as in transgenic mice continuously expression angptl2 in adipose (Tabata *et al*. 2009) or skin tissue (Aoi *et al*. 2011), is associated with adipose and skin tissue inflammation, respectively (Aoi *et al*. 2011; Tabata *et al*. 2009). In addition, serum angptl2 also showed circadian expression (Kitazawa *et al.* 2011). Taken together, angptl2 is a circadian gene, and disruptions of its regulation may lead to diseases.

1.3.3.4. Angptl2 in AT1R recycling

Another physiological and important role of angptl2 is its involvement in the trafficking and recycling of the AT1R in the cytosol (Guo *et al.* 2001; Guo *et al.* 2003; Guo *et al.* 2006), linking angptl2 with angII signaling and part of the RAS. Because of this particular role, angptl2 has also been called angiotensin II receptor-associated protein 1 (ARAP1) by other teams (Guo *et al.* 2001; Guo *et al.* 2003; Guo *et al.* 2006; Doblinger *et al.* 2012; Mederle *et al.* 2013). Angptl2 was found localized with AT1R in the mouse kidney vasculature, and down-regulation of angptl2 was accompanied by slight reduction in AT1R expression in cultured mesangial cells (Doblinger *et al*. 2012). In the same

study, it was shown that renal angptl2 expression was suppressed by angII in a dose-dependent manner, suggesting angII regulation of renal angptl2 expression (Doblinger *et al*. 2012). Overexpression of angptl2 in the renal proximal tubule of mice resulted in salt-sensitive increase in blood pressure, which was reversed by ACE inhibitor perindopril or AT1R antogonist losartan (Guo *et al*. 2006). Interestingly, global angptl2 KO mice are normotensive (Mederle *et al.* 2013), suggestive of compensatory mechanisms in the systemic vasopressor systems to maintain blood pressure (Mederle *et al.* 2013). However, there is no direct evidence currently showing angptl2 regulation of blood pressure and further investigations are necessary to clarify this role. Mechanistically, both *in vivo* and *in vitro* studies have shown that angptl2 acts as a positive modulator of vascular AT1R by binding to the receptor's intracellular C-terminal region, which plays a crucial role for receptor internalization, desensitization, and phosphorylation, thereby facilitating its recycling to the plasma membrane (Guo *et al*. 2003; Guo *et al*. 2001).

Collectively, angptl2 exhibits physiological roles such as angiogenesis, tissue repair and remodeling, as well as AT1R recycling.

1.3.4. Pathological roles of angptl2

Just like most other proteins, there exists a tightly regulated level of angptl2 to maintain cell homeostasis (Kadomatsu *et al.* 2014). However, when there is excess activation of angptl2, homeostasis is disrupted, leading to chronic inflammation and tissue damages (Kadomatsu *et al.* 2014). In the following section, pathological roles of angptl2 will be discussed.

1.3.4.1. Angptl2 in rheumatoid arthritis

Rheumatoid arthritis, a chronic inflammatory disorder, is characterized by synovitis and ultimately, destruction of the joints (Firestein 2003). During rheumatoid arthritis, there is recruitment of activated B and T lymphocytes, monocytes and macrophages, plasma cells, as well as mast cells to the joint, to act as sources of inflammatory cytokines, including interleukin-1 (IL-1), interleukin-6 (IL-6), and TNFα (Brennan and McInnes 2008). Importantly, abundant gene and protein expression of angptl2 was found in synoviocytes of patients with rheumatoid arthritis (Okada *et al*.

2010). Likewise, angptl2 levels in synovial fluid was higher in patients with rheumatoid arthritis than those with osteoarthritis, a form of arthritis with lesser degree of inflammation, without differences in serum levels of angptl2 between the two groups (Okada *et al.* 2010). Further analysis by immunofluorescent staining indicated that angptl2 was produced by fibroblast- and macrophage-like synoviocytes in the rheumatoid arthritis synovium (Okada *et al*. 2010). Angptl2 also promoted chemotaxis of both monocytes and ECs in rheumatoid arthritis fluid, which may be associated to its signaling through integrins (Okada *et al*. 2010). In conjunction, angptl2 may also mediate angiogenesis, as described previously (Kim *et al*. 1999b; Kubota *et al*. 2005), resulting in the common feature of vascular inflammation in rheumatoid arthritis. Collectively, angptl2 plays a potential role in the pathogenesis of rheumatoid arthritis by mediating inflammatory vascular remodeling and macrophage recruitment to the joints.

1.3.4.2. Angptl2 in cancer

With chronic inflammation emerging as a key player in cancer development, and that ER stress and hypoxia are common in cancer progression and metastasis (Bi *et al.* 2005), angptl2 has been implicated in several cancer cell types, including breast cancer (Endo *et al*. 2014), lung cancer (Endo *et al.* 2012; Sasaki *et al.* 2012), sarcoma (Teicher 2012) and osteosarcoma (Odagiri *et al.* 2014), leukemia (Zheng *et al*. 2012), skin cancer (Aoi *et al*. 2011; Aoi *et al*. 2014), and very recently, hepatic cancer (Gao *et al.* 2014).

In breast cancer patients, increased serum levels of angptl2 was able to reflect clinical progression of disease, and could potentially be a biomarker for breast cancer metastasis (Endo *et al.* 2014). Furthermore, expression of angptl2 was found in correlation with carcinogenesis frequency in a mouse model (Aoi *et al*. 2011). In this study, Aoi *et al*. found that angptl2 expression in mouse skin tissues was positively correlated to a chronic inflammatory status as well as ROS levels, and was more susceptible to carcinogenesis (Aoi *et al*. 2011). The authors concluded that angptl2 'primed' the microenvironment into a pro-inflammatory one that becomes more susceptible to DNA damage and genomic instability (Aoi *et al*. 2011). This is illustrated in Figure 14. These authors also demonstrated that, by promoting epithelial-to-mesenchymal transition *via* activation of the tumour growth factor (TGF)-β-Smad

pathway, and lymphoangiogenesis, angptl2 increased metastasis to lymph nodes as well as distant secondary organs (Aoi *et al*. 2011). Chronic inflammation in the skin tissues of transgenic mice overexpressing angptl2 also showed infiltration by inflammatory cells such as activated macrophages and neutrophils (Aoi *et al*. 2011), and was speculated as the source of ROS, which is able to inactivate DNA repair enzymes (Colotta *et al.* 2009). Subsequent to this study, angptl2 involvement in cancer development was further strengthened by studies in human lung tumour tissues – its expression in lung tumour tissues from cancer patients was significantly higher that that found in non-tumour lung tissues from the same cancer patients (Endo *et al.* 2012). Additionally, continuous anti-oxidant N-acetylcysteine (NAC) treatment in an angptl2-induced chemically-induced squamous cell carcinoma model attenuated inflammation as well as ROS accumulation (Aoi *et al.* 2014), further emphasizing the pro-inflammatory role of angptl2. In osteosarcoma cell lines, it was suggested that angptl2 was acting through integrins $\alpha 5\beta 1$, p38 MAPK and MMP-9 to increase metastasis (Odagiri *et al.* 2014), and that demethylation of the gene promoter region of angptl2 was associated with increased angptl2 expression (Odagiri *et al.* 2014). In the same study by Odagiri *et al*., it was additionally reported that a cleaved form of angptl2 resulting from TLL1 protease cleavage did not enhance tumour metastasis *in vitro* in osteosarcoma cell lines, and only the full-length angptl2 was capable in promoting tumour progression (Odagiri *et al.* 2014). It was additionally proposed by the authors that the lack of TLL1 in cancer cells may explain the deleterious effect of angptl2 (Odagiri *et al*. 2014).

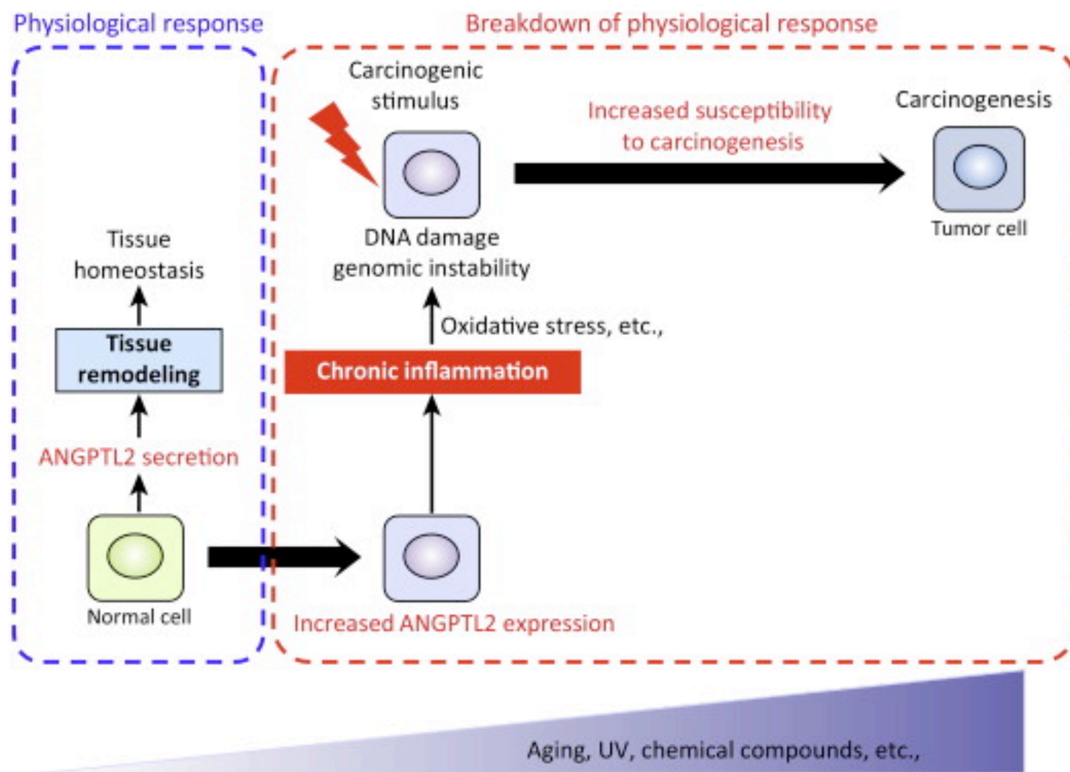

Figure 14. Proposed mechanism linking angptl2 and carcinogenesis. Physiological secretion of angptl2 contributes to tissue homeostasis maintenance. Chronic stresses such as aging can increase production of angptl2 and result in chronic inflammation, which can induce oxidative stress and genomic instability, ultimately increasing susceptibility to carcinogenesis.

Reprinted with the permission from Elsevier: Trends Endocrinol Metab. Kadomatsu T, Endo M, Miyata K, Oike Y. Diverse roles of ANGPTL2 in physiology and pathophysiology. *Trends Endocrinol Metab*. **25**, 245-254. Copyright (2014).

Interestingly, data from another group showed angptl2 as a putative tumour suppressor in ovarian cancer (Kikuchi *et al.* 2008). By genome-wide analysis of an ovarian cancer cell line, Kikuchi *et al*. identified a novel homozygous loss of angptl2 (9q33.3), which was absent in normal epithelial cells (Kikuchi *et al*. 2008), suggesting angptl2's anti-tumour effects. Indeed, restoring angptl2 expression or adding angptl2 in cell medium inhibited ovarian cell growth, while knocking down angptl2 had the opposite effect (Kikuchi *et al*. 2008). Of note, the lack of angptl2 immunoreactivity in cancer cells correlated with poorer survival in the early stages of disease, while the

opposite was true for advanced stages of disease (Kikuchi *et al*. 2008), suggesting angptl2 could act in a stage-dependent manner. However, the mechanism that regulates angptl2 silencing in these ovarian cells, leading to its inactivation, remains unknown (Kikuchi *et al*. 2008).

In the context of pathological angiogenesis seen in cancer settings (Folkman 1971), angptl2 seems to also play a crucial role. A microarray analysis of resistant tumour cells in response to anti-VEGF therapy unexpectedly showed angptl2 as a potential pro-angiogenic candidate that may be involved in tumour refractoriness to anti-VEGF therapy (Crawford *et al.* 2009). Consistent with this, in chemically induced carcinogenesis and xenograft tumour mouse models, both angptl2-deficient mice and angptl2-KD tumour cells showed lower levels of tumour angiogenesis (Aoi *et al*. 2011). In contrast, overexpression of angptl2 in mice resulted in augmented tumour angiogenesis (Aoi *et al*. 2011). Lastly, and as mentioned before, angptl2 was found to bind to LILRB2, allowing it to promote leukemia development by supporting *ex vivo* expansion of haematopoietic stem cells (Zheng *et al*. 2012).

Taken together, most studies have proposed angptl2 as a likely player in cancer pathophysiology including tumour angiogenesis, with the exception of the study by Kikuchi *et al*. (Kikuchi *et al*. 2008), suggesting angptl2 as a potential therapeutic target in cancer treatment.

1.3.4.3. Angptl2 in obesity and insulin resistance

It is now clear that obesity is considered a chronic inflammatory disorder (Lumeng and Saltiel 2011), where adipocytes and macrophages could act as sources of pro-inflammatory cytokines (Kanda *et al.* 2006). Evidence of angptl2 involvement in obesity and associated insulin resistance was first demonstrated in 2009 (Tabata *et al*. 2009), proposing angptl2 as an adipokine, or adipocyte-derived cytokine (Ouchi *et al.* 2011), for the first time. In this seminal report, angptl2 was shown to be produced by adipocytes, where it is abundantly expressed, and particularly in visceral adipose tissues (Tabata *et al*. 2009). Much like in cancer cells, angptl2 expression and production were increased by ER stress and hypoxia in obesity (Tabata *et al*. 2009). In human, serum levels of angptl2 positively correlated with inflammatory states and insulin resistance

levels (Tabata *et al*. 2009), and was also associated with type 2 diabetes development (Doi *et al.* 2012). In line with this, both gene and protein expressions of angptl2 in glomeruli from renal biopsies was up-regulated in diabetic patients compared to control (Sun *et al.* 2007). On the other hand, anti-diabetic treatment with pioglitazone in type 2 diabetic and obese patients for 3 months (Tabata *et al*. 2009) or weight reduction in overweight but non-diabetic men (Muramoto *et al.* 2011) resulted in lower circulating angptl2 levels. In comparing WT and angptl2 KO mice fed a HFD for 8 weeks, it was shown that whereas WT mice developed systemic insulin resistance as examined by glucose and insulin tolerance tests, KO mice only developed mild resistance, as reflected by greater insulin sensitivity in both the skeletal muscle and liver (Tabata *et al*. 2009). Moreover, they gained significantly less weight compared to the WT mice, and expressed lower adipocytokines and macrophage markers in adipose tissues (Tabata *et al*. 2009). On the contrary, overexpressing angptl2 in adipose tissues in mice promoted local inflammation in the adipose tissue as there were increased gene expressions of inflammatory cytokines such as IL-6 and TNFα; these mice also developed systemic insulin resistance and displayed glucose intolerance and insulin resistance, as reflected by blunted insulin signaling in both skeletal muscle and liver, without significant weight differences compared to WT mice (Tabata *et al*. 2009).

In cultured ECs, angptl2 acted on α5β1 integrins (Tabata *et al*. 2009), which are known to activate inflammatory gene expressions *via* the NFκB pathway (Klein *et al.* 2002). Indeed, angptl2 stimulated NFκB nuclear translocation and inhibitor of kappa B (IκB) degradation, induced expression of adhesion molecules and promoted monocyte migration (Tabata *et al*. 2009). Taken together, this study suggests that adipocyte-derived angptl2 acts as an inflammatory mediator by promoting vascular remodeling and macrophage recruitment into adipose tissue, thereby playing a key role in insulin resistance and the pathogenesis of diabetes, as illustrated in Figure 13 previously.

Of note, another group showing that angptl2 is a circadian gene, as mentioned before, suggested the contrary (Kitazawa *et al.* 2011). Angptl2 was reduced in diabetic db/db mice and HFD-fed mice at feeding time, and administration of a recombinant angptl2 improved insulin sensitivity, lowered levels of glucose, TG and FFA in these mice, suggesting an anti-diabetic role of angptl2 (Kitazawa *et al*. 2011). To date, this has

been the only study associating angptl2 with anti-diabetic properties and it was proposed that angptl2 is involved in the progression of diabetes (Kitazawa *et al*. 2011), rather than its pathogenesis (Tabata *et al*. 2009). Using 3T3-L1 cells of adipocytes, it was found that angptl2 expression was highest during prematurity of cells and diminished in fully mature adipoctyes, suggesting its role in regulating adipocyte differentiation (Kitazawa *et al*. 2011). Moreover, knocking down the angptl2 gene using siRNA disrupted insulin signaling *via* de-activation of the Akt downstream pathway, specifically by increasing expression of tribbles homolog 3 (Trib3) (Kitazawa *et al*. 2011), an inhibitory protein of Akt activation (Du *et al.* 2003). Discrepancies on the role of angptl2 on insulin sensitivity between these studies may be due to different mouse models used, HFD-induced insulin resistance (Tabata *et al*. 2009) and the db/db mouse model (Kitazawa *et al*. 2011). Each model represents distinct disease state, as a HFD would induce insulin resistance as the mice develop, while db/db mice establish severe insulin resistance at 8 weeks of age (Kitazawa *et al*. 2011). It was proposed that angptl2 could play divergent roles in different stages in the pathogenesis of diabetes (Kitazawa *et al*. 2011).

In cultured, fully matured 3T3-L1 adipocytes, Zheng *et al*. found evidence that exogenous addition of TNFα induced angptl2 expression, likely through activation of transcription factor FoxO1 (Zheng *et al.* 2011), whose signaling is associated with production of pro-inflammatory cytokine IL-1β *via* NFκB (Su *et al.* 2009). On the other hand, insulin suppressed angptl2 gene expression, which was impaired by PI3K inhibitor (Zheng *et al*. 2011). Altogether, these data indicate that under a pro-inflammatory or insulin-resistance environment, where FoxO1 activity is increased, expression of its target gene angptl2 is up-regulated, suggesting a potential role of angptl2 in obesity-induced inflammation and insulin resistance.

In another obesity-related chronic inflammatory disorder, such as chronic kidney disease, evidence of angptl2 implication was found. In a general Japanese population, elevated serum levels of angptl2 was associated with the likelihood of chronic kidney disease after adjusting for known cardiovascular risk factors and events (Usui *et al.* 2013). However, whether angptl2 could directly impair kidney function remains to be elucidated (Usui *et al*. 2013). Angptl2 levels were also higher in microvascular lesions of diabetic glomerulopathy (Sun *et al.* 2007). Similarly, serum angptl2 levels also

independently correlated with albumin-to-creatinine ratio in diabetic patients (Li *et al.* 2013), and could be used to identify diabetic patients with nephropathy in early phase (Li *et al.* 2013). Consistent with this, the Hisayama Study also reported a positive correlation between serum angptl2 and the development of type 2 diabetes in a general Japanese population of 2164 individuals ranging from 40 to 79 year-old followed up for 7 years (Doi *et al.* 2012), suggesting angptl2 as an independent risk factor for diabetes (Doi *et al.* 2012). Collectively, there is accumulating evidence further supporting the involvement of angptl2 as a causal factor in diabetes.

In summary, it seems that most studies support the role of angptl2 in regulating insulin sensitivity, and that high levels of angptl2 could predict the development of insulin resistance and diabetes. Therefore, lowering levels of angptl2 could be a potential strategy against obesity-mediated insulin resistance and diabetes.

1.3.4.4. Angptl2 in vascular remodeling

In view of obesity being a worldwide pandemic (Flier 2004) and is closely linked to CVDs (Castelli 1998), some investigators have turned their interests to adipose tissue biology. As already mentioned, adipocytes dynamically secrete bioactive factors, also known as adipokines, including pro- and anti-inflammatory adipokines, in response to the environment (Guilherme *et al.* 2008). Besides visceral adipose tissues secreting these adipokines (Guilherme *et al*. 2008), perivascular adipose tissues that surround the outer layer of arterial vessels also do the same (Meijer *et al.* 2011). There are recent studies suggesting that an imbalance of perivascular adipocyte pro- and anti-inflammatory adipokine production can result in vascular remodeling that is associated with CVDs (Iacobellis *et al.* 2008; Takaoka *et al.* 2009). In a recent study, angptl2 expression in human perivascular adipose tissue was reported (Tian *et al.* 2013), which was positively correlated to adiponectin, an anti-inflammatory adipokine, in non-coronary heart disease patients (Tian *et al*. 2013), but also positively correlated to TNFα in coronary heart disease patients (Tian *et al*. 2013). This suggests that angptl2 cooperates with pro-inflammatory TNFα and prevents the anti-inflammatory activity of adiponectin (Tian *et al*. 2013). In the same study, angptl2-deficient mice that underwent an endovascular wire injury in the femoral artery showed attenuated vascular neointimal thickening compared

to WT littermates (Tian *et al*. 2013). Perivascular adipose tissue-derived angptl2 up-regulated expressions of genes involved in inflammation and ECM degradation, including TNFα and MCP-1 (Tian *et al*. 2013). There was also increased MMP-2 activity in the vascular tissue of mice transplanted with visceral adipose tissue of transgenic mice overexpressing angptl2 (Tian *et al*. 2013). Interestingly, angptl2 expression in perivascular adipose tissues of atherosclerosis-prone and hypercholesterolemic ApoE KO mouse was significant greater than that in WT mice (Tian *et al*. 2013), and angptl2 expression was also increased with age (Tian *et al*. 2013). Altogether, coronary risk factors including aging and hypercholesterolemia could increase perivascular adipose tissue angptl2 expression, which could in turn contribute to vascular remodeling (Tian *et al*. 2013).

1.3.4.5. Angptl2 in inflammatory tissue disorders

A recent study by Nakamura *et al*. showed that angptl2 expression was abundant in hypertrophied ligamentum flavum tissue fibroblasts (Nakamura *et al.* 2014). Ligamentum flavum hypertrophy is an inflammatory disorder that results in lumbar spinal canal narrowing as there is mechanical compression of the nerve root (Beamer *et al.* 1973; Sairyo *et al.* 2007). In the study by Nakamura *et al*., *in vitro* experiments in a chamber attached to a stretching apparatus using fibroblasts isolated from patients undergoing lumbar surgery demonstrated that angptl2 expression in hypertrophied fibroblasts was induced by mechanical stretching *via* activation of calcineurin/nuclear factor of activated T cell (NFAT) pathways (Nakamura *et al*. 2014), and correlated with that of TGF-β1 (Nakamura *et al*. 2014). The mRNA of TGF-β1 receptors also increased following angptl2 treatment in fibroblasts (Nakamura *et al*. 2014). Most likely, mechanical stretching stress could, *via* activating mechanosenstive ion channels and increasing intracellular Ca^{2+}, activate the calcineurin/NFAT pathways, which have been reported to induce angptl2 expression in tumour cells (Endo *et al*. 2012). Nakamura *et al*. speculated that when the level of mechanical loading is physiological, optimal amounts of angptl2 acts as a tissue remodeling factor to maintain homeostasis; however, when the level of mechanical loading becomes pathological, as in patients with lumbar spinal canal stenosis, excess angptl2 would promote irreversible pathological remodeling and

degeneration in ligamentum flavum tissues, leading to hypertrophy (Nakamura *et al*. 2014).

Angptl2 has also been implicated in another inflammatory tissue disorder, namely dermatomyositis (Ogata *et al.* 2012), an autoimmune disease characterized by chronic skin and muscle tissues inflammation that lead to skin eruption and muscle weakness (Callen 2000). Abundant gene and protein expression of angptl2 were reported in skin eruptions of patients presented with dermatomyositis, along with evidence that angptl2 was derived from skin cells, likely as a result of hypoxia and ER stress, and activates the NFκB inflammatory cascade through the integrin α5β1, as an autocine or paracrine (Ogata *et al*. 2012).

In summary, angptl2 expression is associated with inflammatory tissue diseases, as demonstrated in ligamentum flavum hypertrophy (Nakamura *et al*. 2014) and dermatomyositis (Ogata *et al*. 2012), suggesting angptl2 as a potential target in treatment against these disorders.

1.3.4.6. Angptl2 in abdominal aortic aneurysm (AAA) development

In 2012, the first evidence of angptl2 involvement in AAA development was demonstrated by Dr. Oike's group (Tazume *et al.* 2012). Clinically, AAA is diagnosed as either ≥1.5 times increase in aortic diameter when compared to a normal adjacent aorta, or a presented aneurysm with ≥30 mm diameter (Schermerhorn 2009). AAA is a chronic inflammatory disease, and is typically characterized by infiltrating inflammatory cells including T-cells, B-cells, macrophages, as well as mast cells (Ocana *et al.* 2003).

In the first part of this particular study, aortic aneurismal lesion tissues from patients presented with AAA were first examined. The major findings of this study were that, indeed, angptl2 was expressed within aortic aneurismal walls of AAA patients, that angptl2 expression was primarily localized at the medial layer of aneurysmal lesions and was co-localized with cells expression CD68, a macrophage marker, but not with those expressing CD20, CD3, or CD15 (Tazume *et al*. 2012). CD20, CD3, and CD15 are markers for B cells (Ernst *et al.* 2005), T cells (van Dongen *et al.* 1988), and neutrophils (Larsen *et al.* 1990), respectively. This suggests that angptl2 is primarily produced within infiltrating macrophages and not within B or T lymphocytes and neutrophils (Tazume *et*

al. 2012). In the second part of the study, mice genetically lacking angptl2 (KO) were used to determine association of angptl2 with chronic inflammation in $CaCl_2$-induced AAA. Male 10- to 12-week-old WT or KO mice underwent peri-aortic application of $CaCl_2$ for 28 days, after which mice were sacrificed for experiments. Furthermore, using the $CaCl_2$-induced AAA mouse model, abundant expression of angptl2 was found at the medial layer of aortic aneurysm in mice with AAA, but not in sham-operated, control mice (Tazume *et al*. 2012). As well, pattern of angptl2 expression was similar to that of Mac2 (Tazume *et al*. 2012), also a macrophage marker. In angptl2 KO mice, AAA development was attenuated, accompanied with less mRNA expressions of TNFα, IL-1β, and IL-6 compared to $CaCl_2$-treated WT mice (Tazume *et al*. 2012). However, the amount of infiltrating macrophages in the lesions between KO and WT mice were similar (Tazume *et al*. 2012), suggesting macrophage-derived angptl2 contributed to AAA development. Authors concluded that macrophage-derived angptl2 could increase inflammatory cytokine production and aorta ECM degradation (Tazume *et al*. 2012).

1.3.4.7. Angptl2 in atherogenesis

A direct role of angptl2 in atherogenesis was first documented in 2013 by our laboratory (Farhat *et al.* 2013). With a previous report showing a 4-fold increase in angptl2 mRNA levels from ECs isolated from arteries of active smokers with CAD *versus* non-smoking CAD subjects (Farhat *et al*. 2008), and that plasma levels of angptl2 were greater in Japanese patients with CAD *versus* control healthy subjects (Tabata *et al*. 2009), the role of angptl2 and the underlying mechanisms were determined in the setting of atherogenesis (Farhat *et al.* 2013). Besides merely being a lipid storage disease, atherosclerosis actually is also a chronic inflammatory disorder (Libby *et al.* 2002). In our study, plasma levels of angptl2 in WT mice was positively correlated with age, which was exacerbated in severely dyslipidemic $LDLr^{-/-}$; $hApoB^{+/+}$ mice (Farhat *et al.* 2013). In these mice where atherosclerosis develops spontaneously (Sanan *et al.* 1998), angptl2 protein expression in atherosclerotic plaque of the aorta increased with age and progression of disease, which correlated with that of F4/80, a marker of macrophages (Farhat *et al.* 2013). Acute exogenous stimulation by recombinant angptl2 on native $LDLr^{-/-}$; $hApoB^{+/+}$ mouse endothelium potently up-regulated expression of ICAM-1 and

P-selectin and induced leukocyte adhesion, paralleled with increased adhesion molecule expression (Farhat *et al.* 2013). Additionally, angptl2 was secreted by ECs and was bound to VSMC (Farhat *et al.* 2013). Interestingly, chronic administration of recombinant angptl2 in young pre-atherosclerotic mice for 4 weeks increased circulating cholesterol levels in addition to plaque formation by 10-fold (Farhat *et al.* 2013), showing for the first time, links among lipid handling, atherogenesis, and angptl2. Taken together, angptl2 acts as a pro-inflammatory factor that participates in various early steps of atherogenesis, by activating ICAM-1 and P-selectin, priming an inflammatory vascular endothelium, thereby promoting leukocyte adhesion onto the endothelium (Farhat *et al.* 2013).

The involvement of angptl2 in atherogenesis was further supported by a subsequent Japanese report showing that in another atherosclerotic mouse model, the ApoE KO mouse, angptl2 protein levels in aortic tissues were similarly and positively correlated with atherosclerosis severity (Horio *et al*. 2014), and that angptl2 was expressed in both ECs and infiltrating macrophages in human tissues (Horio *et al*. 2014). The authors demonstrated that angptl2 activated pro-inflammatory NFκB signaling in ECs, which then increased monocyte and macrophage chemotaxis (Horio *et al*. 2014). Conversely, angptl2 deficiency in double-KO of ApoE and angptl2 in mice resulted in significantly smaller atherosclerotic lesions than single ApoE KO littermates, while expressions of pro-inflammatory markers, such as VCAM-1, E-selectin, TNFα, IL-6, IL-1β, were lower compared to littermates (Horio *et al*. 2014). Furthermore, exogenous stimulation by angptl2 resulted in increased mRNA expressions of VCAM-1, ICAM-1, and E-selectin, as well as activation of the integrin α5β1/NFκB inflammatory cascade in human coronary artery ECs, and increased IκB degradation (Horio *et al*. 2014). Collectively, the two studies (Farhat *et al*. 2013; Horio *et al*. 2014) show consistent evidence supporting the pro-inflammatory primary role of angptl2 in the pathogenesis of athersclerosis.

1.3.4.8. Angptl2 in endothelial dysfunction

As endothelial dysfunction is an early indicator of atherosclerosis (Celermajer *et al.* 1992; Bonetti *et al.* 2003), a few investigators have questioned whether angptl2 was

involved in regulating endothelial function in their studies (Horio *et al*. 2014; Tabata *et al*. 2009). In the study by Tabata *et al*., as previously described, obesity induced angptl2 expression in adipocytes, which was proposed to contribute to vascular inflammation as well as monocyte migration, as summarized in Figure 15.

Although endothelial function was not directly tested in this study, *in vitro* demonstration that recombinant angptl2 in cultured ECs stimulated nuclear translocation of NFκB and degradation of IκB *via* integrin α5β1 and Rac1 activation provided the clues that angptl2 was directly involved in endothelial inflammation (Tabata *et al*. 2009). Previous reports have shown that Rac1, a G-protein in the Rho family involved in cell migration and adhesion (Bar-Sagi and Hall 2000; Burridge and Wennerberg 2004), can activate NFκB (Sulciner *et al.* 1996; Perona *et al.* 1997). NFκB activation, in turn, has been proposed to play a critical pro-inflammatory and pro-oxidant role in the suppression of endothelial-dependent dilation in aging (de Winther *et al.* 2005; Csiszar *et al.* 2008). Therefore, angptl2 stimulation of NFκB nuclear translocation *via* Rac1 activation in ECs may suggest one of a downstream pathway by which angptl2 may regulate endothelial function.

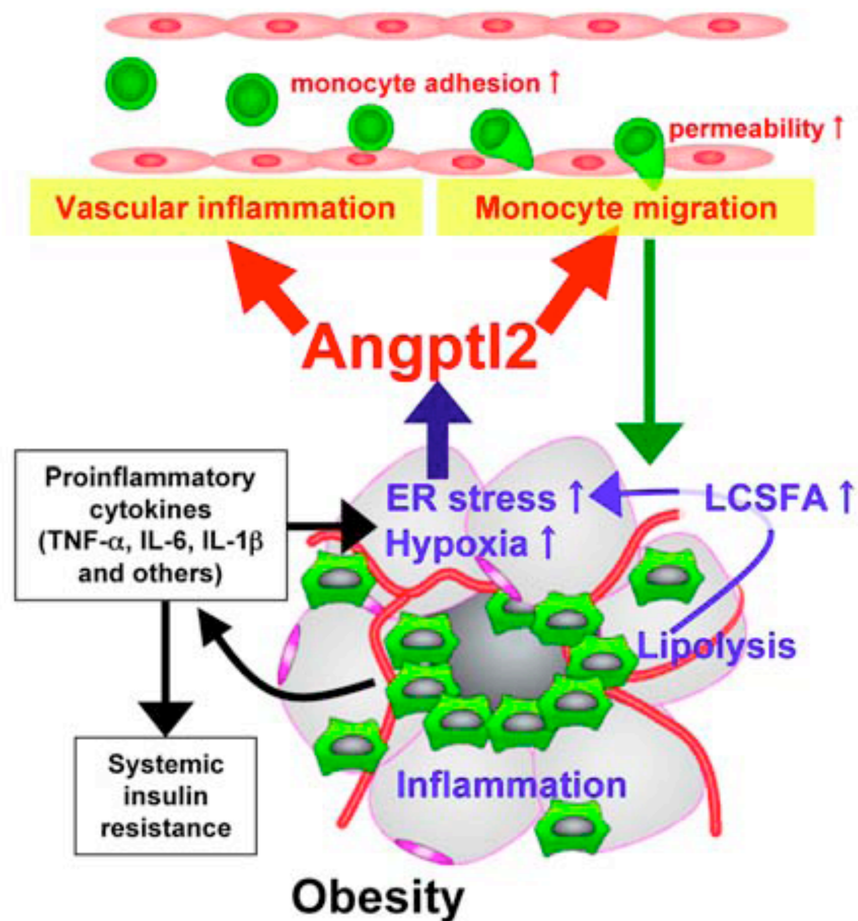

Figure 15. A proposed model of adipocyte-derived angptl2 contribution to inflammation, insulin resistance, and vascular dysfunctions. With weight gain and obesity, adipocyte size increases and oxygen supply decreases from surrounding blood vessels, which results in hypoxia and ER stress. This is also caused by an increase in long chain saturated fatty acid (LCSFA) within adipocytes. Together, they contribute in the induction of angptl2 expression. In turn, excess angptl2 causes vascular inflammation while circulating monocytes attach to the inflamed endothelium. Angptl2 also promotes monocyte migration into adipocytes, which in turn augment local inflammation and worsens systemic insulin resistance.

Reprinted with the permission from Elsevier: Cell Metab. Tabata M, Kadomatsu T, Fukuhara S, Miyata K, Ito Y, Endo M, Urano T, Zhu HJ, Tsukano H, Tazume H, Kaikita K, Miyashita K, Iwawaki T, Shimabukuro M, Sakaguchi K, Ito T, Nakagata N, Yamada T, Katagiri H, Kasuga M, Ando Y, Ogawa H, Mochizuki N, Itoh H, Suda T, Oike Y. Angiopoietin-like protein 2 promotes chronic adipose tissue inflammation and obesity-related systemic insulin resistance. *Cell Metab*. **10**, 178-188. Copyright (2009).

In the same study, Tabata *et al*. reported improved insulin signaling in peripheral tissues including the skeletal muscle in HFD-fed angptl2 KO compared to HFD-fed WT mice, as reflected by significantly increased tyrosine phosphorylation of the insulin receptor β and serine phosphorylation of Akt (Tabata *et al*. 2009). As insulin, besides mediating glucose uptake in the skeletal muscle, is also able to induce endothelial-dependent and NO-mediated vasodilation (Laakso *et al.* 1990; Dimmeler *et al.* 1999; Wheatcroft *et al.* 2003), high angptl2 expression, as in HFD-fed WT mice (Tabata *et al*. 2009), may lead to blunted insulin-mediated endothelial-dependent dilation. Thus, by extrapolation, there may be a possible link between angptl2 and endothelial dysfunction.

Another piece of indirect evidence proposing a link between angptl2 and endothelial dysfunction was presented by our laboratory (Farhat *et al*. 2013). In this study focusing on the role of angptl2 in atherogenesis, it was first demonstrated that acute stimulation with recombinant angptl2 induced leukocyte adhesion in native ECs isolated from atherosclerotic dyslipidemic mice but not WT mice (Farhat *et al* 2013). Chronic infusion of recombinant angptl2 in these mice for 4 weeks increased gene expressions of inflammatory TNFα and IL-6 in freshly isolated aortic ECs (Farhat *et al*. 2013). In addition, it was shown that angptl2 was mainly produced by ECs and not VSMCs (Farhat *et al*. 2013). Again, endothelial function was not directly assessed in this study, but there has been strong evidence tying inflammation and endothelial dysfunction together (Faraci 2005; Forstermann and Munzel 2006; Harrison *et al.* 2006; Rask-Madsen and King 2007). Thus, the pro-inflammatory role of angptl2 in ECs could potentially contribute to endothelial dysfunction.

Evidence directly linking angptl2 and endothelial dysfunction was presented by Horio *et al*. recently (Horio *et al*. 2014). Transgenic mice expressing endothelial angptl2 driven by the tie-2 promoter exhibited endothelial dysfunction possibly as a result of decreased NO production (Horio *et al*. 2014). This was demonstrated by diminished ACh-mediated vasodilation in the aorta in the transgenic compared to WT mice, which was associated with lower levels of phosphorylated eNOS (Ser1177) relative to total eNOS (Horio *et al*. 2014). On the other hand, HFD-fed angptl2 deficient mice showed less severe endothelial dysfunction in the aorta than WT mice, which was associated with

higher levels of phosphorylated eNOS (Ser1177) relative to total eNOS (Horio *et al*. 2014), suggesting a role of angptl2 in endothelial function regulation, at least in the aorta.

Taken together, a few studies have indirectly examined the role of angptl2 in the regulation of endothelial function, but to date, only 1 study has directly shown angptl2 involvement in endothelial dysfunction in the aorta (Horio *et al*. 2014). Coupled with findings that angptl2 is produced by ECs (Farhat *et al*. 2013), it is highly possible that this pro-inflammatory protein could contribute to endothelial dysfunction, which may extend to other vascular beds besides the aorta, and is the main question of the work herein.

1.3.5. Reported molecular pathways associated with angptl2

The recently identified angptl2 is truly a protein with diverse roles both physiologically and pathophysiologically (Kadomatsu *et al.* 2014), and some of the molecular pathways that are associated with angptl2 have been reported.

Angptl2 binds to integrin α5β1 and activates NFκB nuclear translocation and IκB degradation (Tabata *et al*. 2009), which leads to NFκB-mediated gene expression of inflammatory cytokines such as TNFα and IL-6 in ECs (Tabata *et al.* 2009; Horio *et al.* 2014), keratinocytes (Ogata *et al.* 2012), synoviocytes (Okada *et al.* 2010), adipocytes (Tabata *et al.* 2009), as well as cancer cells (Odagiri *et al.* 2014). This particular NFκB-dependent pathway has also been demonstrated in ECs to induce expressions of ICAM-1 and VCAM-1 following stimulation with angptl2 (Tabata *et al.* 2009; Horio *et al.* 2014), which are known adhesion molecules involved in monocyte adhesion (Libby *et al.* 2002), suggesting angptl2 involvement in atherogenesis (Farhat *et al.* 2013; Horio *et al.* 2014). In aortic aneurysms, angptl2 increased MMP-9 expression in wall tissues and contributed to ECM degradation (Tazume *et al.* 2012), which was likely mediated by the NFκB signaling cascade as well (Tazume *et al.* 2012), as NFκB has been reported regulate MMP-9 transcription (Bond *et al.* 1998).

Angptl2 also induces oxidative stress in the setting of carcinogenesis in skin tissues (Aoi *et al.* 2014). Likely, angptl2 activates the NFκB pro-inflammatory signaling pathway to activate infiltrated macrophages and neutrophils (Aoi *et al.* 2014), which act as sources of ROS (Klaunig and Kamendulis 2004). In turn, ROS may promote

phosphorylation of transcription factors c-Jun and activated transcription factor 2 (ATF2) leading to increased target gene expression (Klaunig *et al.* 2011), including inflammatory genes such as IL-6 (Reimold *et al.* 2001) as well as angptl2 (Endo *et al.* 2012), suggesting a possible regulatory mechanism of angptl2 transcription (Aoi *et al.* 2014).

As well in cancer settings, angptl2 was reported to activate the TGFβ-Smad pathway (Aoi *et al.* 2011), which has been shown to play a critical role in promoting epithelial-to-mesenchymal transition (Massague 2008; Ikushima and Miyazono 2010). In particular, angptl2 overexpression was associated with increased gene expressions of TGFβ1, TGFβ2, and their respective receptors, as well as increased phosphorylation of their effector, Smad2 in squamous cell carcinoma (Aoi *et al.* 2011). Activation of the same TGFβ-Smad pathway by angptl2 was also reported in fibroblasts, which likely contributed to hypertrophy in ligamentum flavum and up-regulation of collagen expression, accelerating lumbar spinal canal stenosis development (Nakamura *et al.* 2014).

Angptl2 was reported to possess anti-apoptotic activities through the PI3K/Akt pathway in ECs (Kubota *et al.* 2005). Stimulation with angptl2 in HUVECs led to phosphorylation of ERK1/2 and Akt but no effects on p38 MAPK and JNK (Kubota *et al.* 2005). On the other hand, knocking down angptl2 with siRNA in adipocytes inhibited insulin-mediated phosphorylation of Akt, FoxO1 and AS160 (Kitazawa *et al.* 2011), again suggesting angptl2 regulation of Akt activity. Additionally, angptl2 siRNA treatment increased mRNA expression of Trib3 (Kitazawa *et al.* 2011), a specific Akt inhibitory protein (Du *et al.* 2003).

Taken together, inflammation, pathological tissue remodeling, and oxidative stress are likely results of molecular pathways downstream of angptl2. In most cases, NFκB seems to be the transcription factor with a key role regulating target proteins downstream of angptl2 in various cell types.

1.3.6. Reported mechanisms regulating angptl2 transcription and expression

The regulation of angptl2 transcription and expression has been investigated in a few recent studies, some of which will be briefly discussed here. In *in vitro* settings, Lee

et al., using murine 3T3-L1 pre- and differentiated adipocytes, as well as macrophages, reported that TGF-β1, a multifunctional cytokine, induced angptl2 expression (Lee *et al.* 2013). Using sequence analysis, the authors found a functional Smad binding element in the angptl2 promoter region (Lee *et al.* 2013). Furthermore, regulation of angptl2 by TGF-β1 depended specifically on the Smad3 protein, a member of the Smad protein family and downstream of the TGF-β1 transduction pathway (Lee *et al.* 2013). Of note, angptl2 promoted epithelial-to-mesenchymal transition in cancer cells (Aoi *et al.* 2011) and ligamentum flavum tissue degeneration in fibroblasts (Nakamura *et al.* 2014) *via* the activation of the same TGFβ-Smad pathway, which may suggest feedback mechanisms between angptl2 and the TGFβ-Smad signaling pathway.

In another study, TNFα induced angptl2 expression in fully matured 3T3-L1 adipocytes likely through activation of transcription factor FoxO1 (Zheng *et al.* 2011). Interestingly, *in vitro* DNA-binding and *in vivo* chromatin immunoprecipitation assays showed a site in the angptl2 promoter for FoxO1 direct binding (Zheng *et al.* 2011). On the other hand, insulin seemed to suppress angptl2 gene expression (Zheng *et al.* 2011), which is in accordance with the findings that insulin inhibits FoxO1 (Nakae *et al.* 2002). Besides insulin, it was reported that inflammatory cytokines including TNFα and interferon γ (IFNγ) also suppressed angptl2 gene expression in cultured mesangial cells (Mederle *et al.* 2013). This was opposite of what was found in adipoctyes (Zheng *et al.* 2011), and may be attributed to the different cell types used.

In addition, an ATF/CREB site was also found in the angptl2 promoter region in cancer cells (Endo *et al.* 2012), which was proposed to be a binding site for the ATF2/c-Jun complex in enhancing NFAT-dependent angptl2 induction (Endo *et al.* 2012). The NFAT nuclear factors have been implicated in the pathogenesis of tumour (Mancini and Toker 2009) and further supporting this, NFAT induction of angptl2 was also observed in ECs (Horio *et al.* 2014) and ligamentum flavum fibroblasts (Nakamura *et al.* 2014). Consistent with these findings, the ATF/CREB binding proteins and/or calcineurin/NFAT pathway have been associated with advanced tumour development (van Dam and Castellazzi 2001; Mancini and Toker 2009). In the same study by Endo *et al*., a putative binding site for NFκB in the angptl2 promoter region in cancer cells was also reported (Endo *et al.* 2012), which may again suggest feedback mechanisms between NFκB and

angptl2, with NFκB promoting angptl2 transcription while angptl2 induces the NFκB pathway, as previously reported (Tabata *et al.* 2009; Horio *et al.* 2014).

Another reported regulatory mechanism of angptl2 transcription is the circadian regulation by the CLOCK and BMAL1 genes, which regulate angptl2 promoter activities (Kadomatsu *et al.* 2013), and disruption of its circadian expression was proposed to lead to diseases (Kitazawa *et al.* 2011). Besides this, other stresses, such as UV light (Aoi *et al.* 2011; Ogata *et al.* 2012; Aoi *et al.* 2014), ER stress and hypoxia (Tabata *et al.* 2009; Endo *et al.* 2012), a HFD (Tabata *et al.* 2009), smoking (Farhat *et al.* 2008), oxidative stress (Aoi *et al*. 2011) and mechanical stress (Nakamura *et al.* 2014) have all been reported to induce angptl2 expression in different cell types.

In summary, expression of angptl2 is regulated by a number of signaling pathways and conditions, most of which involve inflammation. A schematic representation of some of the known pathways that induce angptl2, and ones that are induced by angptl2, is shown in Figure 16.

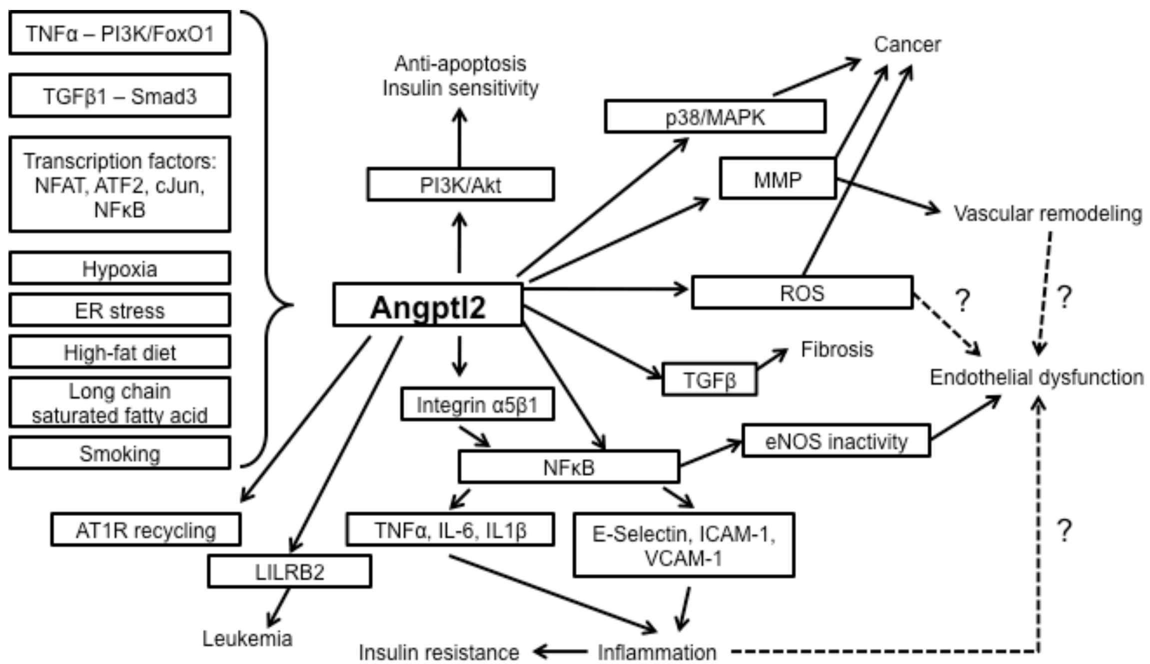

Figure 16. Schematic representation of a selection of reported pathways that induce angptl2 expression and the pathways that are in turn induced by angptl2. The potential links between angptl2 and endothelial dysfunction are questioned.

2. Research Overview

The endothelium, although consists of only a simple monolayer of cells, is critical in the maintenance of vascular homeostasis through secretion of multiple mediators. As the endothelium is optimally situated at the interface of circulating blood and the vascular vessel wall, it can respond to a vast spectrum of stimuli, either physical or chemical, and regulate permeability, vascular tone, inflammation and cellular adhesion, and VSMC proliferation. Also because of its geographical location, the endothelium is easily subjected to external stress stimuli resulting in increased oxidative stress and inflammation, leading to endothelial dysfunction, an obligatory first step towards CVD. With increasing biological age, vascular aging concurrently takes place, whereby the function of the endothelium declines, as reflected by decreased NO bioavailability and alteration of vasodilatory pathways (Thorin and Thorin-Trescases 2009). This process can be accelerated in the presence of risk factors such as obesity and related dyslipidemia and metabolic disorders, hypertension, and smoking, which further favor oxidative stress production and endothelial dysfunction. Initially at early manifestation when dysfunction is still reversible, the endothelium is able to adapt and maintain vascular tone; however, at a certain point, the damage becomes irreversible and CVD occurs. Thus, endothelial function can be a clinical biomarker to identify patients at risk of future CVD. Despite that, however, the benefits of individual endothelial function measurement still do not outweigh the costs and there still needs to be better biomarkers to distinguish endothelial dysfunction at an early, reversible stage.

The recently identified pro-inflammatory and pro-oxidative angptl2 has been implicated in a number of chronic inflammatory disorders (Kadomatsu *et al.* 2014) and its circulating levels have been positively correlated to increased CVD risks (Tabata *et al.* 2009; Doi *et al.* 2012; Usui *et al.* 2013). As there is only one recent study providing direct evidence that increased angptl2 could result in endothelial dysfunction associated with lower eNOS function, better characterization of angptl2 function in regulating endothelial function as a result of various EDRFs is clearly warranted. The general purpose of this work is, therefore, to determine the effect of angptl2 in endothelial function, and, whether angptl2 knock-down could protect against premature vasculature aging induced by various risk factors in a mouse model.

2.1. Study #1: Does angptl2 knock-down protect against obesity-induced endothelial dysfunction?

2.1.1. Background

Obesity, often caused by excess caloric intake, is a well established cardiovascular risk factor that contributes majorly to endothelial dysfunction (Xu and Zou 2009). In 2009, Dr. Oike's group identified adipocyte-derived angptl2 as a key inflammatory mediator underlying the pathogenesis of obesity-induced insulin resistance (Tabata *et al.* 2009). Consistent with this, our laboratory also recently reported that EC-derived angptl2 exerted pro-inflammatory effects in mediating atherogenesis in mice, as well as heightened circulating angptl2 levels in CAD patients (Farhat *et al.* 2013). Altogether, previous findings suggest that high angptl2 expression could lead to endothelial dysfunction.

2.1.2. Hypothesis

The knock-down of angptl2 could protect against obesity-induced endothelial dysfunction in mice.

2.1.3. Specific aims

1) To determine the acute effects of exogenous recombinant angptl2 protein on endothelial function in the femoral artery isolated from mice.
2) To generate a global angptl2 KD mouse and to characterize its endothelial function by dissecting out contribution of various EDRFs to ACh-induced vasodilation in the femoral artery, compared to WT littermates.
3) To compare and evaluate endothelial function in different vascular beds, namely the small resistance mesenteric artery and the larger conductance femoral artery, in KD and WT mice fed with a 3-month HFD or regular chow.
4) To evaluate the metabolic profile, their inflammatory and metabolic gene expressions in the liver, skeletal muscle, and adipose tissues from these mice treated with a HFD.

2.2. Study #2: Does angptl2 knock-down protect against angII-induced endothelial dysfunction?

2.2.1. Background

Given the pro-inflammatory and pro-oxidative role of angptl2 (Kadomatsu *et al.* 2014), and the data generated from the first study, it is clear that angptl2 is involved in the regulation of endothelial function. Little is known, however, if angptl2 could have an impact on the cerebral vascular endothelium. Cerebral arteries are classified as resistance arteries and are highly sensitive to oxidative stress (Chrissobolis *et al.* 2011).

2.2.2. Hypothesis

Knock-down of angptl2 could protect against pro-inflammatory and pro-oxidative effects of angII and thus angII-induced endothelial dysfunction in the cerebral artery.

2.2.3. Specific aims

1) To evaluate and compare cerebral endothelial function and the relative contribution of EDRFs between WT and angptl2 KD mice.
2) To evaluate and compare cerebral endothelial function between WT and KD mice with or without a chronic infusion of angII.
3) To determine the pathways involved in angII-induced endothelial dysfunction in these mice.

3. Articles

3.1. Article 1

Title: Lack of angiopoietin-like-2 expression limits the metabolic stress induced by a high-fat diet and maintains endothelial function in mice.

This article was accepted for publication on the 9th of June, 2014 and published in the *Journal of American Heart Association* on the 15th of August, 2014.

3.1.1. Contribution of co-authors

Carol Yu: Conceived, designed and performed the experiments, analyzed the data, interpreted the results, prepared the figures, wrote the manuscript and approved the final version of the manuscript.

Xiaoyan Luo: Performed the experiments (mesenteric arteries), interpreted the results, prepared the figures and approved the final version of the manuscript.

Nada Farhat: Generated the angptl2 knock-down mouse model, prepared the recombinant angptl2 protein and approved the final version of the manuscript.

Caroline Daneault: Measured mouse liver TG levels and approved the final version of the manuscript.

Natacha Duquette: Performed the tail-cuff experiments in mice and approved the final version of the manuscript.

Cécile Martel: Prepared the recombinant angptl2 protein, measured LPL activities in mouse liver, edited the manuscript and approved the final version of the manuscript.

Jean Lambert: Performed statistical analysis of data and approved the final version of the manuscript.

Nathalie Thorin-Trescases: Conceived and designed the experiments, interpreted the results, edited the manuscript and approved the final version of the manuscript.

Christine Des Rosiers: Provided expertise in the metabolic aspect of the study and approved the final version of the manuscript.

Eric Thorin: Conceived and designed the experiments, analyzed the data, interpreted the results, edited the manuscript and approved the final version of the manuscript.

Journal of the American Heart Association
OPEN ACCESS

American Heart Association | American Stroke Association

Lack of Angiopoietin−Like−2 Expression Limits the Metabolic Stress Induced by a High−Fat Diet and Maintains Endothelial Function in Mice

Carol Yu, Xiaoyan Luo, Nada Farhat, Caroline Daneault, Natacha Duquette, Cécile Martel, Jean Lambert, Nathalie Thorin-Trescases, Christine Des Rosiers and Éric Thorin

J Am Heart Assoc. 2014;3:e001024; originally published August 15, 2014;
doi: 10.1161/JAHA.114.001024
The *Journal of the American Heart Association* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Online ISSN: 2047-9980

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://jaha.ahajournals.org/content/3/4/e001024

Subscriptions, Permissions, and Reprints: The *Journal of the American Heart Association* is an online only Open Access publication. Visit the Journal at http://jaha.ahajournals.org for more information.

Downloaded from http://jaha.ahajournals.org/ at Institut de Cardiologie de Montreal on November 11, 2014

American Heart Association | American Stroke Association

Lack of Angiopoietin-Like-2 Expression Limits the Metabolic Stress Induced by a High-Fat Diet and Maintains Endothelial Function in Mice

Carol Yu, MSc; Xiaoyan Luo, MSc; Nada Farhat, PhD;* Caroline Daneault, MSc; Natacha Duquette, BSc; Cécile Martel, PhD; Jean Lambert, PhD; Nathalie Thorin-Trescases, PhD; Christine Des Rosiers, PhD; Éric Thorin, PhD

***Background*—**Angiopoietin-like-2 (angptl2) is produced by several cell types including endothelial cells, adipocytes and macrophages, and contributes to the inflammatory process in cardiovascular diseases. We hypothesized that angptl2 impairs endothelial function, and that lowering angptl2 levels protects the endothelium against high-fat diet (HFD)-induced fat accumulation and hypercholesterolemia.

***Methods and Results*—**Acute recombinant angptl2 reduced ($P<0.05$) acetylcholine-mediated vasodilation of isolated wild-type (WT) mouse femoral artery, an effect reversed ($P<0.05$) by the antioxidant *N*-acetylcysteine. Accordingly, in angptl2 knockdown (KD) mice, ACh-mediated endothelium-dependent vasodilation was greater ($P<0.05$) than in WT mice. In arteries from KD mice, prostacyclin contributed to the overall dilation unlike in WT mice. After a 3-month HFD, overall vasodilation was not altered, but dissecting out the endothelial intrinsic pathways revealed that NO production was reduced in arteries isolated from HFD-fed WT mice ($P<0.05$), while NO release was maintained in KD mice. Similarly, endothelium-derived hyperpolarizing factor (EDHF) was preserved in mesenteric arteries from HFD-fed KD mice but not in those from WT mice. Finally, the HFD increased ($P<0.05$) total cholesterol–to–high-density lipoprotein ratios, low-density lipoprotein–to–high-density lipoprotein ratios, and leptin levels in WT mice only, while glycemia remained similar in the 2 strains. KD mice displayed less triglyceride accumulation in the liver ($P<0.05$ versus WT), and adipocyte diameters in mesenteric and epididymal white adipose tissues were smaller ($P<0.05$) in KD than in WT fed an HFD, while inflammatory gene expression increased ($P<0.05$) in the fat of WT mice only.

***Conclusions*—**Lack of angptl2 expression limits the metabolic stress induced by an HFD and maintains endothelial function in mice. **(*J Am Heart Assoc*. 2014;3:e001024 doi: 10.1161/JAHA.114.001024)**

Key Words: adipokines • endothelium-derived relaxing factors • inflammation • isolated arteries

Dietary imbalance is well known to cause obesity favoring with time, the development of insulin resistance, dyslipidemia, diabetes and ultimately atherosclerosis.[1] Altered levels of cholesterol, especially high low-density lipoprotein (LDL)–to–high-density lipoprotein (HDL) ratios, as well as heightened insulin levels in diabetes, promote inflammation and endothelial dysfunction, which are at the root of atherogenesis.[2] Recently, a member of the angiopoietin-like (angptl) family, angiopoietin-like-2 (angptl2), has been identified as 1 of the key inflammatory mediators that regulate obesity-related insulin sensitivity,[3] dyslipidemia, and atherogenesis.[4,5] Angptl2 seems to play a major pro-inflammatory role in a variety of pathologies, including atherosclerosis,[4,5] diabetes,[6] abdominal aortic aneurysm,[7] neointimal hyperplasia,[8] rheumatoid arthritis,[9] dermatomyositis,[10] and even tumor progression.[11] Increased angptl2 expression has been reported in endothelial cells from chronic atherosclerotic smokers,[12] while its circulating level correlates with adiposity,[3] C-reactive protein levels,[3] and tumor necrosis factor (TNF)α levels.[8] Little is known, however, about the role of angptl2 in lipid profiling and endothelial function. It was suggested that angptl2 replenishment could restore insulin sensitivity and improve lipid levels with decreased serum triglycerides (TGs) and free fatty acids (FFAs) in genetically diabetic (*db/db*) mice[13]; in contrast, another study in overweight subjects revealed lower circulating angptl2 levels with lifestyle intervention in association with changes in TG

From the Departments of Pharmacology and Surgery (C.Y., N.F., É.T.), Social and Preventive Medicine (J.L.), and Department of Nutrition (C.D.R.), Faculty of Medicine, Université de Montréal, Montreal, Quebec, Canada; Montreal Heart Institute, Research Center, Montreal, Quebec, Canada (C.Y., X.L., N.F., C.D., N.D., C.M., N.T.-T., C.D.R., É.T.).

*Dr Nada Farhat is currently located at Pharsight Corporation Canada, Montréal, Quebec, Canada.

Correspondence to: Carol Yu, MSc, Montreal Heart Institute, Research Center, 5000 rue Bélanger, Montreal, Quebec, Canada H1T 1C8.

Received April 16, 2014; accepted June 9, 2014.

© 2014 The Authors. Published on behalf of the American Heart Association, Inc., by Wiley Blackwell. This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.

Downloaded from http://jaha.ahajournals.org/ at Institut de Cardiologie de Montreal on November 11, 2014

metabolism.[14] Our recent study, which used infusion of recombinant angptl2 in preatherosclerotic young mice, further increased cholesterol and LDL in the plasma,[4] suggesting deleterious effects of angptl2 on lipid profiling. In contrast, no changes in lipid profiles of angptl2 knockout mice fed a high-fat diet (HFD) compared with wild-type (WT) mice were reported.[3,5] Taken together, there are discrepancies in recent literature regarding the role of angptl2 in lipid handling. In terms of endothelial function, a recent study demonstrated that endothelium-specific overexpression of angptl2 in mice led to vasodilatory dysfunction and vascular inflammation.[5] In the same study, it was also shown that in angptl2-deficient mice, HFD-induced endothelial dysfunction was ameliorated in the aorta, which was associated with greater expression levels of phospho–endothelial nitric oxide synthase (eNOS) compared with WT mice.[5] With the knowledge that endothelial cells express and produce angptl2[4,5,12] and that the endothelium is a dynamic and complex organ, with diverse phenotypes depending on vascular beds, it is important to better characterize the role of angptl2 in regulating endothelial function.

Healthy vascular function involves the balance of endothelium-derived relaxing factors (EDRFs) and contracting factors, while imbalance of these contributes to endothelial dysfunction. There are 3 major EDRFs—nitric oxide (NO),[15] prostacyclin (PGI_2),[16] and endothelium-derived hyperpolarizing factor (EDHF).[17] In conductance arteries, NO is the main EDRF, while in resistance arteries, the main contributor to dilation is EDHF.[18] Reduced endothelium-dependent vasodilation due to impairment of 1 of these EDRFs contributes to endothelial dysfunction,[19,20] which can ultimately lead to cardiovascular disease. We and others reported, however, that the expression of the EDHF pathway compensated for the decreased NO- and PGI_2-dependent vasodilatory contribution in femoral arteries from dyslipidemic mice[21] and carotid arteries from rabbits[22] but then deteriorated with age. In $eNOS^{-/-}$ mice, the loss of NO during acetylcholine (ACh)- and flow-mediated vasodilation is compensated by the expression of EDHF,[23,24] while the contribution of PGI_2 is increased.[25] Additional compensatory pathways such as increased contribution of dilatory H_2O_2 have also been reported in coronary arteries from patients with coronary artery disease, which may eventually contribute to the endothelial dysfunction associated with metabolic stress.[18] The vascular endothelium is therefore plastic and adapts to the metabolic environment up to a decompensation phase revealing its irreversible damage and dysfunction. Based on the pro-inflammatory[4,5] and pro-oxidative[26] properties of angptl2, we hypothesized that angptl2 modulates endothelial function and that lowering angptl2 levels protects the endothelium against HFD-induced fat accumulation and hypercholesterolemia.

To test our hypothesis, we examined EDRF contribution and lipid handling using our newly generated angptl2 knockdown (KD) mice, fed either a regular diet (RD) or an HFD, an established method to induce adiposity, metabolic stress, and endothelial dysfunction.[27] Our results suggest that lowering angptl2 is beneficial for the vascular endothelium by maintaining its respective EDRF contribution in conductance and resistance arteries, in addition to a more favorable lipid profile in KD mice fed an HFD. To the best of our knowledge, this is the first report of the impact of angptl2 in the contribution of the various EDRFs and their resistance against a stress induced by an HFD.

Materials and Methods

Animals

All animal experiments were performed in accordance with the "Guide for the Care and Use of Experimental Animals of the Canadian Council on Animal Care" and the "Guide for the Care and Use of Laboratory Animals" of the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the Montreal Heart Institute Ethics Committee (ET 2010-62-1). Generation of the angptl2 KD mouse model was achieved through a microinjection of a construct generated via retroviral gene trap vectors (developed at Texas A&M Institute for Genomic Medicine) performed in C57Bl/6J mice (Figure 1A) purchased from The Jackson Laboratory. A β-geo cassette was inserted between bp 5305 and 5390 of the *angptl2* gene. KD mice were subsequently bred at the Institute for Research in Immunology and Cancer (Montreal, Quebec, Canada). All mice used in this study were genotyped by PCR analysis of genomic DNA isolated from ear clips to select both KD and WT animals (see Table 1 for primer sequences). Negligible levels of angptl2 mRNA and protein levels were confirmed in various tissues (Figure 1B and 1C). In characterizing fasting plasma profile and endothelial function in mice at 3 to 4 months of age, $angptl2^{+/+}$ littermates were used as WT and no significant differences were observed between $angptl2^{+/+}$ littermates and C57Bl/6J WT mice. Subsequently, WT mice purchased from The Jackson Laboratory were used for the diet study. Male mice were used for all experiments. Mice were fed ad libitum either a regular diet (RD, 2018; Harlan Teklad Laboratories) or a high-fat diet (HFD, TD.88137; Harlan Teklad Laboratories), starting at 3 months until 6 months of age. Mice were kept under standard conditions (24°C; 12:12-hour light/dark cycle), and during the 3 months of diet treatment, blood pressure and heart rate were recorded weekly by using tail-cuff plethysmography (Kent Scientific Corporation), after training to limit stress, as previously described.[28] Mice were fasted 16 hours before sacrifice for

Downloaded from http://jaha.ahajournals.org/ at Institut de Cardiologie de Montreal on November 11, 2014

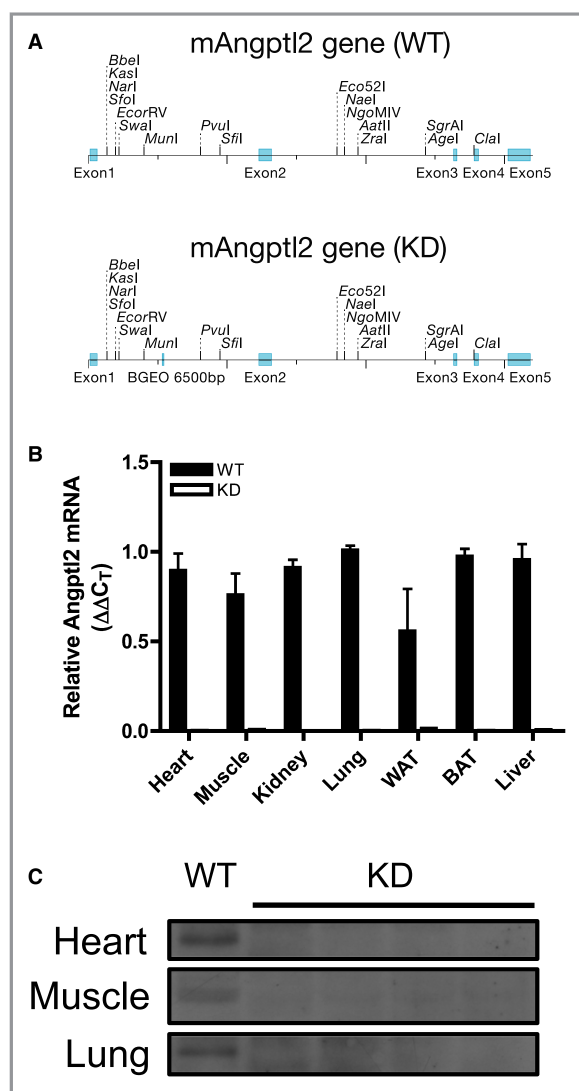

Figure 1. A, Schematic representation showing insertion of a promoterless trapping β-geo cassette of 6500 bp in size into the mouse angptl2 locus, downstream of exon 1; top and bottom representation show angptl2 wild-type (WT) and knock-down (KD) scheme, respectively. B, Verification of angptl2 knock-down in various mouse tissues by qPCR analysis; white adipose tissue (WAT), brown adipose tissue (BAT); n=3 to 4. C, Verification of angptl2 knock-down in various mouse tissues by Western blot. qPCR indicates quantitative polymerase chain reaction.

experiments by using terminal anesthesia (44 mg/kg ketamine and 2.2 mg/kg xylazine). Plasma and tissues (liver, adipose tissues, heart, soleus muscle) were collected and kept at −80°C, while the femoral artery and mesenteric arterial bed were harvested and placed in ice-cold physiological saline solution (pH 7.4, in mmol/L: NaCl 119, KCl 4.7, KH_2PO_4 1.18, $MgSO_4$ 1.17, $NaHCO_3$ 24.9, $CaCl_2$ 1.6, EDTA 0.023, and glucose 10) for endothelial function studies.[21,29] Liver and adipose tissue samples were immediately fixed in formaldehyde and paraffin embedded for subsequent hematoxylin-eosin (H&E) staining. A segment of the femoral artery was immediately embedded in OCT for subsequent dihydroethidium (DHE, D7008; Sigma Aldrich) staining.

Plasma Parameters

Plasma lipid profile (total cholesterol, LDL cholesterol, HDL cholesterol), glucose, and TG levels were measured at the Biochemistry Laboratory of the Montreal Heart Institute (Montreal, Quebec, Canada). Adiponectin (MRP300; R&D Systems), leptin (90030; Crystal Chem), FFAs (K612-100; BioVision), fasting insulin (80-INSMSU-E01; Alpco Diagnostics), and angptl2 (sE91919Mu; Uscn Life Science Inc) were quantified by using ELISA kits according to the manufacturers' protocol.

Western Blots

Proteins isolated from mouse tissues (heart, soleus muscle, and lung) in lysis buffer (50 mmol/L Tris-HCl, pH 7.45, 5 mmol/L EDTA, 10 mmol/L EGTA, 1% v/v Triton) were subjected to SDS-PAGE followed by Western blotting to detect angptl2 (1:200, AF2084; R&D Systems).

Endothelial Function of the Femoral Artery According to Pressurized Arteriography

Segments of 2 to 3 mm of the left or right gracilis artery were dissected in ice-cold physiological saline solution; surrounding fat and tissues were removed, after which the segment was cannulated at both ends (average internal diameter=283±3 μm; 165 segments) and pressurized at 80 mm Hg under no-flow conditions in a pressurized arteriograph (Living Systems Instrumentation) as described previously.[21] The artery segment was aerated with 12% O_2/5% CO_2/83% N_2 and equilibrated at 37°C for 45 minutes before the addition of phenylephrine (PE, 1 to 3 μmol/L) to obtain preconstriction of 30% to 50% of maximal diameter and single cumulative concentration-response curves to ACh (1 nmol/L to 30 μmol/L). The acute effects (1 hour) of angptl2-Glutathione S-transferase (GST) (50 nmol/L) on ACh-induced dilation, combined or not with the antioxidant *N*-acetylcysteine (NAC; 10 μmol/L), were assessed and compared with exposure to an equivalent aliquot of the last dialysis bath used for purification of the recombinant protein (Tris-buffered Saline EDTA [TBSE]; 50 mmol/L Tris-base, 150 mmol/L NaCl, 1 mmol/L EDTA). Recombinant angptl2-GST protein was produced as detailed previously.[4] For other studies of endothelial function, N^{ω}-nitro-L-arginine (LNNA, 100 μmol/L), indomethacin (Indo, 10 μmol/L), or the combination of both drugs was placed in the bath throughout equilibration and experiment, to inhibit NOS or cyclooxygenase or to reveal

Downloaded from http://jaha.ahajournals.org/ at Institut de Cardiologie de Montreal on November 11, 2014

Table 1. Primer Sequences Used in Quantitative RT-PCR

Gene	Forward Sequence (5′ to 3′)	Reverse Sequence (5′ to 3′)
Angplt2	GATCCAGAGTGACCAGAATC	TCTCAGGCTTCACCAGGTAG
Angptl2 V76	CTTGCAAAATGGCGTTACTTAAGC	CCAATAAACCCTCTTGCAGTTGC
TNFα	TGATCCGCGACGTGGAACTGG	CGACGTGGGCTACAGGCTTGTCA
IL-6	CCATAGCTACCTGGAGTACATGA	GTCCTTAGCCACTCCTTCTGTGA
TGFβ	ATTCCTGGCGTTACCTTGG	CCTGTATTCCGTCTCCTTGG
LPL	GGCTCTGCCTGAGTTGTAGAA	TCACTCGGATCCTCTCGATGA
Adiponectin	GTCAGTGGATCTGACGACACCAA	ATGCCTGCCATCCAACCTG
Leptin	CAGGATCAATGACATTTCACACAC	CTGGTCCATCTTGGACAAACTC
Cyclophilin A	CCGATGACGAGCCCTTGG	GCCGCCAGTGCCATTATG
HSL	GGCACAGACCTCTAAATCCC	CCGCTCTCCAGTTGAACC
SREBP1c	GAACAGACACTGGCCGAGATG	GAGGCCAGAGAAGCAGAAGAGAAG
SREBP2	GTTCTGGAGACCATGGAG	AAACAAATCAGGGAACTCTC
Angptl3	AGCACCAAGAACTACTCCCC	ATAAACGGCAGAGCAGTCGG
Angptl4	TCCGTGGGGACCTTAACTGT	GTAGCGGCCCTTCCATGTTT
Citrate synthase	GCCAGTGCTTCTTCCACGAAT	CATGCCACCGTACATCATGTC
Cyp7a1	AACGATACACTCTCCACCTTTG	CTGCTTTCATTGCTTCAGGG
PPARα	CTATTCGGCTGAAGCTGGTGTA	CAGGTCGTGTTCACAGGTAAGA
HMG-CoA reductase	AGTACATTCTGGGTATTGCTGG	ACTCGCTCTAGAAAGGTCAATC
CD36	GGCCAAGCTATTGCGACATGA	CAGATCCGAACACAGCGTAGA
FXR	TGGAGAACTCAAAATGACTCAGG	CTTTTGTAGCACATCAAGCAGG
Sirtuin-1	ATCCAGCTCAGGTGGAGGAAT	TTGACCGATGGACTCCTCACT
PPARγ	CCTGAAGCTCCAAGAATACC	GGTTCTTCATGAGGCCTGTT

FXR indicates farnesoid X receptor; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme-A; HSL, hormone-sensitive lipase; LPL, lipoprotein lipase; PPAR, peroxisome proliferator-activated receptor; SREBP, sterol regulatory element binding protein; TGF, transforming growth factor; TNF, tumor necrosis factor; IL, interleukin.

EDHF, respectively. To study vascular smooth muscle cell function, the endothelium was removed by passing an air bubble through the lumen of the artery and confirmed by loss of response to ACh. After incubation, PE (0.1 nmol/L to 30 μmol/L) was used to preconstrict the artery, followed by single cumulative concentration-response curves to sodium nitroprusside (0.1 nmol/L to 30 μmol/L).

Fluorescence Studies

Isolated femoral artery segments of 2 mm from 6-month-old WT or angptl2 KD mice fed with either an RD or HFD were incubated in oxygenated physiological saline solution at 37°C along with 10 μmol/L 4,5-diaminoflorescein diacetate (DAF-2, a fluorescent dye for NO detection[30]) with or without LNNA (100 μmol/L) for 30 minutes, after which the artery segment was washed 3 times with physiological saline solution, preconstricted with 3 μmol/L PE, and dilated with 1 μmol/L ACh. During the experiment, changes in fluorescence intensities reflecting NO production during vasodilation were measured, as previously described.[30]

Oxidative Stress Quantification in the Femoral Artery

Frozen femoral artery segments in OCT were cut into 7-μm-thick sections, and sections were double stained with 5 μmol/L DHE and 2 μmol/L To-Pro-3 (T3605; Molecular Probes), as previously described.[31] DHE fluorescence was visualized by using confocal microscopy (Zeiss LSM 510; Carl Zeiss; objective ×20) in which DHE was excited with the HeNe laser at 543 nm and resulting emitted light was collected between 565 and 615 nm, while acquisition settings were kept constant for all samples. ImageJ (National Institutes of Health) software was used to analyze DHE fluorescence intensities based on this equation: $I=\Sigma\ I/A$, where I is the DHE-fluorescence intensity, Σ I is the summation of To-Pro-3 nuclei stain intensities, and A is the total area of the nuclei.

Downloaded from http://jaha.ahajournals.org/ at Institut de Cardiologie de Montreal on November 11, 2014

Endothelial Function of Mesenteric Arteries According to Wire Myography

Mesenteric arteries (third-order branches) were dissected in ice-cold physiological saline solution. Segments of 2 mm in length were mounted on 20-μm tungsten wires in microvessel myographs (IMF, University of Vermont, Burlington) as previously described.[29] Mesenteric segments were first equilibrated for 30 to 45 minutes; then, their contractility was tested with a KCl–physiological saline solution (40 mmol/L), followed by 2 washout periods. They were further incubated for 30 to 45 minutes with or without LNNA (100 μmol/L), then preconstricted with a half-maximal effective concentration dose of thromboxane A_2 analog U46619 (0.1 to 10 nmol/L), followed by dose-response curves to ACh (0.1 nmol/L to 3 μmol/L). To study vascular smooth muscle cell function, we mechanically removed the endothelium by gentle rubbing with human hair and confirmed the removal by the loss of response to ACh. After incubation, single cumulative concentration-response curves to PE (0.1 nmol/L to 3 μmol/L), followed by single cumulative concentration-response curves to sodium nitroprusside (0.1 nmol/L to 30 μmol/L), were performed.

Quantification of TGs in Mouse Livers

FFAs from the liver were quantified as previously reported[32,33] including tissue lipid extraction[34] and separation into TG and phospholipid classes by using an aminoisopropyl column (Varian).[35] FFAs were *trans*-methylated according to a modified protocol previously described by Lepage and Roy.[36] Gas chromatography–mass spectometry was operated in chemical ionization mode using ammonia as the reagent gas and was internally validated. FFAs were identified according to their retention time and *m/z* ratio; concentrations were determined by using calibration curves with internal and external standards.

Adipocyte Size Analysis

Isolated epididymal and mesenteric adipose tissues embedded in paraffin sections for H&E staining were visualized by using light microscopy, and ImageJ was used to measure the mean diameter of 30 to 50 adipocytes. The average of 3 separate images was used to calculate mean adipocyte diameter.

Real-Time Quantitative Polymerase Chain Reaction

Total RNA was extracted from various tissues using the RNeasy mini-kit (Qiagen Canada) according to the manufacturer's protocol. Reverse transcription reaction (100 ng) was performed as previously described[4,37] using the Moloney murine leukemia virus reverse transcriptase (200 U; Invitrogen). Quantitative polymerase chain reaction (qPCR) was performed using the EvaGreen qPCR Mastermix (Mastermix-LR; Applied Biological Materials Inc). Primers of target genes were designed using the Clone Manager software (Table 1). The ΔΔCT method was used for analysis of relative gene expression using cyclophilin A as the housekeeping gene. For each pair of primers used, its optimal concentration, cDNA template concentration, and annealing temperature were optimized by performing standard curves that yielded efficiencies of 100±10%.

Statistical Analysis

Results are presented as mean±SEM, and n indicates number of mice. EC_{50} is the half-maximum effective concentration for each concentration-response curve, estimated by using Graph Pad Prism 5.0 software according to the variable slope sigmoidal dose-response curve formula:

$$Y = \text{Bottom} + ([\text{Top} - \text{Bottom}])/(1 + 10^{[\log EC_{50} - X]} \times \text{Hillslope})$$

where bottom is the Y value at the bottom plateau, top is the Y value at the top plateau, and Hillslope describes the steepness of the curve. E_{max} is the maximal ACh-induced dilation at the maximal dose tested. Normality tests were first performed for all groups using the d'Agostino–Pearson omnibus test. If sample sizes were normally distributed, parametric tests were performed: the unpaired Student *t* test was used to compare 2 groups and the 2-way ANOVA followed by Bonferroni posttest were performed for comparison of more than 2 groups. When groups did not follow a Gaussian distribution, nonparametric tests were performed: the Mann–Whitney U test was used to compare 2 groups and the Kruskal–Wallis test followed by the Dunn's posttest were used for comparison of more than 2 groups. When "n" was too low to test for normality, the z-score method (Y–mean/SD) was used for each individual datum (Y) followed by normality test using the d'Agostino–Pearson test. If the transformed data sets followed a normal distribution, parametric tests were used; if data sets did not follow a normal distribution, nonparametric tests were used. In all cases, $P<0.05$ was considered statistically significant.

Results

Acute Addition of Angptl2 Led to Impaired Endothelial Function in the Femoral Artery, Which Was Reversed by Addition of the Antioxidant NAC

The effect of an acute addition of angptl2 on endothelial function in the femoral artery was first investigated.

Downloaded from http://jaha.ahajournals.org/ at Institut de Cardiologie de Montreal on November 11, 2014

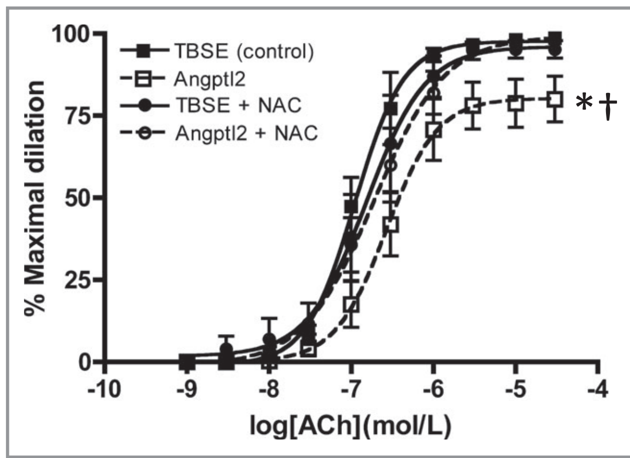

Figure 2. Vascular reactivity of pressurized femoral arteries measured by ACh-mediated dilation in 3- to 4-month-old WT (n=4 to 5) with addition of Tris-buffered saline EDTA (control) or angptl2-Glutathion S-transferase (50 nmol/L) with or without antioxidant NAC (10 µmol/L). The z-score method followed by the d'Agostino–Pearson omnibus test was used to test normality of data sets, after which the 1-way ANOVA followed by the Bonferonni posttest were used. *$P<0.05$ vs control; †$P<0.05$ vs + NAC. ACh indicates acetylcholine; Angptl2, angiopoietin-like-2; GST, Glutathion S-transferase; NAC, N-acetylcysteine; TBSE, Tris-buffered saline EDTA; WT, wild-type.

ACh-induced vasodilation in 3- to 4-month-old WT mice was significantly reduced after acute addition of 50 nmol/L angptl2-GST (E_{max} [%]: TBSE=99±1 and angptl2=80±7, $P<0.05$, n=4 to 5), as shown in Figure 2. Because angptl2 has been demonstrated to have pro-oxidative properties in cancer cells,[26] we next asked whether an antioxidant could reverse the detrimental effects of angptl2 on endothelial function. Indeed, endothelial dysfunction caused by the acute angptl2 addition was completely reversed by NAC (10 µmol/L; E_{max} [%]: angptl2=80±7 and angptl2+NAC=98±1, $P<0.05$, n=4 to 5) with no overall effects by NAC alone without angptl2 addition (E_{max} [%]: TBSE=99±1 and TBSE+NAC=95±3, n=4 to 5). These data suggest that angptl2 induces deleterious effects on endothelial function, at least partially via its pro-oxidative properties.

Phenotype of Angptl2 KD Mice

To further study the pathophysiological role of angptl2, we generated an angptl2 KD mouse model. Angptl2 KD mice were born alive following a Mendelian pattern and were grossly comparable to WT littermates. Verification of angptl2 depletion was determined by quantification by qPCR and Western blot (Figure 1B and 1C). Young mice from 3 to 4 months of age showed similar fasting plasma parameters, with higher (P=0.04; n=13) insulin levels in KD mice (Table 2) but with normal glucose and insulin tolerance (data not shown). Surprisingly, KD mice (n=28) showed significantly slower heart rate compared with WT mice (n=24) at this age, while systolic and diastolic blood pressures were similar for the 2 strains (Table 3).

Table 2. Parameters of Fasting Plasma in 3- to 4-Month-Old WT and Angptl2 KD Mice

	WT	KD
Weight, g	24.7±0.4 (10)	23.4±1.1 (8)
Glucose, mmol/L	10.7±1.4 (10)	13.4±0.9 (8)
Triglycerides, mmol/L	0.63±0.09 (10)	0.52±0.06 (8)
Adiponectin, µg/mL	7.3±0.2 (8)	7.4±0.4 (7)
Leptin, ng/mL	6.3±2.2 (8)	5.7±0.9 (8)
FFAs, mmol/L	0.55±0.05 (10)	0.51±0.06 (8)
Cholesterol—total, mmol/L	2.85±0.17 (10)	2.78±0.17 (8)
Cholesterol—HDL, mmol/L	2.65±0.13 (10)	2.60±0.13 (8)
Cholesterol—LDL, mmol/L	0.12±0.02 (6)	0.15±0.03 (5)
Cholesterol—total/HDL	1.08±0.01 (10)	1.06±0.01 (8)
LDL/HDL	0.04±0.01 (6)	0.05±0.01 (5)
Insulin, ng/mL	0.18±0.04 (13)	0.29±0.04 (13)‡

Data presented as mean±SEM of (n) mice. The Mann–Whitney U test was used. FFA indicates free fatty acid; HDL, high-density lipoprotein; KD, knockdown; LDL, low-density lipoprotein; WT, wild-type.
‡$P<0.05$ vs WT.

Angptl2 KD Mice Had Better Endothelial Function in the Femoral Artery as Measured by Vasodilation to ACh

Mice from 3 to 4 months of age were used to study vascular endothelial function in the femoral artery (Figure 3). Under control conditions (Figure 3A), the maximal dilation to ACh resulting from the combination of NO, PGI_2, and EDHF was slightly greater in KD (n=8) than in WT mice (n=7), as shown by the higher E_{max} and a tendency for a higher sensitivity indicated by their EC_{50} values (P=0.06, Table 4). Inhibition of

Table 3. Measurements of Heart Rate and Blood Pressures by Tail-Cuff Plethysmography in 3- to 4-Month-Old WT and Angptl2 KD Male Mice

	WT	KD
Heart rate, bpm	680±9 (24)	640±11 (28)‡
Systolic blood pressure, mm Hg	153±3 (24)	155±3 (28)
Diastolic blood pressure, mm Hg	122±3 (24)	119±3 (28)

Data presented as mean±SEM of (n) mice. Data sets were tested for normality using the d'Agostino–Pearson normality test and the unpaired Student t test was used.
KD indicates knockdown; WT, wild-type.
‡$P<0.05$ vs WT.

Downloaded from http://jaha.ahajournals.org/ at Institut de Cardiologie de Montreal on November 11, 2014

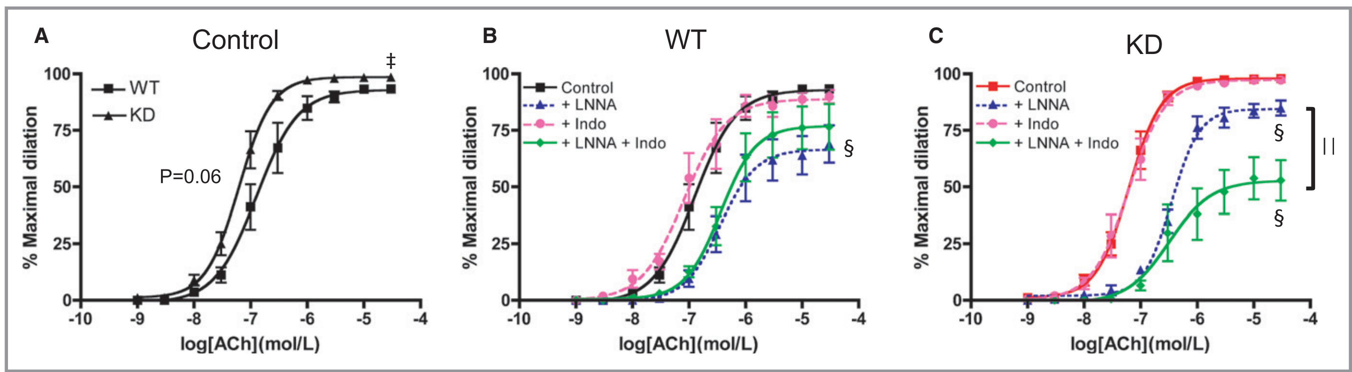

Figure 3. Vascular reactivity as measured by ACh-mediated dilation in femoral arteries of WT (n=7) and angptl2 KD (n=8) mice at 3 to 4 months of age in no-drug control condition (A) and in the presence of LNNA (100 μmol/L), indomethacin (Indo, 10 μmol/L), or their combination in arteries isolated from WT mice (B) and KD mice (C). The z-score method followed by the d'Agostino–Pearson omnibus test was used to test normality of data sets, after which the unpaired Student *t* test (A) and the 1-way ANOVA followed by the Bonferonni posttest were used (B and C). ‡$P<0.05$ vs WT; §$P<0.05$ vs control; ||$P<0.05$ vs +LNNA. ACh indicates acetylcholine; KD, knockdown; LNNA, N^{ω}-nitro-L-arginine; WT, wild-type.

NOS by LNNA reduced this response in arteries isolated from WT littermates and KD mice (Figure 3B and 3C, Table 4), while inhibition of cyclooxygenase with Indo had no impact on ACh-induced vasodilation (Figure 3B and 3C, Table 4). In the presence of LNNA and Indo, combined activities of NO and PGI_2 are abolished, revealing that of EDHF[38,39]; these experimental conditions revealed a lower EDHF contribution in KD mice (Figure 3B and 3C). In WT littermates, compared with LNNA alone, where both PGI_2 and EDHF are present, incubation with LNNA and Indo, where only EDHF is present, did not result in additional effects (Figure 3B, Table 4), suggesting that PGI_2 per se played no role in ACh-mediated dilation in WT littermates. In contrast, in KD mice, E_{max} decreased significantly in the presence of LNNA combined with Indo (Figure 3C, Table 4), suggesting that PGI_2 contributed significantly to ACh-induced femoral artery dilation in KD mice but not WT littermates in the absence of NO. As there are interactions and compensations among the 3 main EDRFs[40] under each individual experimental condition, PGI_2 contribution to vasodilation could only be revealed following NO inhibition in KD mice. Overall, these results suggest that angptl2 KD results in better endothelial function, which was associated with greater contribution of PGI_2 and less EDHF to vasodilation, at least in young and healthy mice.

Table 4. Efficacy (E_{max}) and Sensitivity (EC_{50}) to Acetylcholine in Femoral Arteries Isolated From 3- to 4-Month-Old WT and Angptl2 KD Male Mice

	WT		KD	
	E_{max} (%)	EC_{50}	E_{max} (%)	EC_{50}
Control	93±2 (7)	6.8±0.2 (7)	98±1 (8)‡	7.2±0.1 (8)
+LNNA	69±8 (7)§	6.3±0.2 (7)	85±3 (8)§	6.4±0.1 (8)§
+Indo	90±3 (7)	7.0±0.2 (7)	97±1 (8)‡	7.2±0.1 (8)
+LNNA +Indo	77±10 (7)	6.4±0.1(7)	53±9 (8)§‖	6.2±0.2 (8)§

Vessels were preconstricted to 30% to 50% of maximal diameter with phenylephrine (1 to 3 μmol/L). E_{max} are expressed as the percentage of the maximal diameter. Data presented as mean±SEM of (n) mice. The z-score method followed by the d'Agostino–Pearson omnibus test was used to test normality of data sets, after which the unpaired Student *t* test (‡) and the 1-way ANOVA followed by the Bonferonni posttest were used (§ and ‖). KD indicates knockdown; WT, wild-type; LNNA, N^{ω}-nitro-L-arginine; Indo, indomethacin.

‡$P<0.05$ vs WT; §$P<0.05$ vs control; ‖$P<0.05$ vs +LNNA.

Endothelial Function in the Femoral Artery Was Preserved in KD Mice Fed an HFD

At 6 months of age, when fed an RD, the sensitivity to ACh under no-drug control condition was, as similarly observed at 3 to 4 months of age, slightly greater in femoral arteries from KD (n=13) compared with WT (n=10) mice, as indicated by a shift to the left of the dose-response curve (Figure 4A and 4B) and by the higher EC_{50} values (Table 5). The efficacies (E_{max}) were identical in both strains (Figure 4, Table 5). For both KD and WT mice fed an RD diet, addition of LNNA to inhibit NOS significantly decreased E_{max} and EC_{50} values similarly (Figure 4, Table 5), suggesting that NO contributed equally for both strains of mice. When fed an HFD (n=9 to 10), both strains of mice displayed similar global responses to ACh, while LNNA significantly decreased the sensitivity of ACh-mediated dilation compared with no-drug condition in KD mice only. In WT mice, LNNA no longer had a significant inhibition on dilation, suggestive of a lower eNOS activity in WT mice fed an HFD. In KD mice, however, LNNA-sensitive ACh-mediated dilations were similar under either diet, suggesting that NO-dependent dilation sensitive to LNNA

Downloaded from http://jaha.ahajournals.org/ at Institut de Cardiologie de Montreal on November 11, 2014

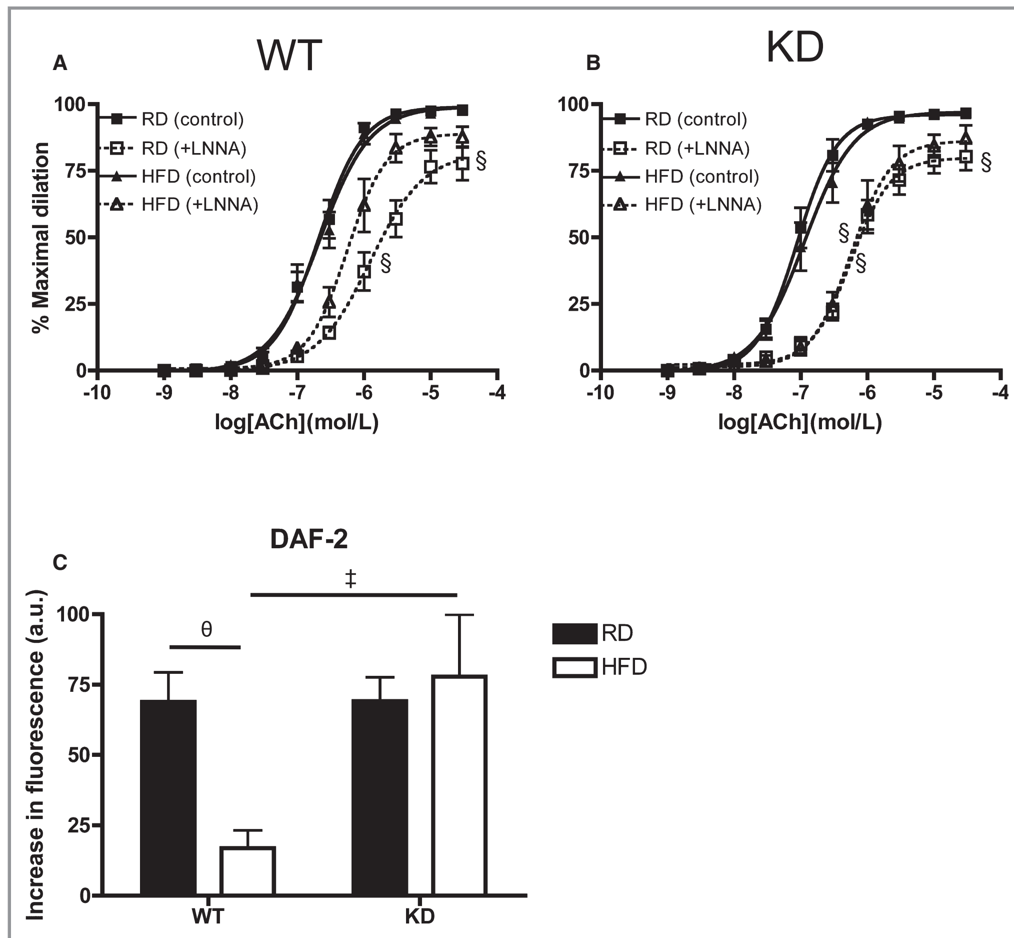

Figure 4. Vascular reactivity as measured by ACh-mediated dilation in femoral arteries under no-drug control condition and with LNNA of 6-month-old (A) WT (n=7 to 10) and (B) angptl2 KD (n=9 to 13) mice fed an RD or HFD. The Kruskal–Wallis test followed by the Dunn's posttest were used for data sets not normally distributed. §*P*<0.05 vs control. C, Production of NO was measured by loading femoral arteries with DAF-2 with average increases in fluorescence intensities during addition of 10 μmol/L ACh (n=6). The z-score method followed by the d'Agostino–Pearson omnibus test was used to test normality of data sets, after which the 2-way ANOVA followed by the Bonferonni posttest were used. ‡*P*<0.05 vs WT; θ*P*<0.05 vs RD. ACh indicates acetylcholine; DAF-2, 4,5-diaminoflorescein diacetate; HFD, high-fat diet; KD, knockdown; LNNA, N^{ω}-nitro-L-arginine; NO, nitric oxide; RD, regular diet; WT, wild-type.

Table 5. Efficacy (E_{max}) and Sensitivity (EC_{50}) to Acetylcholine in Femoral Arteries Isolated From 6-Month-Old WT and Angptl2 KD Male Mice Fed a Regular (RD) or High-Fat Diet (HFD)

	WT+RD		WT+HFD		KD+RD		KD+HFD	
	E_{max} (%)	EC_{50}	E_{max} (%)	EC_{50}	E_{max} (%)	EC_{50}	E_{max} (%)	EC_{50}
Control	98±1 (10)	6.7±0.1 (10)	98±1 (9)	6.7±0.1 (9)	97±1 (13)	7.0±0.1 (13)‡	96±1 (10)	6.9±0.1 (10)
+LNNA	78±6 (9)§	5.9±0.1 (9)§	88±4 (7)	6.3±0.1 (7)	80±5 (12)§	6.2±0.1 (12)§	87±5 (9)	6.2±0.1 (9)§
+Indo	92±6 (10)	6.8±0.2 (10)	96±2 (8)	6.8±0.1 (8)	97±1 (13)	6.9±0.1 (13)	96±1 (10)	7.0±0.1 (10)
+LNNA+Indo	74±7 (10)§	5.9±0.1 (10)§	81±7 (8)	6.2±0.1 (8)§	65±7 (13)§	5.9±0.2 (13)§	74±6 (9)	6.2±0.1 (9)§

Vessels were preconstricted to 30% to 50% of maximal diameter with phenylephrine (1 to 3 μmol/L). E_{max} are expressed as the percentage of the maximal diameter. Data presented as mean±SEM of (n) mice. The Kruskal–Wallis test followed by the Dunn's posttest were used. KD indicates knockdown; WT, wild-type; LNNA, N^{ω}-nitro-L-arginine; Indo, indomethacin. ‡*P*<0.05 vs WT+RD; §*P*<0.05 vs control (within respective groups).

Downloaded from http://jaha.ahajournals.org/ at Institut de Cardiologie de Montreal on November 11, 2014

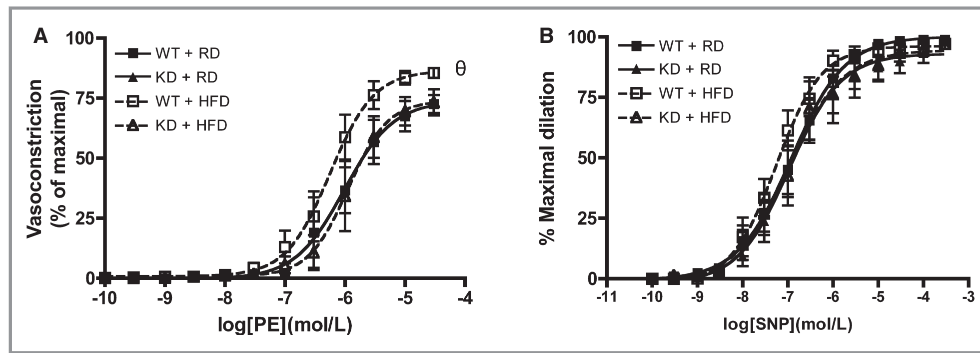

Figure 5. Vascular smooth muscle cell function was assessed in femoral arteries from WT and angptl2 KD mice, by (A) vasoconstriction to phenylephrine (PE), and (B) dilation to sodium nitroprusside (SNP) in the absence of the endothelium. Data are mean±SEM of n=4 to 6 mice, and compared using the Mann–Whitney *U* test. θ$P<0.05$ vs RD. KD indicates knockdown; RD, regular diet; WT, wild-type.

was preserved in KD mice. To further confirm preservation of NO in HFD-fed angptl2 KD mice compared with WT mice, we loaded femoral arteries with a fluorescent dye specific for NO production, DAF-2, as previously described.[30] In agreement with our functional data, a 3-month HFD significantly reduced ACh-induced changes in NO-associated fluorescence only in WT (n=6), while it was preserved in angptl2 KD mice (n=6) (Figure 4C). Artery preincubation with Indo alone or in combination with LNNA to reveal contribution of EDHF did not show differences in ACh-mediated dilation across groups (Table 5). Previously observed contribution of PGI_2 in KD mice was lost at 6 months of age under either diet regimen (Table 5). Furthermore, while vasoconstriction to PE was slightly but significantly greater in HFD-fed WT mice compared with all other groups of mice (Figure 5A), endothelium-independent dilation to sodium nitroprusside was similar across all groups of mice (Figure 5B). Taken together, these data suggest that in angptl2 KD mice treated with an HFD, eNOS-derived NO production is preserved in the femoral arteries.

In our experimental models, the HFD did not change DHE staining, a marker of oxidative stress, in femoral arteries from both strains of mice (Figure 6).

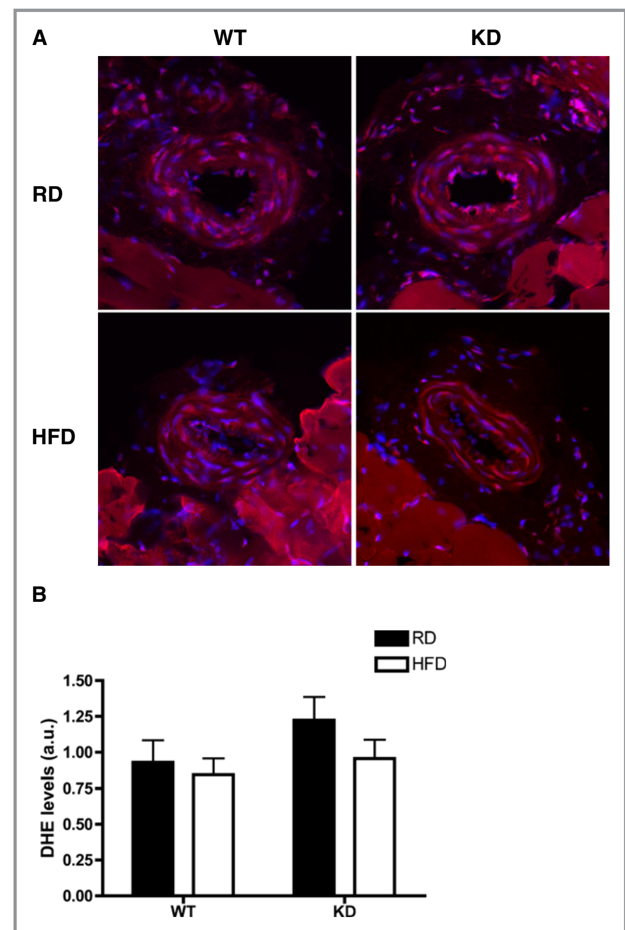

Figure 6. A, DHE staining in femoral arteries of WT and KD mice fed an RD or HFD, and (B) quantifications of DHE intensities in femoral arteries; n=3 to 5. DHE indicates dihydroethidium; HFD, high-fat diet; KD, knockdown; RD, regular diet; WT, wild-type.

An HFD Recruited a Compensatory NO Pathway in the Mesenteric Arteries of WT, Which Was Absent in KD mice

In mesenteric arteries, global endothelial function measured by ACh-mediated vasorelaxation was similar for the 2 strains of mice (Figure 7A). After an HFD, global endothelial function remained similar as well (Figure 7B). Interestingly, deciphering out contribution of NO using LNNA showed that under the RD regimen, there was no significant functional consequence of inhibiting NO with LNNA in WT mice (n=6; Figure 7C), confirming, as reported previously,[41] that in these mesenteric resistance arteries, EDHF is most likely the main vasodilator. When fed an HFD, however, LNNA significantly reduced ACh-induced relaxation (Figure 7D, Table 6) in WT mice (n=6),

Downloaded from http://jaha.ahajournals.org/ at Institut de Cardiologie de Montreal on November 11, 2014

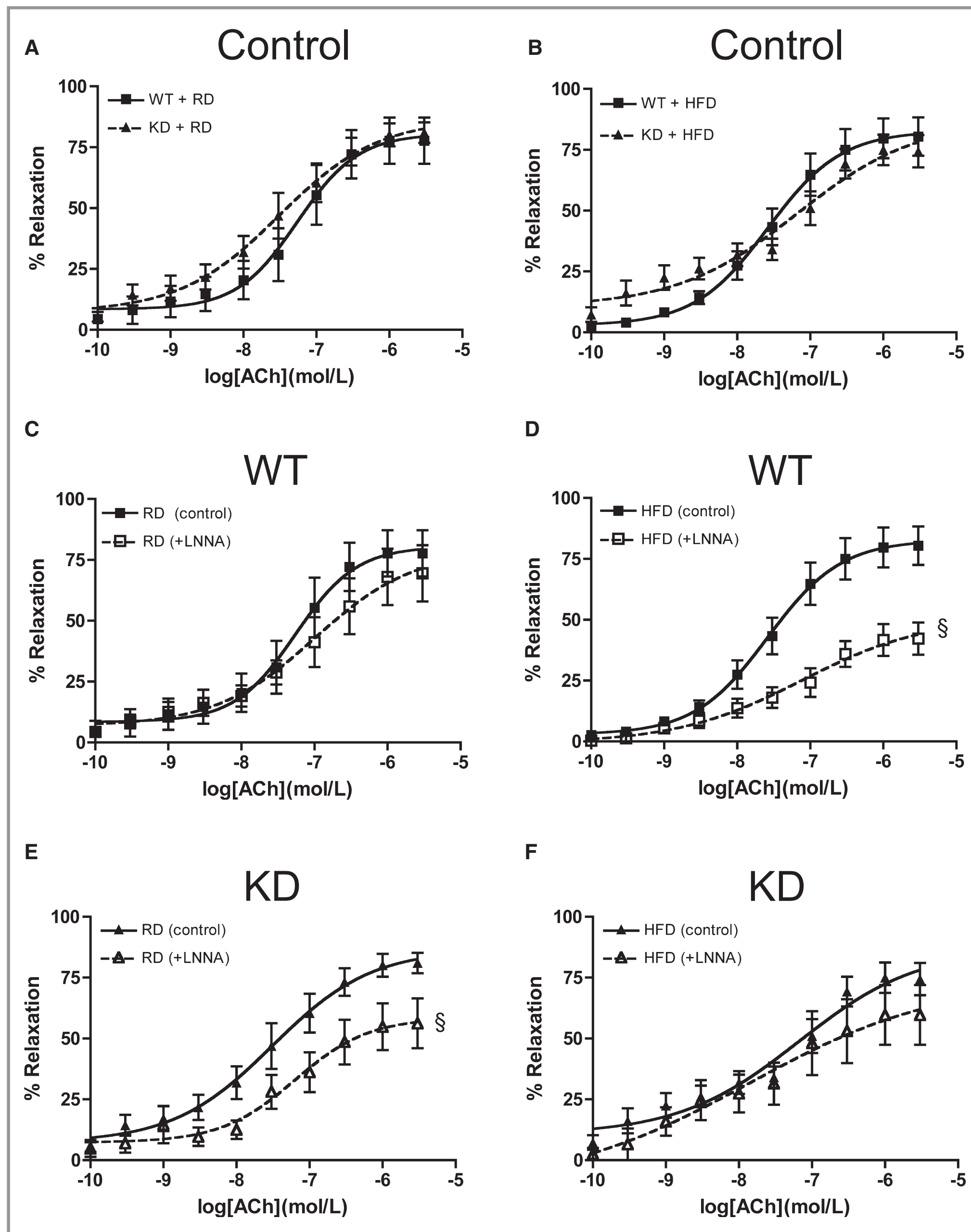

Figure 7. ACh-mediated relaxation in mesenteric arteries of 6-month-old mice fed an (A) RD, (B) HFD under no-drug control condition and with LNNA in WT (n=6) fed an (C) RD or (D) HFD, and in KD (n=6 to 10) fed an (E) RD or (F) HFD. The z-score method followed by the d'Agostino–Pearson omnibus test was used to test normality of data sets, after which the 2-way ANOVA followed by the Bonferonni posttest were used. §*P*<0.05 vs control. ACh indicates acetylcholine; HFD, high-fat diet; KD, knockdown; LNNA, Nω-nitro-L-arginine; RD, regular diet; WT, wild-type.

suggesting a decrease in EDHF-mediated relaxation but compensated by NO. In contrast, LNNA (n=8) unexpectedly reduced ACh-induced relaxation in angptl2 KD mice fed an RD (Figure 7E, Table 6), suggesting that both NO and EDHF contribute to the relaxation. The effect of LNNA (n=5) was, however, absent in KD mice fed an HFD (Figure 7F, Table 6), suggesting that the contribution of EDHF was preserved in mesenteric arteries of KD mice. Of note, in both strains, Indo did not modify ACh-induced relaxation (data not shown). Unlike in the femoral artery, vasoconstriction to PE was similar among all groups of mice (Figure 8A); furthermore, endothelium-independent relaxation to sodium nitroprusside was not different between groups (Figure 8B). Combined, these data suggest that in mesenteric arteries from angptl2

Downloaded from http://jaha.ahajournals.org/ at Institut de Cardiologie de Montreal on November 11, 2014

Table 6. Efficacy (E_{max}) and Sensitivity (EC_{50}) to Acetylcholine in Mesenteric Arteries Isolated From 6-Month-Old WT and angptl2 KD Male Mice Fed a Regular (RD) or High-Fat Diet (HFD)

	WT+RD		WT+HFD		KD+RD		KD+HFD	
	E_{max} (%)	EC_{50}	E_{max} (%)	EC_{50}	E_{max} (%)	EC_{50}	E_{max} (%)	EC_{50}
Control	78±10 (6)	7.3±0.1 (6)	80±8 (6)	7.6±0.2 (6)	81±4 (10)	7.5±0.3 (8)	74±7 (6)	7.2±0.2 (5)
+LNNA	70±12 (6)	7.0±0.3 (3)	42±7 (6)§	7.4±0.3 (6)	56±10 (8)§	7.4±0.4 (7)	60±12 (5)	8.1±0.4 (5)

Vessels were preconstricted with thromboxane A_2 analog U46619 (0.1 to 10 μmol/L). E_{max} values are expressed as the percentage of the maximal diameter. Data presented as mean±SEM of (n) mice. The z-score method followed by the d'Agostino–Pearson omnibus test was used to test normality of data sets, after which the 2-way ANOVA followed by the Bonferonni posttest were used. KD indicates knockdown; WT, wild-type; LNNA, N^{ω}-nitro-L-arginine.
§$P<0.05$ vs control (within respective groups).

KD mice treated with an HFD, the EDHF contribution is preserved, while in WT mice NO compensates for the reduced contribution of EDHF.

KD Mice Had a Better Lipid Profile Than WT Mice Fed an HFD

We examined the phenotypic profiles of WT and KD mice after a 3-month RD or HFD diet treatment (Table 7). Similar to mice 3 to 4 months of age, 6-month-old KD mice fed the RD had similar body weight compared with age-matched WT. After being fed the HFD for 3 months, both WT and KD mice significantly gained weight but KD mice weighed less than WT mice (Table 7). During the 3-month diet treatment, food intake was also similar in the 2 strains of mice fed either diet (data not shown). However, after the HFD, circulating leptin level rose significantly only in WT mice, while fasting plasma glucose, TGs, adiponectin, FFAs, and cholesterol (HDL, total cholesterol) were similar for the 2 strains (Table 7). Although total cholesterol and HDL levels increased in WT and KD mice after HFD, LDL levels increased only in WT, but not in KD mice, so that total cholesterol-to-HDL and LDL-to-HDL ratios increased significantly only in WT mice (Table 7). Fasting insulin in WT mice significantly elevated after HFD, while they were not different between strains. In addition, serum angptl2 was higher in WT mice fed an HFD compared with RD (149±18 versus 77±7 ng/mL, n=3 to 4, P=0.057, Mann–Whitney U test). These results indicate that after exposure to an HFD, the lipid profile was better in angptl2 KD compared with WT mice.

KD Mice Had Similar Blood Pressures as WT Mice but a Lower Resting Heart Rate

While systolic and diastolic blood pressures did not differ between 2 strains of mice and were not affected by the HFD (Table 8), the previously mentioned lower basal heart rate in KD mice at 3 months of age (Table 3) was maintained (n=7 to 10) at 6 months of age for the RD-fed group (Table 8).

Lower Liver TG Levels and Prevention of Upregulation of TNFα Gene Expression in KD Mice Fed an HFD

Because we observed a better lipid profile on an HFD treatment in KD mice, we examined the phenotype of the liver, a major organ involved in lipid handling. Liver sections of KD mice (n=4) fed an HFD showed less lipid droplet

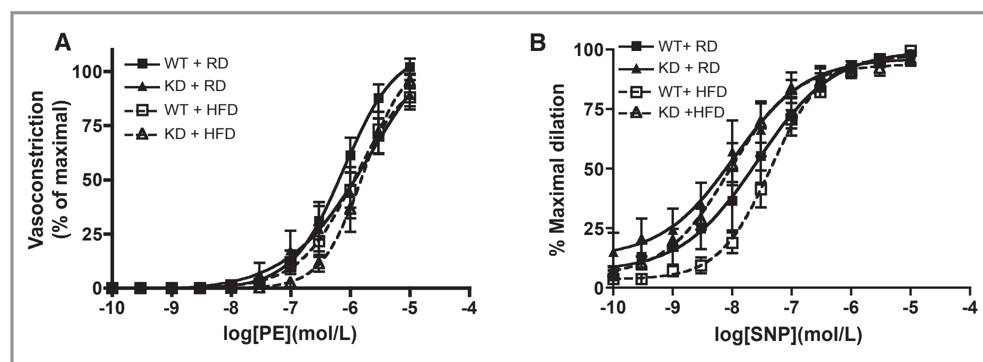

Figure 8. Vascular smooth muscle cell function was assessed in mesenteric arteries from WT and angptl2 KD mice, by (A) vasoconstriction to phenylephrine (PE) and (B) dilation to sodium nitroprusside (SNP) in the absence of the endothelium. Data are mean±SEM of n=4 to 6 mice. KD indicates knockdown; WT, wild-type.

Downloaded from http://jaha.ahajournals.org/ at Institut de Cardiologie de Montreal on November 11, 2014

Table 7. Parameters of Fasting Plasma in 6-Month-Old WT and Angptl2 KD Mice Fed a Regular Diet (RD) or a 3-Month High-Fat Diet (HFD)

	WT		KD	
	RD	HFD	RD	HFD
Weight, g	26.3±0.9 (10)	33.1±1.4 (9)$^{\theta}$	24.2±0.5 (13)	29.0±1.6 (11)$^{‡\theta}$
Glucose, mmol/L	17.0±1.1 (10)	17.4±1.7 (9)	13.5±1.1 (12)	17.1±1.5 (11)
Triglycerides, mmol/L	0.45±0.04 (10)	0.49±0.05 (9)	0.41±0.03 (12)	0.49±0.03 (11)
Adiponectin, μg/mL	8.5±0.4 (5)	10.1±0.3 (5)	9.7±1.1 (5)	8.5±0.7 (5)
Leptin, ng/mL	2.1±0.7 (5)	20.2±2.1 (5)$^{\theta}$	2.3±0.5 (5)	7.5±2.3 (5)
FFA, mmol/L	0.39±0.05 (7)	0.50±0.05 (5)	0.39±0.03 (7)	0.44±0.06 (7)
Cholesterol—total, mmol/L	2.7±0.1 (10)	3.9±0.2 (9)$^{\theta}$	2.7±0.2 (12)	3.9±0.3 (11)$^{\theta}$
Cholesterol—HDL, mmol/L	2.5±0.1 (10)	3.3±0.1 (9)$^{\theta}$	2.4±0.2 (12)	3.5±0.2 (11)$^{\theta}$
Cholesterol—LDL, mmol/L	0.15±0.02 (9)	0.41±0.11 (8)$^{\theta}$	0.13±0.01 (12)	0.25±0.05 (11)
Cholesterol—total/HDL	1.07±0.02 (10)	1.17±0.03 (9)$^{\theta}$	1.12±0.02 (12)	1.12±0.01 (11)
LDL/HDL	0.06±0.01 (9)	0.12±0.03 (8)$^{\theta}$	0.06±0.01 (11)	0.08±0.02 (11)
Insulin, ng/mL	0.10±0.02 (9)	0.27±0.03 (9)$^{\theta}$	0.14±0.02 (12)	0.30±0.08 (10)

Data presented as mean±SEM of (n) mice. The Kruskal–Wallis test followed by the Dunn's posttest were used for data sets not normally distributed; 2-way ANOVA followed by the Bonferroni posttest was used to compare normally distributed data sets. FFA indicates free fatty acid; HDL, high-density lipoprotein; KD, knockdown; LDL, low-density lipoprotein; WT, wild-type.
$^{\theta}P<0.05$ vs RD (within the same strain); $^{‡}P<0.05$ vs WT (within the same treatment).

accumulation compared with WT mice (n=5) fed an HFD (Figure 9A). This was associated with significantly lower levels of liver TGs measured with the use of HPLC in KD mice (n=4) fed an HFD compared with WT mice (n=5) (Figure 9B). Phospholipid levels remained similar among all groups (data not shown). Expressions of hepatic genes were examined using qPCR (Figure 9C through 9F): notably, hepatic angptl2 gene expression was significantly greater after HFD in the WT mice (n=7; Figure 9C). In terms of inflammatory markers, TNFα mRNA levels significantly increased in HFD-fed WT mice (n=7) but did not change in KD mice (n=6; Figure 9D), while transforming growth factor-β increased similarly after HFD (Figure 9E) and interleukin-6 gene expression was unchanged and similar between the 2 strains (Figure 9F). The expression of essential genes coding for proteins involved in the regulation of lipid metabolism was not different between groups (Figure 10). Collectively, these data suggest that an HFD promoted inflammation that was associated with increased TG levels in the liver of WT mice, which was less severe in angptl2 KD mice.

Smaller Adipocyte Size in Fat Depots and Prevention of Inflammatory Gene Expression in Epididymal White Adipose Tissue of HFD-fed KD Mice

Efficiency of fat storage in adipose tissue is highly related to lipid profiling.[42] Under basal conditions when fed an RD, adipocyte cell size as measured by cell diameter (μm) in mesenteric white adipose tissue (mWAT) (Figure 11A and 11B) and epididymal WAT (eWAT) (Figure 11C and 11D) was similar between the 2 strains (n=3 to 6). Remarkably, when

Table 8. Measurements of Heart Rate and Blood Pressures by Tail-Cuff Plethysmography in 6-Month-Old WT and angptl2 KD Mice Fed a Regular Diet (RD) or a 3-Month High-Fat Diet (HFD)

	WT		KD	
	RD	HFD	RD	HFD
Heart rate, bpm	719±16 (10)	760±11 (6)	624±29 (7)‡	713±18 (8)
Systolic blood pressure, mm Hg	145±6 (10)	146±5 (6)	146±2 (7)	153±5 (8)
Diastolic blood pressure, mm Hg	111±7 (10)	116±6 (6)	113±3 (7)	123±5 (8)

Data presented as mean±SEM of (n) mice. The Mann–Whitney *U* test was used. KD indicates knockdown; WT, wild-type.
$^{‡}P<0.05$ vs WT.

Downloaded from http://jaha.ahajournals.org/ at Institut de Cardiologie de Montreal on November 11, 2014

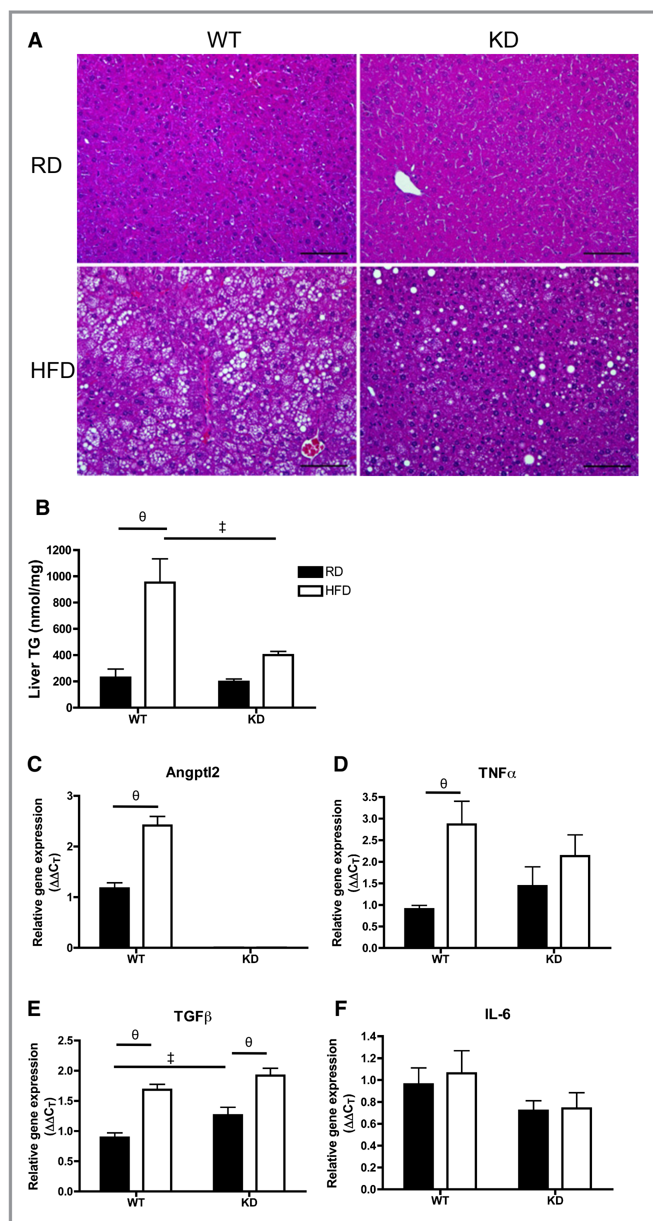

Figure 9. A, H&E-stained liver sections (scale bar=100 μm) and (B) quantification of triglyceride (TG) content in liver of 6-month-old WT and angptl2 KD mice fed a regular diet (RD) or a 3-month high-fat diet (HFD); n=4 to 5. C through F, Quantitative RT-PCR of mRNAs encoding for angptl2 and various inflammatory markers in liver of WT or angptl2 KD mice fed an RD or HFD; n=6 to 7. The z-score method followed by the d'Agostino–Pearson omnibus test was used to test normality of data sets, after which the 2-way ANOVA followed by the Bonferonni posttest were used. ‡$P<0.05$ vs WT; θ$P<0.05$ vs RD. KD indicates knockdown; RT-PCR, real-time quantitative polymerase chain reaction; TGF, transforming growth factor; WT, wild-type.

fed an HFD, adipocytes in mWAT and eWAT both significantly increased in size only in WT (n=5) (Figure 11A through 11D). Analysis by qPCR in eWAT (Figure 11E through 11J) revealed that unlike in the liver, angptl2 mRNA levels did not change after an HFD in WT mice (n=7; Figure 11E). Lipoprotein lipase (LPL) mRNA expression, although not different between RD and HFD for both strains, was significantly lower in HFD-fed KD compared with WT mice (n=7; Figure 11F). Analysis of LPL activity, however, revealed no differences between the strains (data not shown). Interestingly, expressions of adiponectin mRNA decreased significantly after an HFD in WT (n=7) but remained unchanged in KD mice (n=7; Figure 11G), while leptin mRNA significantly increased only in eWAT of WT mice (n=7; Figure 11H) and corresponded to a similar pattern found in plasma leptin levels (Table 7). Similarly, proinflammatory gene markers transforming growth factor-β and TNFα gene expressions were significantly increased in HFD-fed WT mice (n=7), but not in KD mice (Figure 11I and 11J). In eWAT, expression of essential genes coding for proteins involved in the regulation of lipid metabolism was not different between groups (Figure 12). Altogether, these data suggest that the inflammation induced by the metabolic stress of an HFD is prevented in adipocytes of angptl2 KD mice.

Discussion

The novel findings in this study are that KD of angptl2 results in (1) better femoral endothelial function via NO/PGI_2 recruitment in young mice, (2) preserved endothelial dilatory function after HFD associated with maintained NO release in the femoral artery (a conductance artery) and EDHF contribution in the mesenteric artery (a resistance artery), (3) a better lipid profile when exposed to the metabolic challenge of an HFD, and (4) a lower inflammatory status of the liver and eWAT. To the best of our knowledge, this is the first report of the impact of angptl2 in the contribution of the various EDRF and their resistance against a stress induced by an HFD.

In the first part of the study, we tested the acute effects of angptl2 on endothelial function. We found that acute addition of angptl2-GST significantly reduced vasodilation in femoral arteries from WT mice, an effect that was reversed with the addition of antioxidant NAC, implicating the pro-oxidative role of angptl2 on deteriorating endothelial function, at least acutely. The endothelium, and ultimately vascular function, is highly sensitive to increased oxidative stress, with NO bioavailability and EDHF activity being major targets.[43] This is the first demonstration of the prooxidative effect of angptl2 on vascular cells and is in accordance with the recent report by Aoi et al that used cancer cells.[26]

The next logical step was to then examine the role of angptl2 in regulating endothelial function chronically. In characterizing the endothelial function in femoral arteries in which NO is the main EDRF, we found that, in addition to NO, PGI_2 unexpectedly contributed to the vasodilation in young healthy KD mice, which may reflect a remodeling of the EDRF induced by angptl2 KD. It is known that NO and PGI_2

Downloaded from http://jaha.ahajournals.org/ at Institut de Cardiologie de Montreal on November 11, 2014

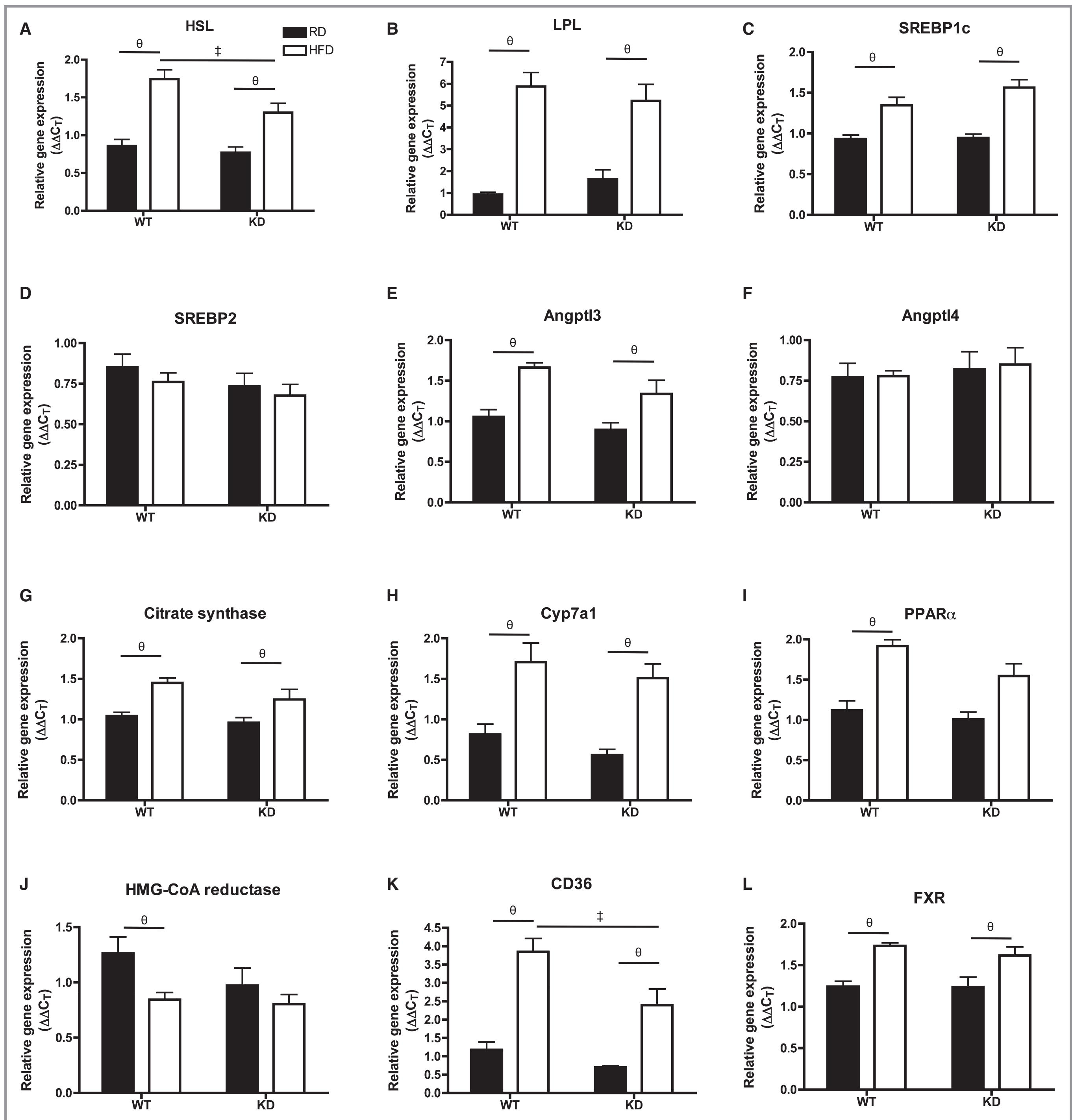

Figure 10. A through L, Expression of genes coding for proteins involved in lipid metabolism regulation in the liver by qPCR; n=6 to 7. Data sets were tested for normality using the z-score method and the d'Agostino–Pearson omnibus test, after which 2-way ANOVA followed by the Bonferroni posttest was used. All except I passed the normality test, where the Krustal–Wallis followed by Dunn's posttest were used. ‡$P<0.05$ vs WT; θ$P<0.05$ vs RD. FXR indicates farnesoid X receptor; HFD, high-fat diet; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme-A; HSL, hormone-sensitive lipase; KD, knockdown; LPL, lipoprotein lipase; PPAR, peroxisome proliferator-activated receptor; qPCR, quantitative polymerase chain reaction; RD, regular diet; SREBP, sterol regulatory element binding protein.

produced by the endothelium play a protective and antiproliferative role as potent inhibitor of platelet function and vasorelaxant.[44] In addition, our data reveal that the contribution of EDHF is greater in femoral arteries isolated from young WT mice compared with KD mice. We previously reported that with age, the contribution of EDHF increases,

Downloaded from http://jaha.ahajournals.org/ at Institut de Cardiologie de Montreal on November 11, 2014

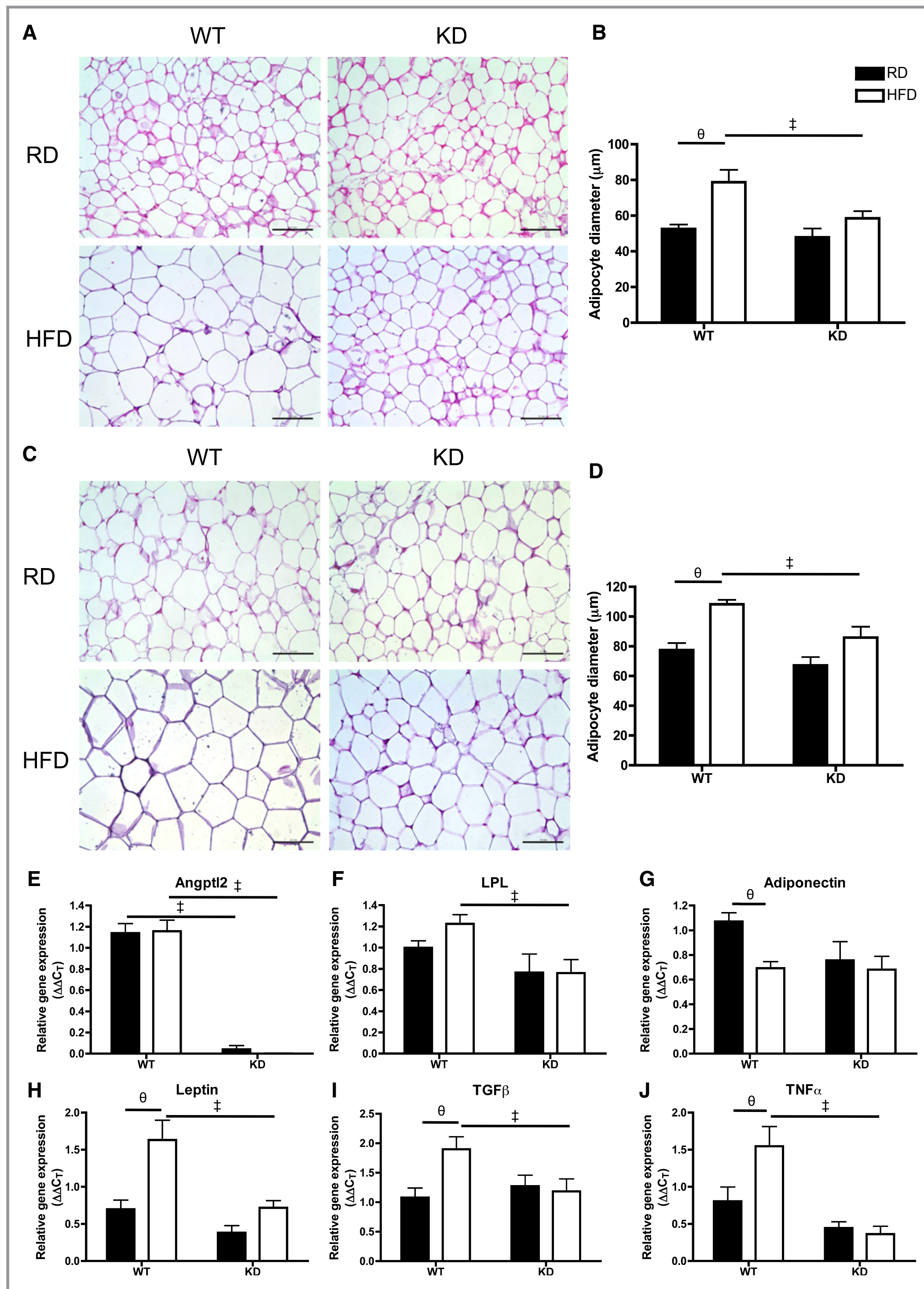

Figure 11. A, Hematoxylin-eosin–stained mesenteric white adipose tissue (mWAT) in different groups (scale bar=100 μm) and (B) quantification of adipocyte size of diameter measurements (average of 3 quantifying analyses was used per animal) in mWAT; n=3 to 5. C, Hematoxylin-eosin–stained epididymal WAT (eWAT) in different groups (scale bar=100 μm) and (D) quantification of adipocyte diameters in eWAT; n=3 to 6. E through J, Gene expression analysis by qPCR in eWAT of WT and KD mice fed an RD or HFD; n=6 to 7. The z-score method followed by the d'Agostino–Pearson omnibus test was used to test normality of data sets, after which the 2-way ANOVA followed by the Bonferonni posttest were used, except in E, where the Kruskal–Wallis followed by Dunn's posttest were used as it did not pass normality test. ‡*P*<0.05 vs WT; θ*P*<0.05 vs RD. HFD indicates high-fat diet; KD, knockdown; qPCR, quantitative polymerase chain reaction; RD, regular diet; WT, wild-type.

Downloaded from http://jaha.ahajournals.org/ at Institut de Cardiologie de Montreal on November 11, 2014

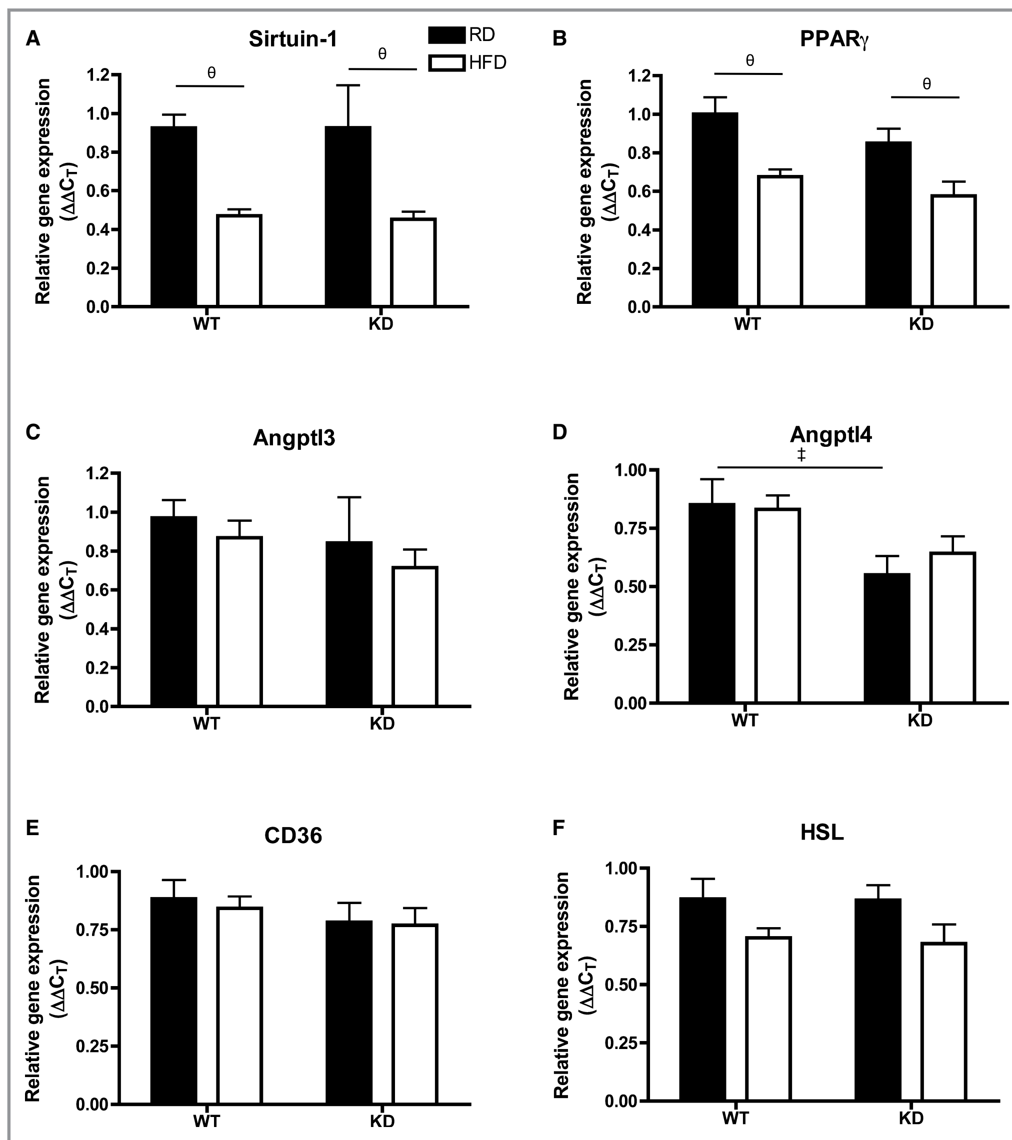

Figure 12. A through F, Expression of genes coding for proteins involved in lipid metabolism regulation in the eWAT by qPCR; n=6 to 7. Data sets were tested for normality using the z-score method and the d'Agostino–Pearson omnibus test, after which 2-way ANOVA followed by Bonferroni's posttest were used. ‡$P<0.05$ vs WT; θ$P<0.05$ vs RD. eWAT indicates epididymal white adipose tissue; HSL, hormone-sensitive lipase; KD, knockdown; PPAR, peroxisome proliferator-activated receptor; qPCR, quantitative polymerase chain reaction; RD, regular diet; WT, wild-type.

compensating for the decline in eNOS activity and/or NO bioavailability.[21] The current results, therefore, suggest that the lack of angptl2 expression may protect endothelial function by influencing the relative contribution of the 3 main EDRFs—NO, PGI_2, and EDHF.

In the second part of the study, we compared the effects of an HFD on endothelial function between WT and KD mice. The current observations are in line with recently reported results demonstrating HFD-fed angptl2 knockout mice with attenuated endothelial dysfunction in isolated descending thoracic aortas,[5] while endothelial cell–derived angptl2 contributed to eNOS inactivation.[5] In the current work, we also reveal the preservation of NO-mediated vasodilation in HFD-fed KD, but not WT mice, as confirmed by monitoring ACh-induced NO release using DAF-2 in femoral arteries. These results complement what has been recently found in aortas of HFD-fed angptl2 knockout mice, where levels of phospho-eNOS was higher than in WT mice.[5] However, the exact mechanism by which NO-mediated dilation is maintained remains unknown. Repeatedly, literature has demonstrated endothelial dysfunction characterized by decreased NO bioavailability due to increased oxidative stress.[18,45] Because we observed complete rescue of endothelial function with NAC after an acute addition of angptl2, we asked if lower oxidative stress

Downloaded from http://jaha.ahajournals.org/ at Institut de Cardiologie de Montreal on November 11, 2014

levels in HFD-fed KD mice could explain our functional data regarding NO. In the present study, however, oxidative stress evaluated by DHE staining did not reveal differences in the femoral artery. Presumably, a 3-month HFD was not as strong a stress on the femoral artery compared with an acute addition of a pharmacological dose of angptl2 directly in the vessel bath. In addition, it was noted that an HFD increased efficacy to PE in denuded femoral arteries from WT, as has been observed by others,[46] but not in KD mice. Despite that difference, low doses of PE were used depending on individual arteries to preconstrict the arteries at 30% to 50% maximal diameter, suggesting that the observed differences in vascular function were not due to different efficacies to PE.

Because the vasodilatory function of the endothelium displays high heterogeneity among various vascular beds, where responses and adaptation to stress also show great diversity, we examined, in parallel, endothelial function in mesenteric resistance arteries. After an RD, 6-month-old mice did not show differences in global endothelial function. In the mesenteric resistance arteries, it has been reported that the main vasodilator is EDHF,[47] which is in accordance with the current results in WT mice where relaxations were insensitive to NOS inhibition. In contrast, inhibiting NOS with LNNA significantly reduced ACh-induced relaxation in RD-fed angptl2 KD mice, suggesting that both EDHF and NO synergistically contributed to relaxation. This could reflect an impact of knocking down angptl2 on EDRF signaling in mesenteric arteries, similar to the remodeling of EDRF observed in femoral arteries from young KD mice in which PGI_2 synergizes with the main EDRF NO.

In mesenteric arteries from WT mice fed an HFD, the contribution of EDHF to the relaxation was significantly reduced but compensated by NO. We previously showed that EDHF was a factor sensitive to mild dyslipidemia-associated oxidative stress[21]; therefore, it is possible that in WT mice challenged by a 3-month HFD, the NOS systems compensates for this impaired EDHF activity at the early stage of obesity. In KD mice fed an HFD, however, LNNA no longer reduced ACh-induced relaxation of isolated mesenteric arteries, suggesting that the EDHF component was preserved. Altogether, in normal WT mice, our results demonstrate that regardless of the arterial bed, the main EDRF is sensitive to the metabolic stress induced by an HFD, and it is efficiently compensated for by a secondary EDRF, maintaining global endothelial function. In the long term, as in aging and chronic obesity, however, adaptive mechanisms may become overwhelmed, leading to permanent endothelial dysfunction, the primary step toward atherosclerosis. With low angptl2 levels, as in the KD mice, the endothelium is more resistant to the stress induced by the HFD, maintaining the functionality of the respective main EDRF. By extrapolation, lowering angptl2 may delay endothelial dysfunction.

We further characterized the impact of knocking down angptl2 on the metabolic stress induced by an HFD. In 2 recent studies focusing on insulin resistance and atherosclerosis, authors did not report differences between the lipid profiles of HFD-fed WT and angptl2 knockout mice.[3,5] Although we previously reported that infusion of purified recombinant angptl2 in young 3-month-old severely dyslipidemic ($LDLR^{-/-}$;$hApoB^{+/+}$) mice further increased LDL cholesterol levels and accelerated atherogenesis,[4] we did not expect the difference in lipid handling between KD and WT mice fed an HFD reported in the present study. Indeed, after a 3-month HFD, total cholesterol-to-HDL and LDL-to-HDL ratios remained unchanged in KD mice, which is in stark contrast with the expected increase in cholesterol levels[3] and with that measured in WT mice. The favorable lipid profile of KD mice fed an HFD was associated with significantly lower levels of TG in the liver, which is in accordance with the results of Tabata et al.[3] In addition, we found that KD mice exhibited a smaller degree of adipocyte hypertrophy in both eWAT and mWAT. There were lower levels of TNFα and transforming growth factor-β mRNA in eWAT from HFD-fed KD mice, likely a consequence of reduced fat accumulation associated with the lack of expression of angptl2,[3] suggesting lower levels of inflammation in adipose tissues of KD mice. In line with this, it has been shown that impaired excess fat storage in adipocytes is closely linked to ectopic fat deposition,[48] as observed in the increased hepatic lipid accumulation in HFD-fed WT but not in KD mice. Unlike Tabata et al,[3] however, we did not observe lower fasting insulin levels in KD mice. Discrepancies may be explained by the modality of genetic inactivation used, being a knockout and ours a KD model. Taken together, the more favorable lipid profile, lower fat accumulation, and proinflammatory gene expression may explain why the endothelium-dependent dilatory function of arteries isolated from KD mice was insensitive to the HFD in contrast to arteries from WT mice.

An unexpected finding in this study was that angptl2 KD mice had lower basal heart rate than WT mice, which was consistent from 3 to 6 months of age. With the HFD, heart rate increased in KD mice but still tended to be lower than that in WT mice. This observation may contribute as well to the better endothelial function in KD mice. Indeed, studies in the middle-aged and elderly have shown that an elevated resting heart rate correlated with subclinical inflammation[49] and lowering basal heart rate in atherosclerosis-prone mice in different studies delayed endothelial dysfunction associated with reduced oxidative stress.[50,51] The beneficial effects of a lower heart rate on the endothelium is suggested to be a combination of sustained shear stress and lower cyclic mechanical stress, resulting in reduced damage to the endothelium.[52]

Downloaded from http://jaha.ahajournals.org/ at Institut de Cardiologie de Montreal on November 11, 2014

Heart rate, blood pressure, and energy expenditure have been shown to be regulated by leptin through central coactivation of the sympathetic nervous system and renin-angiotensin system (see review in Mark [53]). In our experimental models, leptin levels did not increase in the blood of KD mice fed an HFD. Therefore, a lack of increase in leptin during HFD in KD mice could participate in limiting the observed changes in heart rate and metabolic phenotype. Despite contrasting leptin levels, food intake during the 3-month diet treatment was not significantly different between the 2 strains of mice. Interestingly, a recent study reported a possible link between adipocyte-derived adiponectin, which shares established interactions with leptin in settings of obesity,[54] and angptl2.[8] In the current study, however, plasma levels of adiponectin were similar among all groups of mice. Taken together, angptl2 may interact with leptin pathways, but this hypothesis needs to be further tested.

To conclude, accumulating reports are starting to highlight the importance of angptl2 involvement in a plethora of pathologies ranging from obesity and cancer to atherosclerosis in the most recent reports.[4,5,11] A common phenomenon underlying the aforementioned pathologies is the presence of an inflammatory environment. Inflammation in the endothelium favors homeostatic imbalances between vasodilators and vasoconstrictors, ultimately leading to endothelial dysfunction. Our data reveal better endothelial function in the arteries of angptl2 KD mice with preserved NO-mediated dilation in femoral arteries and preserved EDHF-dilation in mesenteric arteries against a 3-month HFD-induced hypercholesterolemia and fat accumulation in the liver and adipose tissues. Because targeting endothelial dysfunction is a rational therapeutic approach in treating patients with cardiovascular disease,[55] further understanding the role of angptl2 in endothelial dysfunction associated with increased inflammation may reveal a new possible target to treatment and prevention of a range of cardiovascular disorders.

Acknowledgments

We would like to thank Maya Mamarbachi and Marie-Élaine Clavet-Lanthier for their technical support in validating the genotype of the mice and processing and staining of tissue samples, respectively.

Sources of Funding

This work was supported by the Canadian Institutes of Health Research (MOP 14496 and 133649 to Dr Thorin) and the Foundation of the Montreal Heart Institute (Dr Thorin). Carol Yu is supported by the Postgraduate Scholarship from the Natural Sciences and Engineering Research Council of Canada. Dr Martel is supported by the Postgraduate Scholarship from the Fonds de la Recherche en Santé du Québec.

Disclosures

None.

References

1. Reaven GM. Banting Lecture 1988. Role of insulin resistance in human disease. *Diabetes*. 1988;1988:1595–1607.
2. Krauss RM, Winston M, Fletcher RN, Grundy SM. Obesity: impact of cardiovascular disease. *Circulation*. 1998;98:1472–1476.
3. Tabata M, Kadomatsu T, Fukuhara S, Miyata K, Ito Y, Endo M, Urano T, Zhu HJ, Tsukano H, Tazume H, Kaikita K, Miyashita K, Iwawaki T, Shimabukuro M, Sakaguchi K, Ito T, Nakagata N, Yamada T, Katagiri H, Kasuga M, Ando Y, Ogawa H, Mochizuki N, Itoh H, Suda T, Oike Y. Angiopoietin-like protein 2 promotes chronic adipose tissue inflammation and obesity-related systemic insulin resistance. *Cell Metab*. 2009;10:178–188.
4. Farhat N, Thorin-Trescases N, Mamarbachi M, Villeneuve L, Yu C, Martel C, Duquette N, Gayda M, Nigam A, Juneau M, Allen BG, Thorin E. Angiopoietin-like 2 promotes atherogenesis in mice. *J Am Heart Assoc*. 2013;2:e000201 doi:10.1161/JAHA.113.000201.
5. Horio E, Kadomatsu T, Miyata K, Arai Y, Hosokawa K, Doi Y, Ninomiya T, Horiguchi H, Endo M, Tabata M, Tazume H, Tian Z, Takahashi O, Terada K, Takeya M, Hao H, Hirose N, Minami T, Suda T, Kiyohara Y, Ogawa H, Kaikita K, Oike Y. Role of endothelial cell-derived Angptl2 in vascular inflammation leading to endothelial dysfunction and atherosclerosis progression. *Arterioscler Thromb Vasc Biol*. 2014; 34:790–800.
6. Doi Y, Ninomiya T, Hirakawa Y, Takahashi O, Mukai N, Hata J, Iwase M, Kitazono T, Oike Y, Kiyohara Y. Angiopoietin-like protein 2 and risk of type 2 diabetes in a general Japanese population: the Hisayama study. *Diabetes Care*. 2012; 36:98–100.
7. Tazume H, Miyata K, Tian Z, Endo M, Horiguchi H, Takahashi O, Horio E, Tsukano H, Kadomatsu T, Nakashima Y, Kunitomo R, Kaneko Y, Moriyama S, Sakaguchi H, Okamoto K, Hara M, Yoshinaga T, Yoshimura K, Aoki H, Araki K, Hao H, Kawasuji M, Oike Y. Macrophage-derived angiopoietin-like protein 2 accelerates development of abdominal aortic aneurysm. *Arterioscler Thromb Vasc Biol*. 2012;32:1400–1409.
8. Tian Z, Miyata K, Tazume H, Sakaguchi H, Kadomatsu T, Horio E, Takahashi O, Komohara Y, Araki K, Hirata Y, Tabata M, Takanashi S, Takeya M, Hao H, Shimabukuro M, Sata M, Kawasuji M, Oike Y. Perivascular adipose tissue-secreted angiopoietin-like protein 2 (Angptl2) accelerates neointimal hyperplasia after endovascular injury. *J Mol Cell Cardiol*. 2013;57:1–12.
9. Okada T, Tsukano H, Endo M, Tabata M, Miyata K, Kadomatsu T, Miyashita K, Semba K, Nakamura E, Tsukano M, Mizuta H, Oike Y. Synoviocyte-derived angiopoietin-like protein 2 contributes to synovial chronic inflammation in rheumatoid arthritis. *Am J Pathol*. 2010;176:2309–2319.
10. Ogata A, Endo M, Aoi J, Takahashi O, Kadomatsu T, Miyata K, Tian Z, Jinnin M, Fukushima S, Ihn H, Oike Y. The role of angiopoietin-like protein 2 in pathogenesis of dermatomyositis. *Biochem Biophys Res Commun*. 2012;418:494–499.
11. Aoi J, Endo M, Kadomatsu T, Miyata K, Nakano M, Horiguchi H, Ogata A, Odagiri H, Yano M, Araki K, Jinnin M, Ito T, Hirakawa S, Ihn H, Oike Y. Angiopoietin-like protein 2 is an important facilitator of inflammatory carcinogenesis and metastasis. *Cancer Res*. 2011;71:7502–7512.
12. Farhat N, Thorin-Trescases N, Voghel G, Villeneuve L, Mamarbachi M, Perrault LP, Carrier M, Thorin E. Stress-induced senescence predominates in endothelial cells isolated from atherosclerotic chronic smokers. *Can J Physiol Pharmacol*. 2008;86:761–769.
13. Kitazawa M, Nagano M, Masumoto KH, Shigeyoshi Y, Natsume T, Hashimoto S. Angiopoietin-like 2, a circadian gene, improves type 2 diabetes through potentiation of insulin sensitivity in mice adipocytes. *Endocrinology*. 2011;152:2558–2567.
14. Muramoto A, Tsushita K, Kato A, Ozaki N, Tabata M, Endo M, Oike Y, Oiso Y. Angiopoietin-like protein 2 sensitively responds to weight reduction induced by lifestyle intervention on overweight Japanese men. *Nutr Diabetes*. 2011;1:1–10.
15. Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*. 1980;288: 373–376.
16. Moncada S, Erusalimsky JD. Does nitric oxide modulate mitochondrial energy generation and apoptosis? *Nat Rev Mol Cell Biol*. 2002;3:214–220.
17. Komori K, Vanhoutte PM. Endothelium-derived hyperpolarizing factor. *Blood Vessels*. 1990;27:238–245.
18. Durand MJ, Gutterman DD. Diversity in mechanisms of endothelium-dependent vasodilation in health and disease. *Microcirculation*. 2013;20:239–247.

Downloaded from http://jaha.ahajournals.org/ at Institut de Cardiologie de Montreal on November 11, 2014

19. Winquist RJ, Bunting PB, Baskin EP, Wallace AA. Decreased endothelium-dependent relaxation in New Zealand genetic hypertensive rats. *J Hypertens*. 1984;2:541–545.
20. Lockette W, Otsuka Y, Carretero O. The loss of endothelium-dependent vascular relaxation in hypertension. *Hypertension*. 1986; 8:II61–II66.
21. Krummen S, Drouin A, Gendron ME, Falck JR, Thorin E. ROS-sensitive cytochrome P450 activity maintains endothelial dilatation in ageing but is transitory in dyslipidaemic mice. *Br J Pharmacol*. 2006;147:897–904.
22. Najibi S, Cowan CL, Palacino JJ, Cohen RA. Enhanced role of potassium channels in relaxations to acetylcholine in hypercholesterolemic rabbit carotid artery. *Am J Physiol*. 1994;266:H2061–H2067.
23. Huang A, Sun D, Smith CJ, Connetta JA, Shesely EG, Koller A, Kaley G. In eNOS knockout mice skeletal muscle arteriolar dilation to acetylcholine is mediated by EDHF. *Am J Physiol Heart Circ Physiol*. 2000;278:H762–H768.
24. Huang A, Sun D, Carroll MA, Jiang H, Smith CJ, Connetta JA, Falck JR, Shesely EG, Koller A, Kaley G. EDHF mediates flow-induced dilation in skeletal muscle arterioles of female eNOS-KO mice. *Am J Physiol Heart Circ Physiol*. 2001;280: H2462–H2469.
25. Sun D, Huang A, Smith CJ, Stackpole CJ, Connetta JA, Shesely EG, Koller A, Kaley G. Enhanced release of prostaglandins contributes to flow-induced arteriolar dilation in eNOS knockout mice. *Circ Res*. 1999;85:288–293.
26. Aoi J, Endo M, Kadomatsu T, Miyata K, Ogata A, Horiguchi H, Odagiri H, Masuda T, Fukushima S, Jinnin M, Hirakawa S, Sawa T, Akaike T, Ihn H, Oike Y. Angiopoietin-like protein 2 accelerates carcinogenesis by activating chronic inflammation and oxidative stress. *Mol Cancer Res*. 2014;12:239–249.
27. Damjanovic M, Barton M. Fat intake and cardiovascular response. *Curr Hypertens Rep*. 2008;10:25–31.
28. Bolduc V, Baraghis E, Duquette N, Thorin-Trescases N, Lambert J, Lesage F, Thorin E. Catechin prevents severe dyslipidemia-associated changes in wall biomechanics of cerebral arteries in LDLr−/−:hApoB+/+ mice and improves cerebral blood flow. *Am J Physiol Heart Circ Physiol*. 2012;302:H1330–H1339.
29. Thorin E, Shreeve SM, Thorin-Trescases N, Bevan JA. Reversal of endothelin-1 release by stimulation of endothelial alpha2-adrenoceptor contributes to cerebral vasorelaxation. *Hypertension*. 1997;30:830–836.
30. Drouin A, Thorin-Trescases N, Hamel E, Falck JR, Thorin E. Endothelial nitric oxide synthase activation leads to dilatory H_2O_2 production in mouse cerebral arteries. *Cardiovasc Res*. 2007;73:73–81.
31. Drouin A, Farhat N, Bolduc V, Thorin-Trescases N, Gillis MA, Villeneuve L, Nguyen A, Thorin E. Up-regulation of thromboxane A(2) impairs cerebrovascular eNOS function in aging atherosclerotic mice. *Pflugers Arch*. 2011;462:371–383.
32. Khairallah RJ, Khairallah M, Gelinas R, Bouchard B, Young ME, Allen BG, Lopaschuk GD, Deschepper CF, Des Rosiers C. Cyclic GMP signaling in cardiomyocytes modulates fatty acid trafficking and prevents triglyceride accumulation. *J Mol Cell Cardiol*. 2008;45:230–239.
33. Gelinas R, Thompson-Legault J, Bouchard B, Daneault C, Mansour A, Gillis MA, Charron G, Gavino V, Labarthe F, Des Rosiers C. Prolonged QT interval and lipid alterations beyond beta-oxidation in very long-chain acyl-CoA dehydrogenase null mouse hearts. *Am J Physiol Heart Circ Physiol*. 2011; 301: H813–H823.
34. Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem*. 1957;226:497–509.
35. Ruiz J, Antequera T, Andres AI, Petron M, Muriel E. Improvement of a solid phase extraction method for analysis of lipid fractions in muscle foods. *Anal Chim Acta*. 2004;520:201–205.
36. Lepage G, Roy CC. Direct transesterification of all classes of lipids in a one-step reaction. *J Lipid Res*. 1986;27:114–120.
37. Gendron ME, Theoret JF, Mamarbachi AM, Drouin A, Nguyen A, Bolduc V, Thorin-Trescases N, Merhi Y, Thorin E. Late chronic catechin antioxidant treatment is deleterious to the endothelial function in aging mice with established atherosclerosis. *Am J Physiol Heart Circ Physiol*. 2010;298: H2062–H2070.
38. Adeagbo AS, Triggle CR. Varying extracellular [K+]: a functional approach to separating EDHF- and EDNO-related mechanisms in perfused rat mesenteric arterial bed. *J Cardiovasc Pharmacol*. 1993;21:423–429.
39. Thorin E, Huang PL, Fishman MC, Bevan JA. Nitric oxide inhibits alpha2-adrenoceptor-mediated endothelium-dependent vasodilation. *Circ Res*. 1998;82:1323–1329.
40. Shimokawa H, Flavahan NA, Lorenz RR, Vanhoutte PM. Prostacyclin releases endothelium-derived relaxing factor and potentiates its action in coronary arteries of the pig. *Br J Pharmacol*. 1988;95:1197–1203.
41. Wigg SJ, Tare M, Tonta MA, O'Brien RC, Meredith IT, Parkington HC. Comparison of effects of diabetes mellitus on an EDHF-dependent and an EDHF-independent artery. *Am J Physiol Heart Circ Physiol*. 2001;281:H232–H240.
42. Scherer PE. Adipose tissue: from lipid storage compartment to endocrine organ. *Diabetes*. 2006;55:1537–1545.
43. Feletou M, Vanhoutte PM. Endothelial dysfunction: a multifaceted disorder (The Wiggers Award Lecture). *Am J Physiol Heart Circ Physiol*. 2006;291:H985–H1002.
44. de Leval X, Hanson J, David JL, Masereel B, Pirotte B, Dogne JM. New developments on thromboxane and prostacyclin modulators part II: prostacyclin modulators. *Curr Med Chem*. 2004;11:1243–1252.
45. Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci USA*. 1990;87:1620–1624.
46. Molnar J, Yu S, Mzhavia N, Pau C, Chereshnev I, Dansky HM. Diabetes induces endothelial dysfunction but does not increase neointimal formation in high-fat diet fed C57BL/6J mice. *Circ Res*. 2005;96:1178–1184.
47. Matoba T, Shimokawa H, Nakashima M, Hirakawa Y, Mukai Y, Hirano K, Kanaide H, Takeshita A. Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in mice. *J Clin Invest*. 2000;106:1521–1530.
48. Unger RH, Orci L. Lipotoxic diseases of nonadipose tissues in obesity. *Int J Obes Relat Metab Disord*. 2000;24(suppl 4):S28–S32.
49. Sajadieh A, Nielsen OW, Rasmussen V, Hein HO, Abedini S, Hansen JF. Increased heart rate and reduced heart-rate variability are associated with subclinical inflammation in middle-aged and elderly subjects with no apparent heart disease. *Eur Heart J*. 2004;25:363–370.
50. Custodis F, Baumhakel M, Schlimmer N, List F, Gensch C, Bohm M, Laufs U. Heart rate reduction by ivabradine reduces oxidative stress, improves endothelial function, and prevents atherosclerosis in apolipoprotein E-deficient mice. *Circulation*. 2008;117:2377–2387.
51. Drouin A, Gendron ME, Thorin E, Gillis MA, Mahlberg-Gaudin F, Tardif JC. Chronic heart rate reduction by ivabradine prevents endothelial dysfunction in dyslipidaemic mice. *Br J Pharmacol*. 2008;154:749–757.
52. Thorin E, Thorin-Trescases N. Vascular endothelial ageing, heartbeat after heartbeat. *Cardiovasc Res*. 2009;84:24–32.
53. Mark AL. Selective leptin resistance revisited. *Am J Physiol Regul Integr Comp Physiol*. 2013;305:R566–R581.
54. Koerner A, Kratzsch J, Kiess W. Adipocytokines: leptin–the classical, resistin–the controversical, adiponectin–the promising, and more to come. *Best Pract Res Clin Endocrinol Metab*. 2005;19:525–546.
55. Bonetti PO, Lerman LO, Lerman A. Endothelial dysfunction: a marker of atherosclerotic risk. *Arterioscler Thromb Vasc Biol*. 2003;23:168–175.

Downloaded from http://jaha.ahajournals.org/ at Institut de Cardiologie de Montreal on November 11, 2014

3.2. Article 2

Title: Knockdown of angiopoietin like-2 protects against angiotensin II-induced cerebral endothelial dysfunction in mice.

This article was accepted for publication on the 12th of December, 2014 by *AJP – Heart and Circulatory Physiology* and is now in press.

3.2.1. Contribution of co-authors

Carol Yu: Conceived, designed and performed the experiments, analyzed the data, interpreted the results, prepared the figures and wrote the manuscript.

Xiaoyan Luo: Performed the experiments (aorta), interpreted the results and prepared the figures.

Natacha Duquette: Performed the tail-cuff experiments in mice.

Nathalie Thorin-Trescases: Conceived and designed the experiments, interpreted the results and edited the manuscript.

Eric Thorin: Conceived and designed the experiments, analyzed the data, interpreted the results and edited the manuscript.

tapraid4/zh4-ahea/zh4-ahea/zh400515/zh41402d15z | xppws | S=5 | 1/5/15 | 10:37 | MS: H-00278-2014 | Ini: 03/BYF/DH

Am J Physiol Heart Circ Physiol 308: H000–H000, 2015.
First published December 15, 2014; doi:10.1152/ajpheart.00278.2014.

Knockdown of angiopoietin like-2 protects against angiotensin II-induced cerebral endothelial dysfunction in mice

Carol Yu,[1,2] Xiaoyan Luo,[1] Natacha Duquette,[1] Nathalie Thorin-Trescases,[1] and Eric Thorin[1,3]
[1]*Montreal Heart Institute, Research Center, Montreal, Quebec, Canada;* [2]*Department of Pharmacology, Faculty of Medicine, Université de Montréal, Montreal, Quebec, Canada; and* [3]*Department of Surgery, Faculty of Medicine, Université de Montréal, Montreal, Quebec, Canada*

Submitted 28 April 2014; accepted in final form 12 December 2014

Yu C, Luo X, Duquette N, Thorin-Trescases N, Thorin E. Knockdown of angiopoietin like-2 protects against angiotensin II-induced cerebral endothelial dysfunction in mice. *Am J Physiol Heart Circ Physiol* 308: H000–H000, 2015. First published December 15, 2014; doi:10.1152/ajpheart.00278.2014.—Angiopoietin like-2 (angptl2) is a circulating pro-inflammatory and pro-oxidative protein, but its role in regulating cerebral endothelial function remains unknown. We hypothesized that in mice knockdown (KD) of angptl2, cerebral endothelial function would be protected against ANG II-induced damage. Subcutaneous infusion of ANG II (200 $ng \cdot kg^{-1} \cdot min^{-1}$, $n = 15$) or saline ($n = 15$) was performed in 20-wk-old angptl2 KD mice and wild-type (WT) littermates for 14 days. In saline-treated KD and WT mice, the amplitude and the sensitivity of ACh-induced dilations of isolated cerebral arteries were similar. However, while endothelial nitric oxide (NO) synthase (eNOS)-derived O_2^-/H_2O_2 contributed to dilation in WT mice, eNOS-derived NO ($P < 0.05$) was involved in KD mice. ANG II induced cerebral endothelial dysfunction only in WT mice ($P < 0.05$), which was reversed ($P < 0.05$) by either *N*-acetyl-L-cysteine, apocynin, gp91ds-tat, or indomethacin, suggesting the contribution of reactive oxygen species from Nox2 and Cox-derived contractile factors. In KD mice treated with ANG II, endothelial function was preserved, likely via Nox-derived H_2O_2, sensitive to apocynin and PEG-catalase ($P < 0.05$), but not to gp91ds-tat. In the aorta, relaxation similarly and essentially depended on NO; endothelial function was maintained after ANG II infusion in all groups, but apocynin significantly reduced aortic relaxation in KD mice ($P < 0.05$). Protein expression levels of Nox1/2 in cerebral arteries were similar among all groups, but that of Nox4 was greater ($P < 0.05$) in saline-treated KD mice. In conclusion, knockdown of angptl2 may be protective against ANG II-induced cerebral endothelial dysfunction; it favors the production of NO, likely increasing endothelial cell resistance to stress, and permits the expression of an alternative vasodilatory Nox pathway.

angiopoietin like-2; endothelium; cerebral arteries; nitric oxide; NADPH oxidases

CARDIOVASCULAR DISEASES, INCLUDING stroke, affect more than 50% of the world population today and remain an unresolved clinical issue. Inflammation and oxidative stress synergize to promote endothelial damage, thereby chronically driving atherogenesis (13, 14). Importantly, the cerebral vascular endothelium is highly sensitive to this deleterious environment (8, 11). Reactive oxygen species (ROS) are signaling molecules generated by the electron transport chain (35) and are by-products of enzymes including the NADPH oxidases (18), xanthine oxidases, and uncoupled endothelial nitric oxide (NO) synthase (eNOS) (19, 28). In parallel, they trigger an innate anti-oxidative system to prevent their potential harmful effects (26, 29). When this system is overwhelmed, however, endothelial damages occur and dysfunction develops, which are the first steps toward atherogenesis.

Recently, a protein identified as angiopoietin like-protein 2 (angptl2), a member of the greater angiopoietin-like family and derived from various cell types including adipocytes (31) and endothelial cells (12), has been implicated in a number of chronic inflammatory disorders such as insulin resistance (31), atherosclerosis (12), and tumor progression (1, 34). On the other hand, upregulation of angptl2 gene expression, among other genes, was recently reported in the aged brain of both rats and human, contributing to poststroke angiogenesis (5). Information of angptl2 involvement in regulating endothelial function, however, is limited. Endothelial cell-derived angptl2 promoted aortic endothelial dysfunction in tie2-angptl2 transgenic mice, as evidenced by lower ACh-mediated aortic relaxations and lower expression of phospho-eNOS (20). Accordingly, in angptl2$^{-/-}$ mice, endothelial function and eNOS phosphorylation were preserved against a severe high-fat diet (20). In line with this, we also observed protection against high-fat diet-induced endothelial dysfunction in angptl2 knockdown (KD) mice, through preservation of NO-mediated dilations in the femoral artery (40). There is no report on cerebral endothelial function, and the specific impact of angptl2 on the regulation of endothelial-derived relaxing factors (EDRFs) is only emerging (40). Through its pro-inflammatory (12, 31) and its pro-oxidative effects (2), we hypothesized that angptl2 contributes to cerebral endothelial dysfunction and the modulation of the EDRFs and that knockdown of angptl2 would increase endothelial cell stress resistance in mouse arteries. To test this hypothesis, we induced endothelial stress by chronic (14 days) infusion of a low dose of ANG II in angptl2 KD mice. We found that knockdown of angptl2 expression was associated with greater NO-dependent dilation, prevented ANG II-induced endothelial dysfunction, and induced an apocynin- and PEG-catalase-sensitive dilatory pathway, suggesting that angptl2 knockdown protects cerebral endothelial function and activates an alternative Nox dilatory pathway in the presence of ANG II, ultimately reinforcing endothelial stress resistance.

METHODS

Animals. All angptl2 KD and wild-type (WT) littermates used in this study were from our colony and were genotyped as previously described (40). Only male mice were used. Mice were kept under standard conditions (24°C; 12-h:12-h light/dark cycle) and were fed ad libitum with regular chow. Mice were euthanized by exsanguination under terminal anaesthesia (44 mg/kg ketamine and 2.2 mg/kg

Address for reprint requests and other correspondence: C. Yu, Montreal Heart Institute, Research Center, 5000 rue Bélanger, Montreal, Quebec, H1T 1C8, Canada

 0363-6135/15 Copyright © 2015 the American Physiological Society

Table 1. *Hemodynamic parameters of 6-month-old WT and angptl2 KD mice treated with chronic subcutaneous infusion of saline or ANG II for 14 days*

	Baseline		*Day 5*				*Day 14*			
			WT		KD		WT		KD	
	WT	KD	Saline	ANG II	Saline	ANG II	Saline	ANG II	Saline	ANG II
Blood pressure, mmHg										
Systolic	138 ± 4 (10)	148 ± 4 (10)	132 ± 5 (4)	132 ± 8 (6)	138 ± 7 (6)	140 ± 11 (4)	124 ± 6 (4)	134 ± 8 (6)	146 ± 8 (6)	143 ± 19 (4)
Diastolic	109 ± 4 (10)	116 ± 4 (10)	102 ± 3 (4)	104 ± 7 (6)	107 ± 7 (6)	107 ± 11 (4)	97 ± 7 (4)	109 ± 6 (6)	115 ± 8 (6)	109 ± 17 (4)
Heart rate, beats/min	587 ± 18 (10)	632 ± 13 (10)	558 ± 37 (4)	612 ± 33 (6)	654 ± 44 (6)	611 ± 23 (4)	616 ± 25 (4)	631 ± 27 (6)	632 ± 26 (6)	578 ± 32 (4)

Values are means ± SE of (*n*) mice. WT, wild-type mice; KD, knockdown mice treated with saline or ANG II for 14 days.

xylazine ip). All animal experiments were performed in accordance with the *Guide for the Care and Use of Experimental Animals of the Canadian Council on Animal Care* and the *Guide for the Care and Use of Laboratory Animals* of the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the Montreal Heart Institute Ethics Committee (ET No. 2010-62-1).

Chronic infusion of ANG II. For ANG II studies, male mice at 20 to 22 wk of age were subcutaneously implanted with an osmotic mini-pump (Alzet 1002; Cupertino, CA) prepared with either saline or 200 $ng \cdot kg^{-1} \cdot min^{-1}$ of ANG II (Abcam; ab120183) for 14 days. At this low dose, ANG II is pro-inflammatory and pro-oxidative, but does not induce changes in blood pressure (6), and therefore promotes endothelial dysfunction independently of its pressor effects. Mice were anesthetized and maintained throughout the procedure by inhalation of isoflurane (1–3% mixed with 97% O_2). For 3 wk before subcutaneous implantation, mice were trained by tail-cuff plethysmography (Kent Scientific, Torrington, CT) with baseline recording and during the 14 days of subcutaneous treatment, blood pressure (systolic, diastolic, and mean arterial pressures) and heart rate were noninvasively recorded on *days 5*, *10*, *12*, and *14* as previously described (3). For reference, blood pressure was also assessed under anesthesia by both Millar catheter and tail-cuff. After euthanasia, blood was drawn from mice by cardiac puncture at terminal anesthesia and plasma was subsequently collected. Mice were weighed, and weights of their hearts and tibia length recorded. The brain was removed from the cranial cavity, and the cerebral artery (posterior cerebral artery) was harvested. The brain was then snap frozen in liquid N_2 and subsequently kept at −80°C. Posterior cerebral arteries and aorta were harvested and placed in ice-cold physiological saline solution [PSS; pH 7.4; (in mmol/l) 119 NaCl, 4.7 KCl, 1.18 KH_2PO_4, 1.17 $MgSO_4$, 24.9 $NaHCO_3$, 1.6 $CaCl_2$, 0.023 EDTA, and 10 glucose] for in vitro endothelial function studies.

Plasma parameters. Glucose, triglyceride, and cholesterol (total, c-LDL, c-HDL) levels were measured by the Biochemistry Laboratory at the Montreal Heart Institute (Montreal, QC, Canada).

Endothelial function of cerebral arteries by pressurized arteriography. Segments of 2 to 3 mm of the posterior cerebral artery were dissected out in ice-cold PSS and cannulated at both ends in a pressurized arteriograph (Living Systems Instrumentation, St. Alban, VT) at 60 mmHg as previously described (10). The artery segment was equilibrated for 45 min, allowing for myogenic tone to develop. Then, a single dose of phenylephrine (PE; 1 to 10 μmol/l) to reach 30% to 50% of maximal diameter was added. A single cumulative concentration-response curve to ACh (0.1 nmol/l to 30 μmol/l) was obtained. To inhibit NOS, NO, cyclooxygenase (COX) enzymes, H_2O_2 production, mitochondrial O_2^- production, to nonspecifically inhibit Nox enzymes, or to specifically inhibit Nox2, N^{ω}-nitro-L-arginine (L-NNA; 100 μmol/l), 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO; 100 μmol/l), indomethacin (Indo; 10 μmol/l), PEG-catalase (100 U/ml), mito-TEMPO (5 μmol/l), apocynin (10 μmol/l), or gp91ds-tat (1 μmol/l; AnaSpec, Fremont, CA) were added, respectively, in the bath during equilibration (30–45 min) and during the experiment. To eliminate oxidative stress, the antioxidant *N*-acetyl-L-cysteine (NAC; 10 μmol/l) was added (37). Of note, we compared endothelial function in mice with and without subcutaneous saline-infused pump implantation and found no significant differences (data not shown), and we verified that NAC has no effect per se on vascular tone (data not shown). During equilibration and experiment, the artery segment was maintained at 37°C and aerated with 12%O_2-5%CO_2-83%N_2, generating an in vitro pO_2 of 150 mmHg, similar to mouse blood pO_2 (10). Dilation to sodium nitroprusside (SNP; 0.1 nmol/l to 30 μmol/l) was also recorded. For vasoconstriction studies, cerebral artery was equilibrated for 45 min before a single cumulative concentration-response curve to ANG II (0.1 nmol/l to 3 μmol/l) was obtained.

Fluorescence studies. Isolated mouse cerebral arteries were pressurized at 60 mmHg and incubated in oxygenated PSS at 37°C with either 5 μmol/l of 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (DCF-DA; a fluorescent dye-based free radical sensor; Molecular Probes) or 10 μmol/l of 4,5-diaminofluorescein diacetate (DAF-2; a fluorescent dye for NO detection; Calbiochem) with or without any inhibitors-PEG-catalase (100 U/ml) or L-NNA (100 μmol/l) to block H_2O_2 and NO production, respectively, for 30 min, after which vessels were washed three times with PSS, preconstricted with PE and dilated with 1 μmol/l ACh. We previously reported that in young healthy mice, ACh-induced cerebrovascular dilation was associated with a rise in DCF-DA fluorescence, a signal that was abolished by L-NNA, PEG-catalase, pyruvate (a H_2O_2 scavenger), or DETC (a SOD inhibitor that increases O_2^-), but not by PTIO (a NO scavenger), demonstrating the specificity of DCF-DA for H_2O_2 in our experimental conditions (10). On the other hand, we also reported that in the presence of excess levels of BH_4, the cofactor of eNOS, ACh-induced dilation was associated with a rise in DAF-2, a signal that was abolished by L-NNA or PTIO, but insensitive to catalase, demonstrating the specificity of DAF-2 for NO (10). Therefore, in our experimental conditions, DCF-DA detects H_2O_2 and DAF-2 detects NO. Parallel changes in cerebral arterial diameter and H_2O_2-fluorescence or NO-fluorescence intensities were recorded during the experiment, as described previously (10).

Table 2. *Baseline blood pressure measured in anesthetized WT and KD mice by tail-cuff plethysmography and by Millar catheter*

	WT	KD
Tail-cuff (under anesthesia)		
Blood pressure, mmHg		
Systolic	110 ± 2 (9)	110 ± 4 (7)
Diastolic	79 ± 2 (9)	81 ± 4 (7)
Millar (under anesthesia)		
Blood pressure, mmHg		
Systolic	99 ± 1 (7)	99 ± 2 (7)
Diastolic	64 ± 1 (7)	67 ± 1 (7)

Values are means ± SE of (*n*) mice.

tapraid4/zh4-ahea/zh4-ahea/zh400515/zh41402d15z | xppws | S=5 | 1/5/15 | 10:37 | MS: H-00278-2014 | Ini: 03/BYF/DH

Table 3. *Morphological parameters of 6-month-old WT and KD mice infused with either saline or a low dose of ANG II*

	WT		KD	
	Saline	ANG II	Saline	ANG II
Mouse weight, g	32.7 ± 0.8 (15)	34.2 ± 0.8 (18)	28.3 ± 1.1* (15)	28.9 ± 0.5* (16)
Heart weight, mg	128 ± 4 (11)	133 ± 4 (12)	132 ± 9 (9)	135 ± 6 (12)
Tibia length, mm	22.1 ± 0.2 (11)	22.5 ± 02 (12)	20.5 ± 0.1* (9)	20.3 ± 0.1* (12)
Heart weight/tibia length	5.8 ± 0.2 (11)	5.9 ± 0.1 (12)	6.4 ± 0.5 (9)	6.7 ± 0.3*(12)
Heart weight/mouse weight	4.0 ± 0.1 (11)	4.0 ± 0.1 (12)	4.6 ± 0.4 (9)	4.8 ± 0.2* (12)

Values are means ± SE of (*n*) mice. *$P < 0.05$ vs. WT.

Structural measurements of cerebral arteries. Passive pressure-diameter measurements were conducted by the pressurized arteriograph in Ca^{2+}-free PSS and 10 μmol/l SNP. Increments of intraluminal pressure of the cerebral artery (20 to 160 mmHg, with a first 20 mmHg step followed by 20-mmHg steps), were recorded to reveal lumen diameter and outer diameter, to calculate structural properties of the cerebral artery. Arterial wall thickness, lumen diameter, and wall-to-lumen ratio were presented at 60 mmHg only to represent structural properties at physiological conditions of the cerebral artery.

Endothelial function of aorta by wire myography. Thoracic aortic rings of 2 mm were mounted on 20 μm tungsten wires in microvessel myographs (IMF; University of Vermont, Burlington, VT) as described previously (32). An optimal basal tension of 1.5 g was determined for vessels while no differences in basal tension were observed among groups (data not shown). The mounted aortic ring was equilibrated for 30 to 45 min, after which the contractility of each arterial ring was determined by a 40 mmol/l KCl-PSS solution, followed by two washout periods, after which vessels were allowed to further incubate for 30 to 45 min in the absence of any drugs, or in the presence of L-NNA (100 μmol/l), indomethacin (10 μmol/l), apocynin (10 μmol/l), or NAC (10 μmol/l). Aortic segments were then preconstricted with a half-maximal effective concentration (EC_{50}) dose of thromboxane A_2 analog 9,11-dideoxy-11α, 9α-epoxymethano-prostaglandin $F_{2\alpha}$ (U46619, 0.1 nmol/l to 10 μmol/l), followed by relaxation curves to ACh (0.1 nmol/l to 30 μmol/l) or SNP (0.1 nmol/l to 30 μmol/l). During equilibration and experiment, aortic segment was aerated with 12%O_2-5%CO_2-83%N_2 at 37°C.

Cerebral vessels isolation and protein analysis by Western blot. Whole brain vessels were isolated from the brain as described previously (3, 9, 24), and 25 μg of proteins in a discontinuous Laemmli buffer were loaded and separated on 10% acrylamide SDS-PAGE gels, transferred onto nitrocellulose membranes, and probed with antibodies against COX-1 (160109; Cayman, Ann Arbor, MI), COX-2 (160106; Cayman), phospho-eNOS (9571; Cell Signalling, Danvers, MA), eNOS (ab66127; Abcam, Cambridge, UK), Nox1 (PA5-27967; ThermoFisher Scientific, Rockford, IL), Nox2 (ab43801; Abcam), Nox4 (NB110-58849; Novus Biologicals, Littleton, CO), and α-actin (A5228; Sigma-Aldrich, St. Louis, MO). Membranes were then probed with respective anti-rabbit (GARGG-500; Peninsula Laboratories, St. Helens, UK) or anti-mouse (715-007-003; Jackson ImmunoResearch, West Grove, PA) secondary antibodies at a dilution factor of 1:10,000.

Statistical analysis. All data presented are means ± SE; *n* is the number of mice. EC_{50} is the half-maximum effective concentration as estimated by the GraphPad Prism 5.0 software for each dose-response curve, based on the variable slope sigmoidal dose-response curve formula: $Y = Bottom + ((Top - Bottom)/(1 + 10\hat{}(logEC_{50} - x)*Hillslope))$, where "bottom" is the value for Y at the plateau bottom, "top" is value for Y at plateau top, and "hillslope" describes the steepness of the dose-response curve. E_{max} is taken at the maximal ACh dose (30 μmol/l) to induce dilation. For all experiments, the unpaired student's *t*-test or 2-way ANOVA followed by Bonferroni post-tests were performed as needed.

RESULTS

Similar hemodynamic parameters in both WT and angptl2 KD mice. Before treatment with ANG II, WT and KD mice showed similar blood pressures and heart rates, assessed by tail-cuff in conscious mice (Table 1) and by tail-cuff and Millar in anesthetized mice (Table 2). Chronic infusion of ANG II for 14 days did not affect the hemodynamic parameters in both WT and KD mice, confirming the low, sub-pressor dose of ANG II (Table 1). Chronic infusion of ANG II did not change heart weight-to-tibia length ratios in either WT or KD mice compared with respective saline-treated animals; however, KD mice had significantly lower body weights (Table 3), in line with literature showing lighter body weights in angptl2-deficient mice (31), and smaller tibia length compared with WT mice with similar heart weights, resulting in significantly higher heart weight-to-mouse weight and heart weight-to-tibia length, respectively, in ANG II-treated KD compared with WT mice (Table 3).

Similar plasma parameters between WT and angptl2 KD mice. Interestingly, nonfasting glucose level was significantly lower in ANG II-treated KD compared with WT mice. Besides

Table 4. *Nonfasting plasma parameters of 6-month-old WT and KD mice infused with either saline or a low dose of ANG II*

	WT		KD	
	Saline	ANG II	Saline	ANG II
Glucose, mmol/l	19.2 ± 1.4 (5)	18.2 ± 0.8 (5)	16.4 ± 1.8 (5)	12.9 ± 0.7* (5)
Triglyceride, mmol/l	0.8 ± 0.2 (5)	0.6 ± 0.1 (5)	1.1 ± 0.2 (5)	0.8 ± 0.1 (5)
Cholesterol				
Total, mmol/l	3.5 ± 0.2 (5)	3.1 ± 0.3 (5)	3.1 ± 0.3 (5)	3.0 ± 0.3 (5)
HDL, mmol/l	3.0 ± 0.1 (5)	2.6 ± 0.2 (5)	2.6 ± 0.2 (5)	2.5 ± 0.3 (5)
LDL, mmol/l	0.14 ± 0.04 (5)	0.23 ± 0.03 (5)	0.18 ± 0.08 (5)	0.15 ± 0.02 (5)
Total/HDL	1.14 ± 0.03 (5)	1.20 ± 0.01 (5)	1.20 ± 0.05 (5)	1.21 ± 0.05 (5)
LDL/HDL	0.05 ± 0.01 (5)	0.09 ± 0.01 (5)	0.07 ± 0.02 (5)	0.07 ± 0.01 (5)

Values are means ± SE of (*n*) mice. *$P < 0.05$ vs. WT.

tapraid4/zh4-ahea/zh4-ahea/zh400515/zh41402d15z | xppws | S=5 | 1/5/15 | 10:37 | MS: H-00278-2014 | Ini: 03/BYF/DH

Table 5. *Spontaneous myogenic tone developed in isolated pressurized cerebral arteries from WT and KD mice infused with either saline or a low dose of ANG II*

	WT		KD	
	Saline	ANG II	Saline	ANG II
Control, %	4.9 ± 3.3 (10)	6.0 ± 2.2 (11)	11.7 ± 5.3 (7)	7.4 ± 2.9 (12)
+ L-NNA, %	24.0 ± 9.2* (4)	35.1 ± 9.2* (5)	33.8 ± 2.5* (4)	29.1 ± 10.7* (4)

Values are means ± SE of (n) mice. *$P < 0.05$ vs. control. L-NNA, N^{ω}-nitro-L-arginine.

that, levels of triglycerides, LDL, and HDL cholesterols remained similar among all groups (Table 4).

Myogenic tone was similar between WT and KD mice. The level of myogenic tone in isolated pressurized cerebral arteries from WT and KD mice was low, similar between strains and unaffected by ANG II treatment, but significantly increased by the NOS inhibitor L-NNA (Table 5). This is in accordance with our previous study (10), where we showed that in young healthy C57Bl6 mice, spontaneous myogenic tone that developed in cerebral arteries was low, but increased in the presence of L-NNA or the NO scavenger PTIO, suggesting that dilatory eNOS-derived NO is produced and counteracts the myogenic tone. The net result is a low level of myogenic tone.

Cerebral vasodilation was preferentially driven by H_2O_2 in WT mice and by NO in angptl2 KD mice. In the control settings, after saline infusion for 14 days in WT and KD mice, cerebrovascular endothelial function was comparable between both strains of mice (Fig. 1, *A* and *B*), as represented by the same efficacies (E_{max}) and sensitivity (EC_{50}) of ACh to dilate (Table 6). When L-NNA was used to inhibit the NOS enzyme,

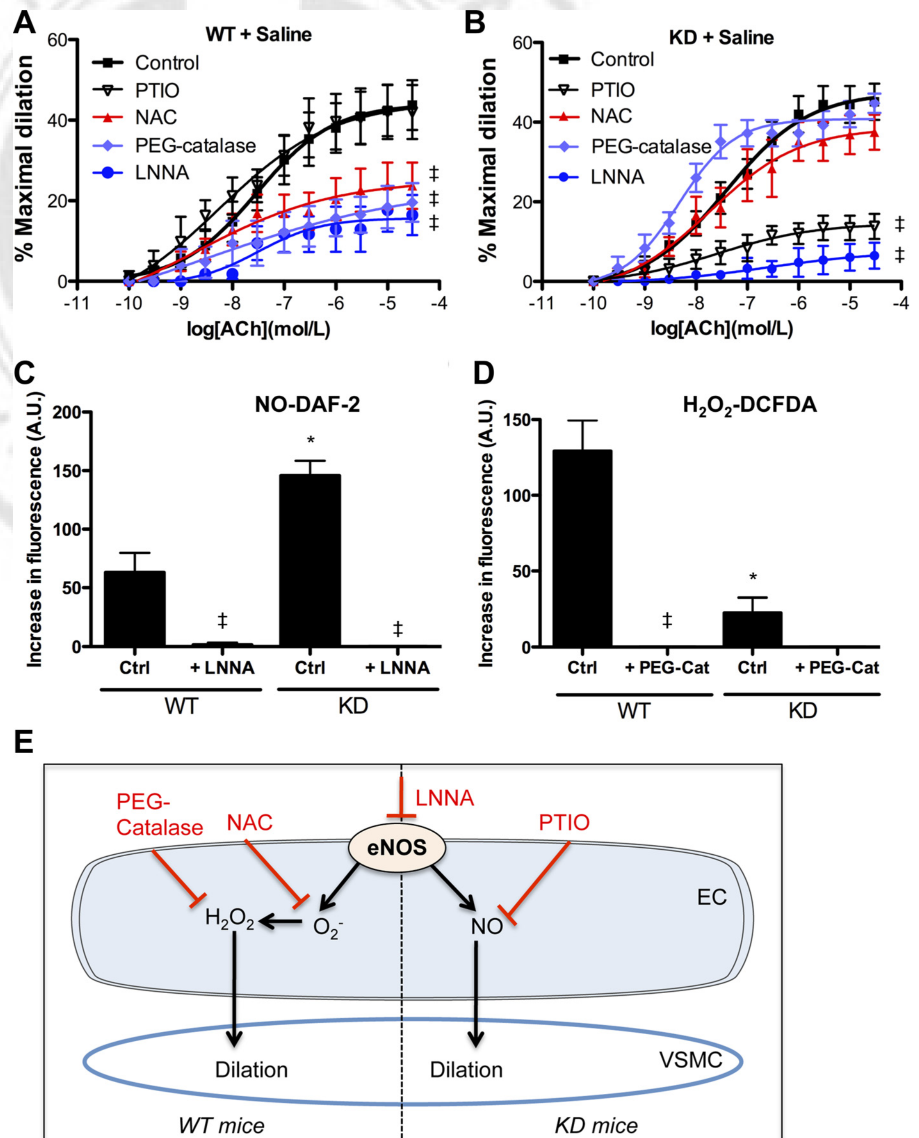

Fig. 1. ACh-mediated endothelium-dependent vasodilation with or without incubation with either *N*-acetyl-L-cysteine (NAC; 10 μmol/l), 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO; 100 μmol/l), PEG-catalase (PEG-cat; 100 U/ml), or N^{ω}-nitro-L-arginine (L-NNA; 100 μmol/l) in pressurized cerebral arteries of 6-mo-old wild-type (WT; *A*) and knockdown (KD; *B*) mice subcutaneously infused with a saline solution (*n* = 4–10). To assess nitric oxide (NO) and H_2O_2 production, pressurized cerebral arteries from 6-mo-old WT mice were loaded with 4,5-diaminofluorescein diacetate (DAF-2; *n* = 4–6; *C*) and 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (DCF-DA; *n* = 5 to 6; *D*), respectively, in the absence or presence of L-NNA or PEG-catalase. Average increases in fluorescence intensities are shown during addition of 10 μmol/l ACh. *$P < 0.05$ vs. WT; ‡$P < 0.05$ vs. no drug control (Ctrl). *E*: schematic dilatory pathways involved in ACh-induced dilation in cerebral arteries from WT and angptl2 KD mice. EC, endothelial cell; VSMC, vascular smooth muscle cell; AU, arbitrary units.

tapraid4/zh4-ahea/zh4-ahea/zh400515/zh41402d15z | xppws | S=5 | 1/5/15 | 10:37 | MS: H-00278-2014 | Ini: 03/BYF/DH

Table 6. *Efficacy (E_{max}) and sensitivity (EC_{50}) to ACh-mediated dilatation in pressurized cerebral arteries of WT and angptl2 KD mice treated with saline in control condition and a sub-pressor dose of ANG II, with or without presence of various inhibitors*

	WT + Saline		WT + ANG II		KD + Saline		KD + ANG II	
	E_{max}, %	EC_{50}	E_{max}, %	EC_{50}	E_{max}, %	EC_{50}	E_{max}, %	EC_{50}
Control	44 ± 6 (10)	7.5 ± 0.4 (10)	16 ± 2*(15)	7.1 ± 0.3 (15)	45 ± 5 (10)	7.6 ± 0.2 (10)	45 ± 4† (17)	8.0 ± 0.2† (18)
+ L-NNA	16 ± 5‡ (4)	7.1 ± 0.6 (4)	13 ± 3 (5)	6.2 ± 0.3 (5)	6 ± 3‡ (5)	6.3 ± 1.3‡ (4)	16 ± 14‡ (3)	5.7 ± 0.8‡ (3)
+ PTIO	42 ± 7 (5)	8.0 ± 0.3 (5)	ND	ND	14 ± 3‡ (6)	8.0 ± 0.6 (6)	ND	ND
+ NAC	24 ± 6‡ (6)	7.7 ± 0.5 (6)	39 ± 5‡ (7)	8.3 ± 0.2‡ (7)	37 ± 4 (5)	7.6 ± 0.4 (5)	42 ± 7 (7)	8.0 ± 0.3 (7)
+ Mito-TEMPO	ND	ND	14 ± 4 (4)	7.4 ± 0.5 (4)	ND	ND	45 ± 5 (5)	7.4 ± 0.1 (5)
+ PEG-Catalase	20 ± 5‡ (5)	7.6 ± 0.5 (5)	21 ± 6 (4)	6.7 ± 0.5 (4)	45 ± 2 (5)	8.3 ± 0.1 (5)	21 ± 2‡§ (5)	7.3 ± 0.2 (5)
+ Apocynin	58 ± 7 (6)	7.9 ± 0.3 (6)	29 ± 5‡* (7)	8.0 ± 0.3 (7)	49 ± 13 (8)	8.0 ± 0.2 (8)	24 ± 5‡ (7)	7.4 ± 0.3 (7)
+ gp91ds-tat	ND	ND	37 ± 6‡ (4)	7.4 ± 0.3 (4)	ND	ND	38 ± 6 (6)	7.8 ± 0.2 (6)
+ Indo	53 ± 8 (8)	7.4 ± 0.2 (8)	41 ± 8‡ (7)	7.7 ± 0.5 (7)	43 ± 7 (7)	8.5 ± 0.1 (7)	48 ± 9 (7)	7.6 ± 0.5 (7)

Values are means ± SE of (*n*) mice. NAC, *N*-acetyl-L-cysteine; Indo, indomethacin; PTIO, 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide; ND, not determined. Two-way ANOVA with Bonferreoni post-test: *$P < 0.05$ vs. WT + Saline; †$P < 0.05$ vs. WT + ANG II; ‡$P < 0.05$ vs. control; §$P < 0.05$ vs. KD + Saline.

response to ACh was significantly blunted in both strains of mice (Fig. 1 and Table 6), suggesting that endothelium-dependent dilatory responses in both WT and KD mice relied on eNOS activation. However, with the use of PTIO, a scavenger of NO, vasodilation in response to ACh was significantly blunted in KD mice, but not affected in WT mice, suggesting a role of eNOS-derived NO in KD mice only (Fig. 1, *A* and *B*; and Table 6). In addition, PEG-catalase significantly reduced ACh-mediated dilation in cerebral arteries from WT mice, but not from KD mice, suggesting a role of eNOS-derived H_2O_2 in WT only (Fig. 1, *A* and *B*; and Table 6). We indeed previously reported the physiological uncoupling of eNOS in cerebral arteries from WT mice, in which eNOS-derived H_2O_2 is the main EDRF (10). NAC, an antioxidant known to scavenge free radicals (30), also reduced the vasodilation induced by ACh in WT mice, but not in KD mice, suggesting that eNOS likely produces O_2^- as the intermediate to more stable H_2O_2 (Fig. 1, *A* and *B*; and Table 6). Altogether, these results suggest that eNOS-derived O_2^-/H_2O_2 contributed to vasodilation in cerebral arteries from WT mice and that, in contrast, eNOS-derived NO was preferentially involved in cerebral artery dilation of KD mice.

The fact that L-NNA almost completely abolished ACh-induced dilation in both WT and KD mice suggests that eNOS is the major source of O_2^-/H_2O_2 and NO, respectively. To further confirm our pharmacological data in WT and KD mice, NO and H_2O_2 production during ACh-induced dilation was assessed by incorporating the fluorescent ROS-reactive dyes, DAF-2 (10) and DCF-DA (7, 10), respectively (Fig. 1, *C* and *D*), in pressurized cerebral arteries of 6-mo-old WT and KD mice. Vasodilations to ACh resulted in significantly greater increase in NO-DAF-2 fluorescence intensities in KD compared with WT mice, a signal that was sensitive to NOS inhibitor L-NNA (Fig. 1*C*). In contrast, vasodilation by ACh resulted in significantly smaller increase in H_2O_2-DCF-DA fluorescence intensities in cerebral arteries of KD mice, which was sensitive to PEG-catalase (Fig. 1*D*). These data confirm that endothelium-dependent dilations of cerebral arteries were mainly driven by eNOS-derived NO in KD mice and by eNOS-derived H_2O_2 in WT mice (Fig. 1*E*).

To compare the downstream effects of NO on vasodilation between WT and KD mice, we tested cerebral vasodilation induced by SNP, an endothelial-independent NO donor, and found that it was similar across all groups of mice (data not shown). This suggests that endothelial function, but not vascular smooth muscle function, is modified by the knockdown of angptl2.

Infusion of a sub-pressor dose of ANG II induced cerebral endothelial dysfunction in WT but not in angptl2 KD mice. Next, we investigated the effects of a sub-pressor dose of ANG II known to induce oxidative stress and inflammation in the cerebrovasculature (6, 38). As shown in Fig. 2 and Table 6, after a 14-day ANG II treatment, WT mice showed, as expected, significantly impaired vasodilation to ACh compared with that of saline-treated control WT mice, whereas cerebral endothelial function was fully preserved in ANG II-treated KD mice compared with saline-treated control KD mice.

Endothelial dysfunction in ANG II-treated WT mice was reversed in the presence of NAC ($P < 0.05$), but not of Mito-TEMPO (Fig. 3*A* and Table 6), suggesting that ANG II-induced endothelial dysfunction was due to increased oxidative stress independent of the mitochondria. PEG-catalase and L-NNA did not affect endothelial dysfunction induced by ANG II (Fig. 3*A* and Table 6), suggesting that ANG II-induced oxidative stress blunted eNOS function. In contrast, endothelial dysfunction in ANG II-treated WT mice was reversed by both apocynin and gp91ds-tat ($P < 0.05$; Fig. 3*B* and Table 6), a

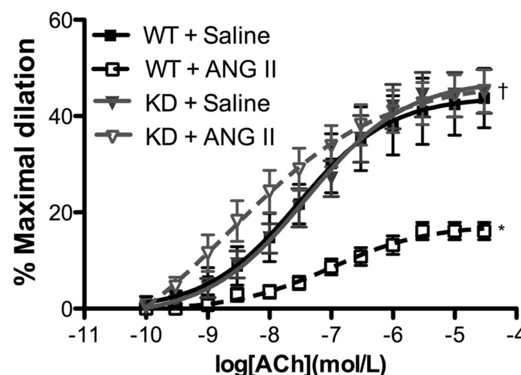

Fig. 2. ACh-mediated endothelium-dependent cerebral vasodilation in WT and angptl2 KD mice treated with either saline or ANG II ($n = 10–16$). *$P < 0.05$ vs. WT + saline; †$P < 0.05$ vs. WT + ANG II.

tapraid4/zh4-ahea/zh4-ahea/zh400515/zh41402d15z | xppws | S=5 | 1/5/15 | 10:37 | MS: H-00278-2014 | Ini: 03/BYF/DH

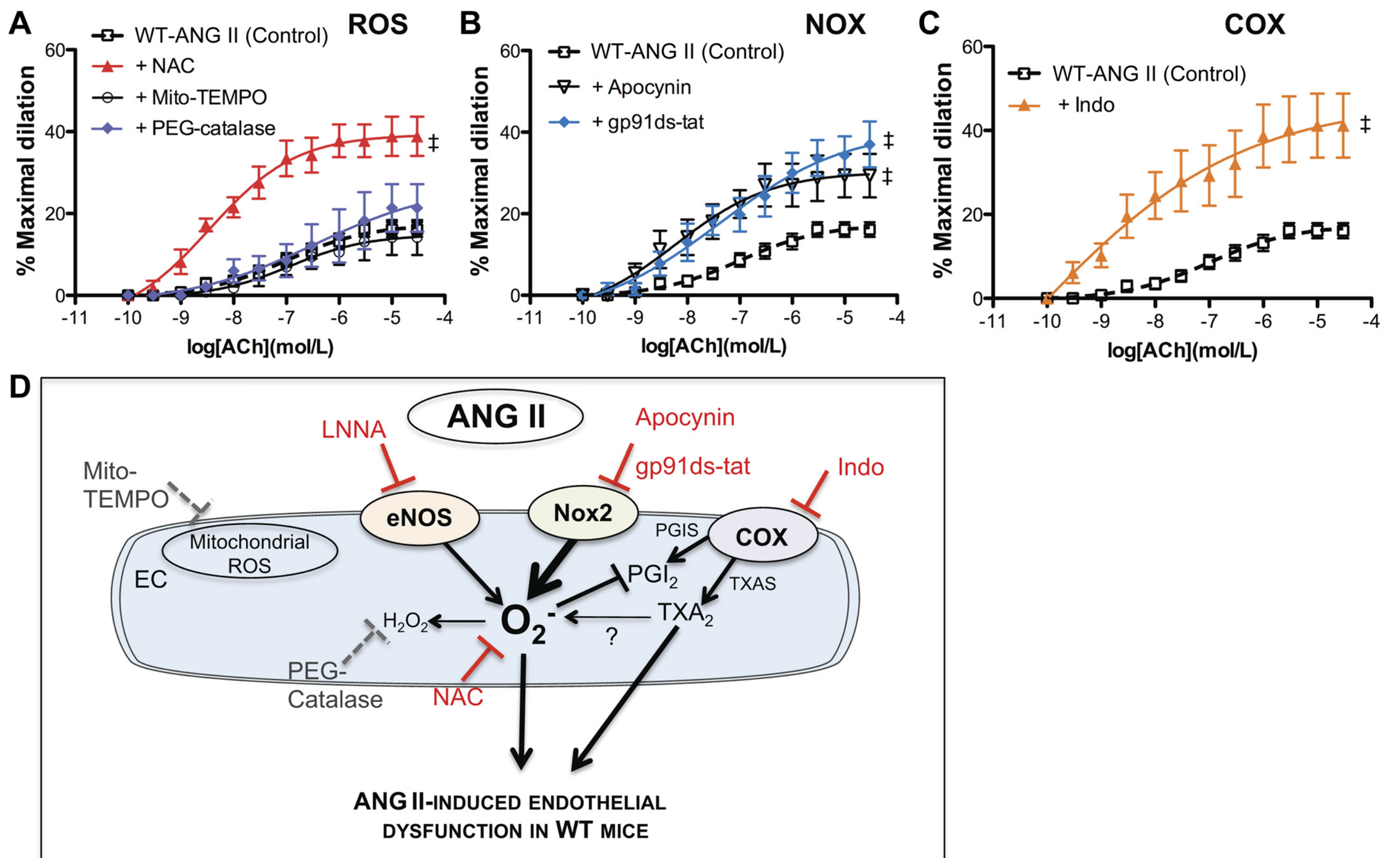

Fig. 3. ACh-mediated endothelium-dependent cerebral vasodilation in ANG II-treated WT mice, with or without incubation with NAC (n = 6 to 7), Mito-TEMPO (n = 4), or PEG-catalase (n = 5) (*A*); apocynin (n = 6 to 7) or gp91ds-tat (n = 5) (*B*); or indomethacin (Indo; n = 7; *C*). ‡$P < 0.05$ vs. control condition. *D*: schematic dilatory pathways involved in ANG II-treated WT mice. PGIS, prostacyclin synthase; PGI_2, prostacyclin; TXAS, thromboxane synthase; TXA_2, thromboxane A_2; ROS, reactive oxygen species.

nonspecific inhibitor of Nox and a specific Nox2 inhibitor, respectively, suggesting that ANG II-induced superoxide generation from Nox2. The difference between saline-treated and ANG II-treated WT mice is the activation of Nox2, likely producing in another cellular compartment large amounts of superoxide that is not converted to dilatory H_2O_2 (insensitive to the effect of PEG-catalase), thus creating a pro-oxidative deleterious environment. Accordingly, indomethacin also reversed endothelial dysfunction in ANG II-treated WT mice ($P < 0.05$; Fig. 3*C* and Table 6), suggesting possible detrimental effects of COX-derived contractile EDCF such as thromboxane A_2. Indeed, COX produces both dilatory (prostacyclin) and contractile (thromboxane A_2) factors, but in the presence of ROS, prostacyclin synthase can be nitrosylated and inactivated (41). In turn, this reveals the contractile component of COX activity, sensitive to indomethacin. Altogether, these data suggest that in cerebral arteries from WT mice, ANG II induced superoxide generation from Nox2, which in turn favored EDCF production and endothelial dysfunction (Fig. 3*D*).

In KD mice, ANG II did not induce cerebral endothelial dysfunction (Fig. 2). NAC or Mito-TEMPO, as expected, did not counteract the vasodilating effects of ACh in these mice (Fig. 4*A* and Table 6), whereas L-NNA reduced ($P < 0.01$) vascular sensitivity to ACh (Table 6), suggesting preserved eNOS function in ANG II-treated KD mice. However, PEG-catalase also significantly reduced ACh-induced dilation (Fig. 4*A* and Table 6), suggesting the involvement of H_2O_2 as a dilatory pathway in ANG II-treated KD mice that was not observed in saline-treated KD mice. Apocynin also significantly decreased the efficacy of ACh to vasodilate ($P < 0.05$), as depicted in Fig. 4*B* and Table 6, whereas the Nox2 inhibitor gp91ds-tat was ineffective. Therefore, the combined contribution of eNOS-derived NO and Nox-derived H_2O_2 likely preserved the dilatory response of cerebral arteries from ANG II-treated KD mice. Indomethacin did not affect the response to ACh, suggesting that neither prostacyclin nor thromboxane A_2 significantly regulates tone (Fig. 4*C* and Table 6). Thus these data support the involvement of H_2O_2 as a complementary dilatory pathway in cerebral arteries from ANG II-treated KD mice (Fig. 4*D*), but not ANG II-treated WT mice.

Of note, vasoconstriction of cerebral arteries in response to acute addition of ANG II in the experimental bath was similar between WT and KD mice (data not shown). In addition, response to SNP in ANG II-infused mice was not different among all the groups (data not shown). Meanwhile, structural properties of cerebral arteries, such as arterial lumen diameter and wall thickness, among different treatment groups of mice of both strains remained similar: arteries from WT mice were structurally similar to those from KD mice (Fig. 5).

tapraid4/zh4-ahea/zh4-ahea/zh400515/zh41402d15z | xppws | S=5 | 1/5/15 | 10:37 | MS: H-00278-2014 | Ini: 03/BYF/DH

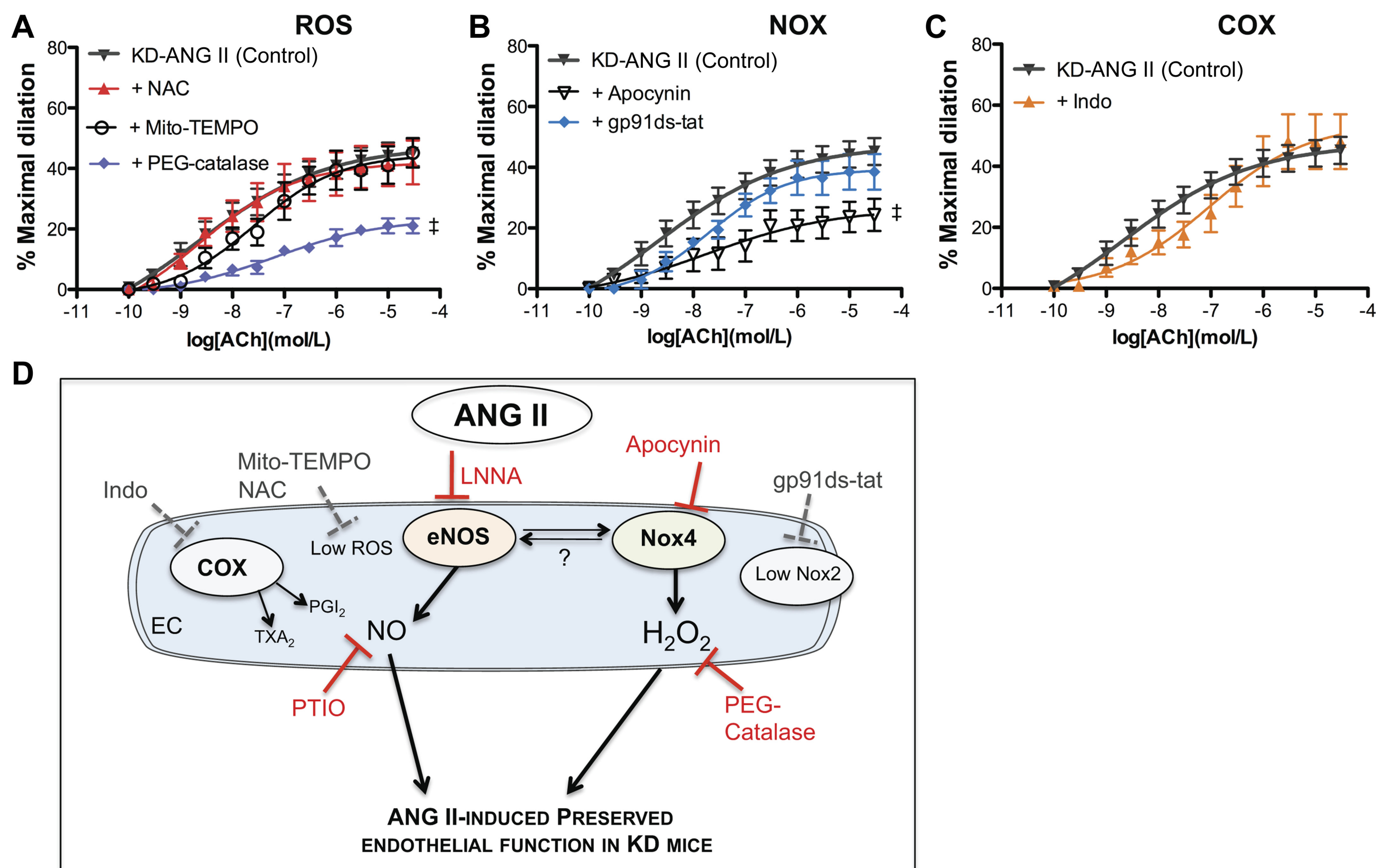

Fig. 4. ACh-mediated endothelium-dependent cerebral vasodilation in ANG II-treated angptl2 KD mice, with or without incubation with NAC (n = 5–7), Mito-TEMPO (n = 5), or PEG-catalase (n = 5) (*A*); apocynin (n = 7 to 8) or gp91ds-tat (n = 5) (*B*); or indomethacin (n = 7; *C*). ‡$P < 0.05$ vs. control condition. *D*: schematic dilatory pathways involved in ANG II-treated angptl2 KD mice. COX, cyclooxygenase; eNOS, endothelial NO synthase.

Endothelial function in the aorta remained unchanged after ANG II infusion, but recruited prostacyclin in angptl2 KD mice. Aortic endothelial function was similar between saline-treated WT and KD mice (Fig. 6, *A* and *B*, and Table 7). In contrast with resistance cerebral arteries, aorta of saline-treated WT and KD mice both relied similarly on NO to relax (Fig. 6, *A* and *B*, and Table 7), as L-NNA significantly reduced ACh-mediated relaxation (Fig. 6, *A* and *B*), while the antioxidant NAC had no effect (Fig. 6, *C* and *D*). Although we observed significant detrimental effects of a chronic sub-pressor dose of ANG II in the cerebral arteries of WT mice, aortic endothelial function after ANG II infusion was unaffected (Fig. 6, *A* and *B*, and Table 7). Unexpectedly, apocynin inhibited dilation in both saline- and ANG II-treated WT mice (Fig. 6*E* and Table 7). On the other hand, and as observed in cerebral arteries, apocynin was only effective in inhibiting relaxation in ANG II-treated, but not saline-treated, KD mice (Fig. 6*F* and Table 7), suggesting again that Nox also contributed to the vasorelaxation in these ANG II-treated KD mice. Furthermore, only in angptl2 KD mice treated with ANG II, indomethacin, a COX inhibitor, significantly reduced efficacy of ACh-induced response (Fig. 6, *G* and *H*, and Table 7), suggesting possible recruitment of prostacyclin to mediate vasodilation. Aortic relaxations to SNP were similar across all groups (data not shown).

Protein expression of Nox4 in cerebral vessels was greater in control KD mice, whereas Nox1 and Nox2 expressions were the same among all groups. Because we observed different responses to various inhibitors in the cerebral endothelial function studies, we further investigated protein expression of several enzymes involved in either vasodilation or ROS generation (Fig. 7). Isolation of cerebral vessels proteins revealed that although basal expression levels of Nox1 and Nox2 were similar across all groups of mice, basal Nox4 protein expression was greater in KD mice treated with saline only (Fig. 7). In addition, the ratio of phospho-eNOS to total eNOS expressions, COX-1, and -2 levels were also similar across all groups (Fig. 7).

DISCUSSION

Our report is the first to suggest that in cerebral arteries, knockdown of angptl2 switches eNOS activity from an O_2^-/H_2O_2 to NO producing enzyme and that this confers, at least partly, endothelial cell resistance to the stress imposed by ANG II. We also show that knockdown of angptl2 likely modifies the intracellular contribution of the Nox pathway, i.e., Nox-derived H_2O_2 (sensitive to apocynin and PEG-catalase) in cerebral arteries from ANG II-treated angptl2 KD mice, versus Nox-derived O_2^- (sensitive to apocynin and gp91ds-tat) in ANG II-treated WT mice. Altogether, these data strongly suggest that knockdown of angptl2 protects the endothelium through remodelling of the EDRFs and the recruitment of an additive dilatory Nox pathway, leading to preserved cerebral endothelial function under ANG II stimulation.

tapraid4/zh4-ahea/zh4-ahea/zh400515/zh41402d15z | xppws | S=5 | 1/5/15 | 10:37 | MS: H-00278-2014 | Ini: 03/BYF/DH

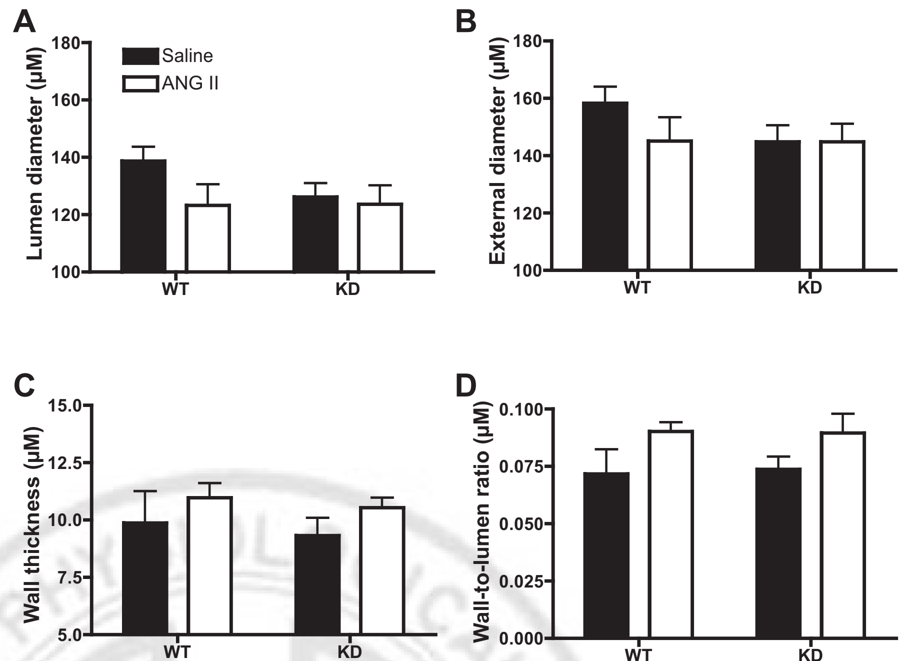

Fig. 5. A chronic sub-pressor dose of ANG II for 14 days did not have significant effects on structural properties of cerebral arteries pressurized at 60 mmHg, represented by lumen diameter (*A*), external diameter (*B*), wall thickness (*C*), and wall-to-lumen ratio (*D*); n = 6–12.

The loss of NO-dependent relaxation, anti-adhesion, and anti-aggregant activities is the main criteria defining endothelial dysfunction. In two recent reports, it was shown that angptl2 promoted endothelial dysfunction in a pro-atherogenic environment, decreased relaxation and expression of phospho-eNOS (20), increased leukocytes adhesion, and ultimately accelerated atherogenesis (12). Our demonstration that knockdown of angptl2 favors NO-dependent dilation in cerebral arteries therefore suggests that angptl2 plays a role in reducing endothelial cell stress resistance. Indeed, endothelium-dependent dilation of isolated cerebral arteries from angptl2 KD mice was sensitive to the NO scavenger PTIO and associated with a greater amount of eNOS-derived NO production in response to ACh. This is in contrast with WT mice, whose cerebral vasodilation has been shown by us to depend mainly on eNOS-derived H_2O_2 (10). We indeed previously demonstrated that in cerebral arteries isolated from young and healthy mice, eNOS was physiologically uncoupled and that H_2O_2 derived from eNOS activity was an EDRF in these arteries (10). This uncoupling of eNOS was not due to low levels of the cofactor BH_4 and did not favor the monomer configuration of eNOS, both conditions observed during pathological eNOS uncoupling (10). In addition, we previously demonstrated that this eNOS-derived H_2O_2-dependent response was absent in cerebral arteries from eNOS$^{-/-}$ mice and was also absent in peripheral arteries such as *gracilis* arteries, showing that this apparent eNOS uncoupling was specific to the cerebrovasculature (10). The finding that cerebral endothelial function in KD was mainly driven by NO suggests that knockdown of angptl2 protects the cerebral endothelium. Moreover, the impact of knocking down angptl2 specifically affected endothelial function as direct stimulation of the NO pathway using SNP in smooth muscle cell led to similar dilatory response in both strains.

ANG II, at a sub-pressor dose, is known to impose low-grade inflammation and oxidative stress, leading to endothelial dysfunction (6). We previously reported that ANG II stimulated free radical production in isolated mouse resistance arteries, as well as COX activity (22). Therefore, protective or reversible effects by NAC and indomethacin were expected and reflect a global disruption of the redox regulation by ANG II. In cerebral arteries from WT mice, ANG II produced endothelial dysfunction that was dependent on the combined effects of oxidative stress and the Nox systems (6, 36), as well as the COX systems, since the addition of either the antioxidant NAC, apocynin, gp91ds-tat, or indomethacin reversed this dysfunction. Thus, in cerebral arteries from WT mice, ANG II induced superoxide generation from Nox2, which in turn favored EDCF production and endothelial dysfunction. We speculated that in angptl2 KD mice, the cerebral endothelium would be less sensitive to ANG II-dependent stress because of the augmented NO production. Our results support this hypothesis, since there was complete prevention of ANG II-induced endothelial dysfunction in cerebral arteries from angptl2 KD mice. It is important to stress that ANG II induced a reversible cerebral endothelial dysfunction, as shown by the normalized dilatory response in the presence of antioxidant NAC, indomethacin, and apocynin in WT mice. It has been reported that angptl2 was involved in plasma membrane recycling of type 1 angiotensin II receptor (AT_1R) (15–17, 34) and that, in isolated perfused kidneys, renal vascular resistance in response to ANG II was lower in angptl2 knock-out mice (25). We observed, in contrast, that vasoconstriction to ANG II was similar in both WT and KD mice. This suggests that it is unlikely that angptl2 directly interacts with the smooth muscle cell ANG II/AT_1R pathway, at least in cerebral arteries.

Apocynin, a nonspecific Nox inhibitor (27), generated opposite responses to ACh-dependent dilation: whereas in ANG II-treated WT mice, apocynin expectedly restored a normal endothelial function (36), it reduced ACh-dependent dilation of cerebral arteries isolated from ANG II-treated KD mice. The more specific Nox2 inhibitor gp91ds-tat was also able to

tapraid4/zh4-ahea/zh4-ahea/zh400515/zh41402d15z | xppws | S=5 | 1/5/15 | 10:37 | MS: H-00278-2014 | Ini: 03/BYF/DH

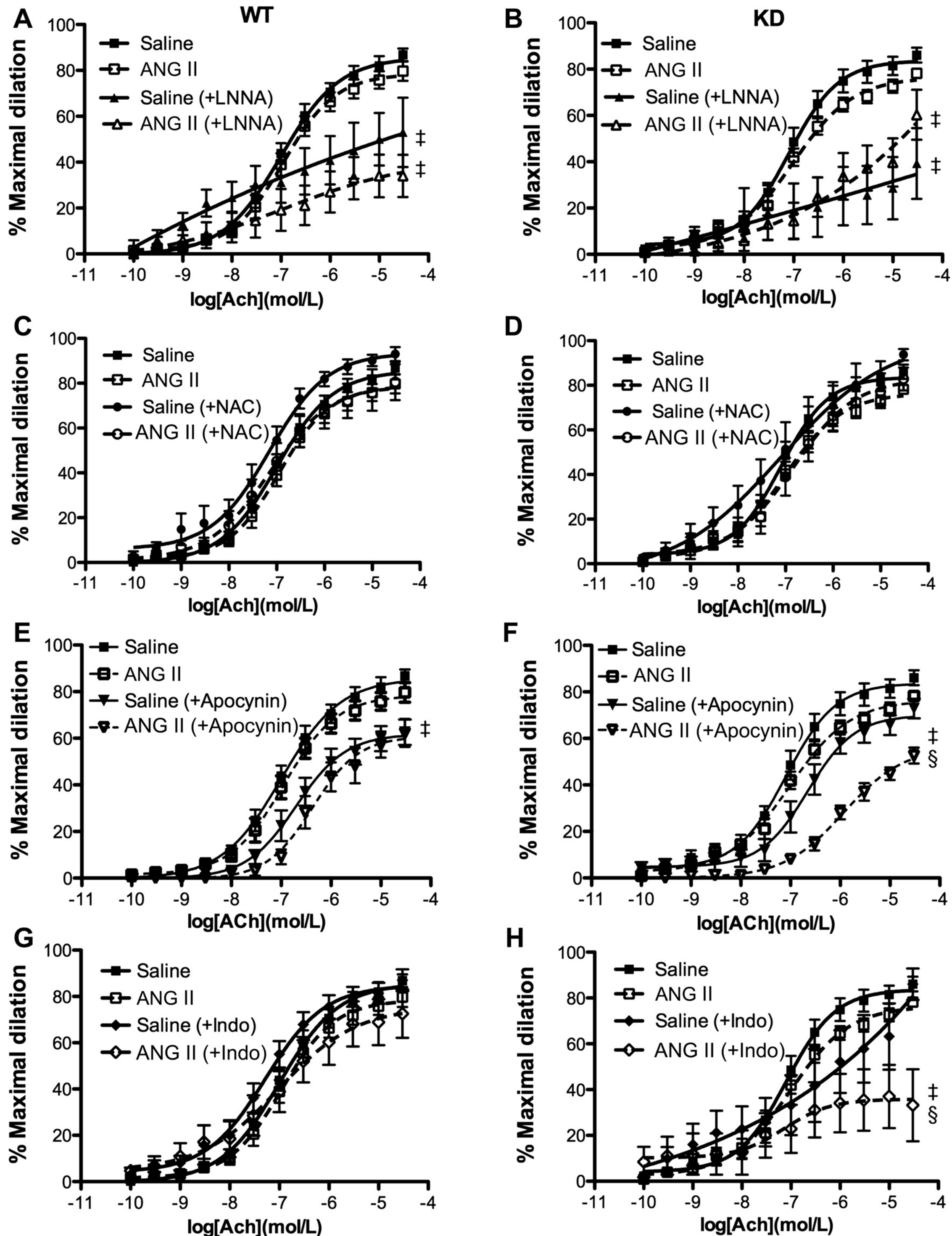

Fig. 6. ACh-mediated endothelium-dependent vasodilation in the aorta of saline or ANG II-treated 6-mo-old WT and angptl2 KD mice with or without incubation with either L-NNA (100 μmol/l, n = 4–7; *A* and *B*), NAC (10 μmol/l, n = 4–7; *C* and *D*), apocynin (10 μmol/l, n = 5–9; *E* and *F*), or indomethacin (10 μmol/l, n = 3–7; *G* and *H*). ‡P < 0.05 vs. no drug control; §P < 0.05 vs. KD + saline.

reverse endothelial dysfunction in ANG II-treated WT, without any effects in KD mice. Altogether, this supports a paradigm that angptl2 regulates the Nox pathway. Because Nox1 and Nox2 produce superoxide and Nox4 produces dilatory H_2O_2 (23), it is possible that knockdown of angptl2 leads to a shift from Nox1/2 to Nox4. This is supported by the lack of effect of NAC in ANG II-treated KD mice, an antioxidant capable of scavenging eNOS- or Nox2-derived O_2^- but not Nox4-derived H_2O_2. In addition, the beneficial effect of Nox2 inhibitor gp91ds-tat in ANG II-treated WT mice only and the inhibitory effect of PEG-catalase in ANG II-treated KD mice only confirm the contribution of Nox2-derived O_2^- in WT mice and the

tapraid4/zh4-ahea/zh4-ahea/zh400515/zh41402d15z | xppws | S=5 | 1/5/15 | 10:37 | MS: H-00278-2014 | Ini: 03/BYF/DH

Table 7. *Efficacy (E_{max}) and sensitivity (EC_{50}) to ACh-mediated relaxation of the aorta of WT and angptl2 KD mice treated with saline in control condition and a sub-pressor dose of ANG II, with or without presence of various inhibitors*

	WT + Saline		WT + ANG II		KD + Saline		KD + ANG II	
	E_{max}, %	EC_{50}	E_{max}, %	EC_{50}	E_{max}, %	EC_{50}	E_{max}, %	EC_{50}
Control	87 ± 3 (9)	7.0 ± 0.1 (10)	80 ± 4 (10)	6.9 ± 0.1 (10)	86 ± 3 (9)	7.1 ± 0.1 (10)	78 ± 2 (10)	7.2 ± 0.2 (11)
+ L-NNA	53 ± 15‡ (5)	NM	34 ± 9‡ (7)	NM	39 ± 15‡ (4)	NM	52 ± 13‡ (7)	NM
+ NAC	93 ± 3 (5)	7.1 ± 0.1 (5)	80 ± 8 (7)	7.2 ± 0.1 (7)	94 ± 3 (4)	7.0 ± 0.5 (5)	83 ± 5 (6)	7.7 ± 1.0 (7)
+ Apocynin	63 ± 5‡ (10)	6.7 ± 0.1 (11)	62 ± 6 (7)	6.1 ± 0.3‡ (7)	73 ± 4 (9)	6.7 ± 0.1 (10)	53 ± 3‡§ (6)	5.7 ± 0.3‡§ (6)
+ Indo	87 ± 5 (5)	7.3 ± 0.1 (6)	73 ± 10 (7)	7.0 ± 0.3 (7)	86 ± 7 (4)	6.0 ± 0.5 (3)	33 ± 16‡§ (6)	7.0 ± 0.3 (3)

Values are means ± SE of (*n*) mice. NM, not measurable. Two-way ANOVA with Bonferreoni posttest: ‡$P < 0.05$ vs. control; §$P < 0.05$ vs. KD + Saline.

contribution of Nox4-derived H_2O_2 in KD mice. We evaluated basal protein expressions of the main Nox enzymes responsible for generating ROS: Nox1, Nox2, and Nox4. However, they were similar among all groups and were unchanged after the infusion of ANG II at a sub-pressor dose, with the exception that Nox4 was expressed at a higher levels in angptl2 KD mice treated with saline. Collectively, protein expressions of the Nox isoforms failed to explain the effects of apocynin and

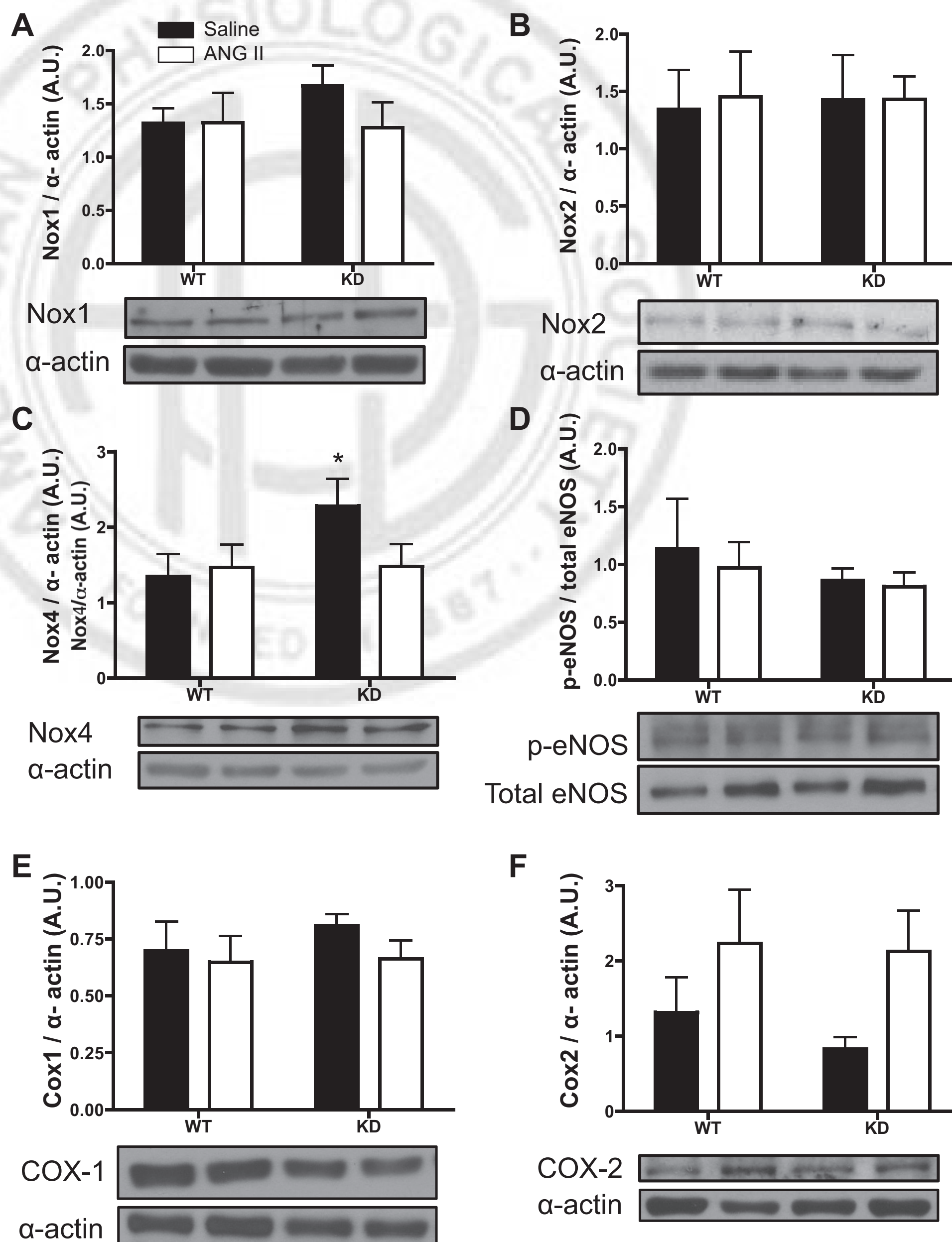

Fig. 7. Protein expression levels of Nox1 (n = 6–8; *A*), Nox2 (n = 4; *B*), Nox4 (n = 8; *C*), phospho-eNOS (p-eNOS; n = 4; *D*), COX-1 (n = 4; *E*), and COX-2 (n = 3 to 4; *F*) in cerebral vessels from WT and KD mice subcutaneously infused with saline (solid bar) or 200 ng·kg^{-1}·min^{-1} ANG II (clear bar). *$P < 0.05$ vs. WT + saline. AU, arbitrary units.

tapraid4/zh4-ahea/zh4-ahea/zh400515/zh41402d15z | xppws | S=5 | 1/5/15 | 10:37 | MS: H-00278-2014 | Ini: 03/BYF/DH

gp91ds-tat to affect cerebral endothelial function in KD mice treated with ANG II. Intriguingly, apocynin has been shown to prevent the translocation of the cytosolic component p47phox to Nox in endothelial cells (21), so that Nox isoform expressions alone in these mice may not reflect functional differences observed in cerebral arteries. To our knowledge, however, our study is the first to propose a link between Nox and angptl2.

Because of the heterogeneity of endothelial function in the vasculature, we studied in parallel the response of the endothelium in the aorta. Under control conditions, both WT and KD mice similarly depended on NO to relax, as shown by parallel inhibition of ACh-mediated dilation by L-NNA and by the lack of inhibitory effect of NAC. Most recently, another group similarly found comparable aortic endothelial function between angptl2 knock-out and WT mice when fed a normal chow (20). In contrast, when mice were exposed to a high-fat diet, endothelial dysfunction induced by the diet was lower in angptl2 knock-out mice than in WT mice, and this protection was associated with a lower eNOS deactivation (20). In our study, endothelial function in the aorta was not affected by ANG II, in either WT or angptl2 KD mice. The fact that the aortic endothelium mostly produces NO in both strains of mice, unlike that of cerebral arteries isolated from WT mice, could explain its greater resistance against the stress induced by ANG II. Apocynin surprisingly reduced ACh-induced relaxation of aorta from WT mice, whether or not treated with ANG II, implicating a differential contribution of Nox in the regulation of vascular tone between cerebral resistance and peripheral conductance arteries from WT mice. Such strong differences are expected based on the important heterogeneity of the endothelium in the vasculature, especially when one considers the unique features of the cerebral circulation (33) and the complexity of the Nox signaling pathways (4). Importantly, the significant inhibitory effect of apocynin in ANG II-treated angptl2 KD mice implies the likely contribution of Nox as a compensatory dilatory pathway when angptl2 is knocked-down. Therefore, as a general feature, we propose that the regulation of the Nox pathways is a target of angptl2 in the endothelium. Nonetheless, ANG II led to a compensatory response of the aortic endothelium, as the nonselective COX inhibitor indomethacin significantly reduced maximal dilation in the aorta of angptl2 KD mice only, indicating possible recruitment of prostacyclin-dependent vasorelaxation, which was not observed in WT littermates. This could be one of the beneficial effects of knocking-down levels of angptl2, since prostacyclin is known for its vascular protective properties.

One of the main limitations of the present study involves recognized deficiencies of the fluoroprobes used, especially DCF-DA. It has been reported that DCF could by itself reduce oxygen and cause production of superoxide at a high rate constant (39), and, therefore, may not be fully specific for H_2O_2. Our group, however, has previously reported that in young and healthy mice, similar to those used in the present study, ACh-induced cerebral vasodilation was associated with a rise in DCF-DA fluorescence, which was sensitive to L-NNA, PEG-catalase, and H_2O_2 scavenger pyruvate, as well as superoxide dismutase inhibitor DETC, but not to NO scavenger PTIO (10), thus demonstrating the greater relative specificity of DCF-DA to H_2O_2 versus NO or its derived reactive nitrogen species.

In conclusion, our results suggest that the knockdown of angptl2 in mice is able to preserve endothelial integrity when challenged with a sub-pressor dose of ANG II. Possible mechanisms may involve different contributions of EDRFs, since there is greater dependence on NO in angptl2 KD mice compared with WT mice that favor O_2^-/H_2O_2 in cerebral vasodilation. In the aorta, knockdown of angptl2 may contribute to recruitment of vasodilating effects of prostacyclin. In addition, knockdown of angptl2 may be associated with beneficial effects of a Nox, likely Nox4, an isoform that predominantly produces dilatory H_2O_2. Taken together, preventing the demonstrated rise of angptl2 through age (20) and atherosclerosis (12, 20) may emerge as a new therapeutic concept to increase endothelial cell stress resistance and delay atherogenesis.

GRANTS

This work was supported by Canadian Institutes of Health Research Grant MOP 14496 (to E. Thorin), the Grant in Aid from the Heart and Stroke Foundation of Canada, and the Foundation of the Montreal Heart Institute. C. Yu is a holder of the Postgraduate Scholarship from the Natural Sciences and Engineering Research Council of Canada.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: C.Y. and E.T. conception and design of research; C.Y., X.L., and N.D. performed experiments; C.Y., X.L., N.D., and N.T.-T. analyzed data; C.Y., X.L., N.T.-T., and E.T. interpreted results of experiments; C.Y., X.L., and N.T.-T. prepared figures; C.Y. drafted manuscript; C.Y., N.T.-T., and E.T. edited and revised manuscript; C.Y., X.L., N.D., N.T.-T., and E.T. approved final version of manuscript.

REFERENCES

1. **Aoi J, Endo M, Kadomatsu T, Miyata K, Nakano M, Horiguchi H, Ogata A, Odagiri H, Yano M, Araki K, Jinnin M, Ito T, Hirakawa S, Ihn H, Oike Y.** Angiopoietin-like protein 2 is an important facilitator of inflammatory carcinogenesis and metastasis. *Cancer Res* 71: 7502–7512, 2011.
2. **Aoi J, Endo M, Kadomatsu T, Miyata K, Ogata A, Horiguchi H, Odagiri H, Masuda T, Fukushima S, Jinnin M, Hirakawa S, Sawa T, Akaike T, Ihn H, Oike Y.** Angiopoietin-like protein 2 accelerates carcinogenesis by activating chronic inflammation and oxidative stress. *Mol Cancer Res* 12: 239–249, 2014.
3. **Bolduc V, Baraghis E, Duquette N, Thorin-Trescases N, Lambert J, Lesage F, Thorin E.** Catechin prevents severe dyslipidemia-associated changes in wall biomechanics of cerebral arteries in LDLr$^{-/-}$:hApoB$^{+/+}$ mice and improves cerebral blood flow. *Am J Physiol Heart Circ Physiol* 302: H1330–H1339, 2012.
4. **Brandes RP, Weissmann N, Schroder K.** Redox-mediated signal transduction by cardiovascular Nox NADPH oxidases. *J Mol Cell Cardiol* 73: 70–79, 2014.
5. **Buga AM, Margaritescu C, Scholz CJ, Radu E, Zelenak C, Popa-Wagner A.** Transcriptomics of post-stroke angiogenesis in the aged brain. *Front Aging Neurosci* 6: 44, 2014.
6. **Capone C, Faraco G, Park L, Cao X, Davisson RL, Iadecola C.** The cerebrovascular dysfunction induced by slow pressor doses of angiotensin II precedes the development of hypertension. *Am J Physiol Heart Circ Physiol* 300: H397–H407, 2011.
7. **Chaytor AT, Edwards DH, Bakker LM, Griffith TM.** Distinct hyperpolarizing and relaxant roles for gap junctions and endothelium-derived H_2O_2 in NO-independent relaxations of rabbit arteries. *Proc Natl Acad Sci USA* 100: 15212–15217, 2003.
8. **Chrissobolis S, Miller AA, Drummond GR, Kemp-Harper BK, Sobey CG.** Oxidative stress and endothelial dysfunction in cerebrovascular disease. *Front Biosci (Landmark Ed)* 16: 1733–1745, 2011.
9. **Drouin A, Farhat N, Bolduc V, Thorin-Trescases N, Gillis MA, Villeneuve L, Nguyen A, Thorin E.** Up-regulation of thromboxane A_2

tapraid4/zh4-ahea/zh4-ahea/zh400515/zh41402d15z | xppws | S=5 | 1/5/15 | 10:37 | MS: H-00278-2014 | Ini: 03/BYF/DH

impairs cerebrovascular eNOS function in aging atherosclerotic mice. *Pflügers Arch* 462: 371–383, 2011.

10. **Drouin A, Thorin-Trescases N, Hamel E, Falck JR, Thorin E.** Endothelial nitric oxide synthase activation leads to dilatory H_2O_2 production in mouse cerebral arteries. *Cardiovasc Res* 73: 73–81, 2007.
11. **Faraci FM, Lentz SR.** Hyperhomocysteinemia, oxidative stress, and cerebral vascular dysfunction. *Stroke* 35: 345–347, 2004.
12. **Farhat N, Thorin-Trescases N, Mamarbachi M, Villeneuve L, Yu C, Martel C, Duquette N, Gayda M, Nigam A, Juneau M, Allen BG, Thorin E.** Angiopoietin-like 2 promotes atherogenesis in mice. *J Am Heart Assoc* 2: e000201, 2013.
13. **Griendling KK, FitzGerald GA.** Oxidative stress and cardiovascular injury: Part I: basic mechanisms and in vivo monitoring of ROS. *Circulation* 108: 1912–1916, 2003.
14. **Griendling KK, FitzGerald GA.** Oxidative stress and cardiovascular injury: Part II: animal and human studies. *Circulation* 108: 2034–2040, 2003.
15. **Guo DF, Chenier I, Lavoie JL, Chan JS, Hamet P, Tremblay J, Chen XM, Wang DH, Inagami T.** Development of hypertension and kidney hypertrophy in transgenic mice overexpressing ARAP1 gene in the kidney. *Hypertension* 48: 453–459, 2006.
16. **Guo DF, Chenier I, Tardif V, Orlov SN, Inagami T.** Type 1 angiotensin II receptor-associated protein ARAP1 binds and recycles the receptor to the plasma membrane. *Biochem Biophys Res Commun* 310: 1254–1265, 2003.
17. **Guo DF, Sun YL, Hamet P, Inagami T.** The angiotensin II type 1 receptor and receptor-associated proteins. *Cell Res* 11: 165–180, 2001.
18. **Haas JA, Lockhart JC, Larson TS, Henrikson T, Knox FG.** Natriuretic response to renal interstitial hydrostatic pressure during angiotensin II blockade. *Am J Physiol Renal Fluid Electrolyte Physiol* 266: F117–F119, 1994.
19. **Hare JM, Stamler JS.** NO/redox disequilibrium in the failing heart and cardiovascular system. *J Clin Invest* 115: 509–517, 2005.
20. **Horio E, Kadomatsu T, Miyata K, Arai Y, Hosokawa K, Doi Y, Ninomiya T, Horiguchi H, Endo M, Tabata M, Tazume H, Tian Z, Takahashi O, Terada K, Takeya M, Hao H, Hirose N, Minami T, Suda T, Kiyohara Y, Ogawa H, Kaikita K, Oike Y.** Role of endothelial cell-derived angptl2 in vascular inflammation leading to endothelial dysfunction and atherosclerosis progression. *Arterioscler Thromb Vasc Biol* 34: 790–800, 2014.
21. **Johnson DK, Schillinger KJ, Kwait DM, Hughes CV, McNamara EJ, Ishmael F, O′Donnell RW, Chang MM, Hogg MG, Dordick JS, Santhanam L, Ziegler LM, Holland JA.** Inhibition of NADPH oxidase activation in endothelial cells by ortho-methoxy-substituted catechols. *Endothelium* 9: 191–203, 2002.
22. **Krummen S, Drouin A, Gendron ME, Falck JR, Thorin E.** ROS-sensitive cytochrome P450 activity maintains endothelial dilatation in ageing but is transitory in dyslipidaemic mice. *Br J Pharmacol* 147: 897–904, 2006.
23. **Lambeth JD, Neish AS.** Nox enzymes and new thinking on reactive oxygen: a double-edged sword revisited. *Annu Rev Pathol* 9: 119–145, 2013.
24. **Li X, Geary GG, Gonzales RJ, Krause DN, Duckles SP.** Effect of estrogen on cerebrovascular prostaglandins is amplified in mice with dysfunctional NOS. *Am J Physiol Heart Circ Physiol* 287: H588–H594, 2004.
25. **Mederle K, Schweda F, Kattler V, Doblinger E, Miyata K, Hocherl K, Oike Y, Castrop H.** The angiotensin II AT_1 receptor-associated protein Arap1 is involved in sepsis-induced hypotension. *Crit Care* 17: R130, 2013.
26. **Milisav I, Poljsak B, Suput D.** Adaptive response, evidence of cross-resistance and its potential clinical use. *Int J Mol Sci* 13: 10771–10806, 2012.
27. **Montezano AC, Touyz RM.** Oxidative stress, Noxs, and hypertension: experimental evidence and clinical controversies. *Ann Med* 44, *Suppl* 1: S2–S16, 2012.
28. **Paravicini TM, Touyz RM.** NADPH oxidases, reactive oxygen species, and hypertension: clinical implications and therapeutic possibilities. *Diabetes Care* 31, *Suppl* 2: S170–S180, 2008.
29. **Ray PD, Huang BW, Tsuji Y.** Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. *Cell Signal* 24: 981–990, 2012.
30. **Sun SY.** *N*-acetylcysteine, reactive oxygen species and beyond. *Cancer Biol Ther* 9: 109–110, 2010.
31. **Tabata M, Kadomatsu T, Fukuhara S, Miyata K, Ito Y, Endo M, Urano T, Zhu HJ, Tsukano H, Tazume H, Kaikita K, Miyashita K, Iwawaki T, Shimabukuro M, Sakaguchi K, Ito T, Nakagata N, Yamada T, Katagiri H, Kasuga M, Ando Y, Ogawa H, Mochizuki N, Itoh H, Suda T, Oike Y.** Angiopoietin-like protein 2 promotes chronic adipose tissue inflammation and obesity-related systemic insulin resistance. *Cell Metab* 10: 178–188, 2009.
32. **Thorin E, Meerkin D, Bertrand OF, Paiement P, Joyal M, Bonan R.** Influence of postangioplasty beta-irradiation on endothelial function in porcine coronary arteries. *Circulation* 101: 1430–1435, 2000.
33. **Thorin E, Shreeve SM.** Heterogeneity of vascular endothelial cells in normal and disease states. *Pharmacol Ther* 78: 155–166, 1998.
34. **Thorin-Trescases N, Thorin E.** Angiopoietin-like-2: a multifaceted protein with physiological and pathophysiological properties. *Expert Rev Mol Med* 16: e17, 2014.
35. **Turrens JF.** Mitochondrial formation of reactive oxygen species. *J Physiol* 552: 335–344, 2003.
36. **Virdis A, Neves MF, Amiri F, Touyz RM, Schiffrin EL.** Role of NAD(P)H oxidase on vascular alterations in angiotensin II-infused mice. *J Hypertens* 22: 535–542, 2004.
37. **Voghel G, Thorin-Trescases N, Farhat N, Mamarbachi AM, Villeneuve L, Fortier A, Perrault LP, Carrier M, Thorin E.** Chronic treatment with *N*-acetyl-L-cysteine delays cellular senescence in endothelial cells isolated from a subgroup of atherosclerotic patients. *Mech Ageing Dev* 129: 261–270, 2008.
38. **Wattanapitayakul SK, Weinstein DM, Holycross BJ, Bauer JA.** Endothelial dysfunction and peroxynitrite formation are early events in angiotensin-induced cardiovascular disorders. *FASEB J* 14: 271–278, 2000.
39. **Wrona M, Wardman P.** Properties of the radical intermediate obtained on oxidation of 2′,7′-dichlorodihydrofluorescein, a probe for oxidative stress. *Free Radic Biol Med* 41: 657–667, 2006.
40. **Yu C, Luo X, Farhat N, Daneault C, Duquette N, Martel C, Lambert J, Thorin-Trescases N, Rosiers CD, Thorin E.** Lack of angiopoietin-like-2 expression limits the metabolic stress induced by a high-fat diet and maintains endothelial function in mice. *J Am Heart Assoc* 3: e001024, 2014.
41. **Zou MH.** Peroxynitrite and protein tyrosine nitration of prostacyclin synthase. *Prostaglandins Other Lipid Mediat* 82: 119–127, 2007.

4. Discussion

Angptl2, a protein first identified merely 15 years ago (Kim *et al.* 1999b), has since been extensively studied in the context of many pathologies ranging from rheumatoid arthritis (Okada *et al.* 2010) and insulin resistance (Tabata *et al.* 2009) to various CVD (Tazume *et al.* 2012; Farhat *et al.* 2013; Horio *et al.* 2014) and even cancer (Aoi *et al.* 2011; Endo *et al.* 2012; Aoi *et al.* 2014; Endo *et al.* 2014; Gao *et al.* 2014; Odagiri *et al.* 2014), summarized in Figure 17. Despite its implication in a myriad of chronic inflammatory diseases, normal levels of angptl2 seem to have physiological effects such as in tissue repair (Kubota *et al.* 2005; Tabata *et al.* 2009) and angiogenesis (Kim *et al*. 1999b). Truly, angptl2 plays diverse roles in many different physiological and pathological environments (Kadomatsu *et al.* 2014), with compelling evidence supporting the pro-inflammatory role of angptl2, especially when in excess (Tabata *et al.* 2009; Aoi *et al.* 2011; Endo *et al.* 2012; Farhat *et al.* 2013; Horio *et al.* 2014; Odagiri *et al.* 2014). Inflammation has been known to be the underlying mechanism in many disease settings and it is highly implicated in the context of endothelial dysfunction (Libby *et al.* 2002), yet the pro-inflammatory role of angptl2 in ECs, endothelial function and its impact on the various EDRFs remains largely unexplored and has only been directly examined in one recent study (Horio *et al.* 2014).

The two studies presented in this work emphasized the role of angptl2 on regulating endothelial function. Since it is now known that different EDRFs participate in the maintenance of vascular tone in different vascular beds, we examined endothelial function in the large conductance arteries such as the aorta and the medium-sized femoral artery, as well as small resistance arteries such as the mesenteric arteries and cerebral arterioles. Owing to the technologies of recombinant protein production (Farhat *et al.* 2013; Farhat *et al.* 2014) as well as specific global gene knock-down in mice (Yu *et al.* 2014), the study of angptl2 in the regulation of endothelial function was possible, and unfolded angptl2 implication in endothelial dysfunction. In addition, using various vascular beds where different EDRFs contribute to endothelial-dependent dilation, it allowed us to study for the first time, the impact of angptl2 on different EDRFs. In this discussion chapter, some unresolved

issues will be discussed, alongside limitations of the presented work and potential future work to address unanswered questions.

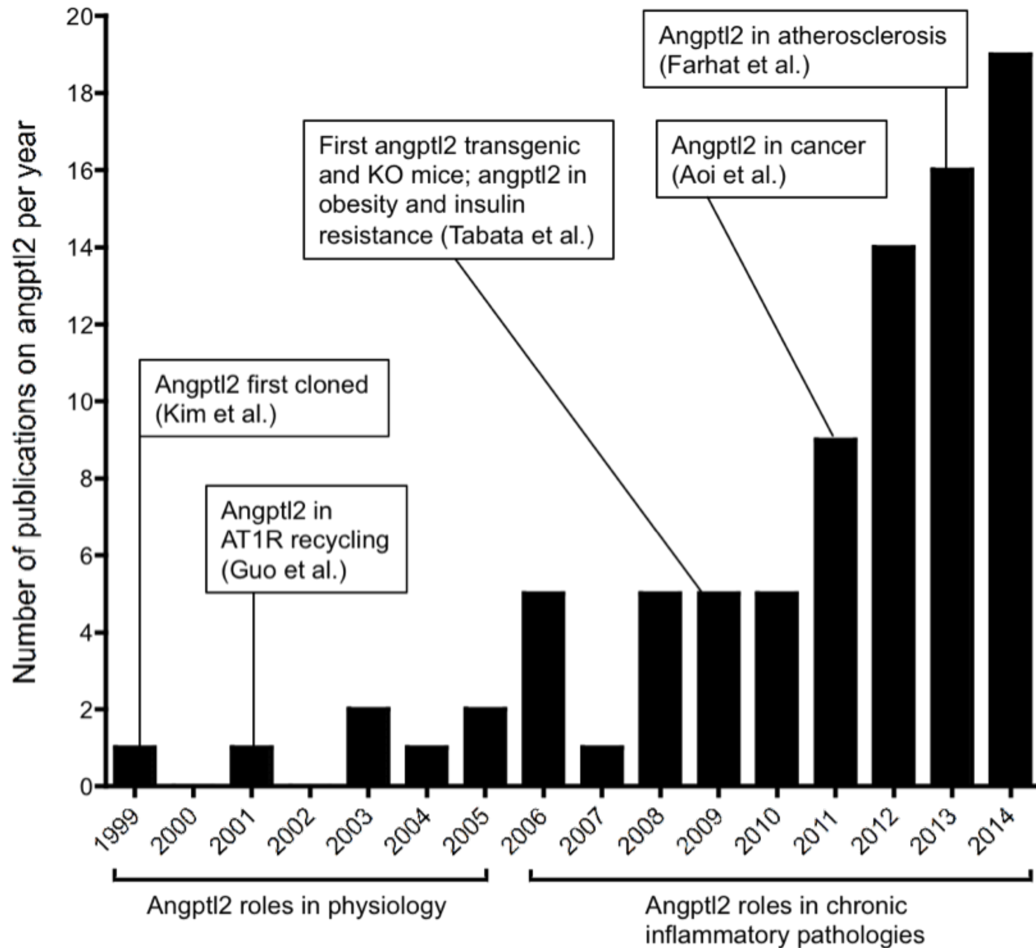

Figure 17. Exponentially growing scientific interests in angptl2 in physiology and pathophysiology.

4.1. Linking it all together: how does angptl2 mechanistically mediate endothelial dysfunction?

In the first study presented in this thesis, a recombinant angptl2 was used to investigate its acute effect on endothelial function measured by ACh-mediated vasodilation in the WT mouse femoral artery, where NO is the main EDRF. Incubation with recombinant angptl2 over the course of experiment indeed led to reduced ACh-mediated vasodilation. This observation led to the conclusion that

angptl2 had a direct deleterious effect on endothelial function. Most likely, at least in the femoral artery, angptl2 acutely exerted pro-oxidative effects directly on the endothelium, as the anti-oxidant NAC was able to acutely and completely reverse the effects of angptl2. The unresolved question is by which molecular pathway angptl2 was able to alter endothelial function? How was NAC able to suppress this dysfunction?

It was reported that in coronary ECs, angptl2 activated the NFκB pro-inflammatory cascade through activation of Rac1, a small Rho GTPase (Tabata *et al.* 2009), which subsequently inactivated eNOS activity (Horio *et al.* 2014). Rac1, besides its established role in innate immunity (Arbibe *et al.* 2000), is also linked to inflammation and ROS production (Sanlioglu *et al.* 2001; Cheng *et al.* 2006; Bedard and Krause 2007; Montezano and Touyz 2012). In particular, it was found to activate NFκB (Perona *et al.* 1997; Sanlioglu *et al.* 2001; Utsugi *et al.* 2006). In turn, NFκB has been linked to suppression of endothelial-dependent vasodilation in aging (de Winther *et al.* 2005; Csiszar *et al.* 2008). Therefore, this could be a potential molecular pathway by which angptl2 suppresses endothelial function in our studies, but this hypothesis will have to be tested. For now, there is preliminary data from our laboratory demonstrating that the recombinant angptl2 protein promotes massive and instantaneous ROS production in cultured human EC (Farhat *et al*. unpublished data). ROS, such as superoxide, as introduced in the introduction (section 1.1.4.1.), can directly decrease NO bioavailability (Mugge *et al.* 1991). Therefore, angptl2 likely induced ROS production in ECs, which then scavenged available NO, leading to endothelial dysfunction. Of interest, one of the many vascular protective properties of NO is the inhibition of NFκB activation (Peng *et al.* 1995; Matthews *et al.* 1996; Spiecker *et al.* 1997). Hence, it is also possible that angptl2 can participate in a feed-forward loop in ECs where it induces ROS production resulting in decreased NO bioavailability, which then loses the function to inhibit NFκB activation, while angptl2 continues to activate NFκB to promote inflammation, resulting in a vicious circle. Meanwhile, the acute reversing effects of NAC on attenuated ACh-mediated vasodilation can also support this hypothesis, as NAC is able to inhibit ROS (Sun 2010) by scavenging free radicals (Aruoma *et al.* 1989) or acting as a precursor for

intracellular cysteine and glutathione by acting as a thiol donor (Zafarullah *et al.* 2003), and has also been reported to inhibit the effects of NFκB through suppression of IκB degradation (Oka *et al.* 2000).

Interestingly, Rac1 is also an essential activator of Nox1 (Cheng *et al.* 2006) and Nox2 (Bedard and Krause 2007; Montezano and Touyz 2012), which both produce $O_2^{-\bullet}$ known to decrease NO bioavailability (Forstermann and Munzel 2006), but Rac1 is not an activator of Nox4 (Bedard and Krause 2007; Montezano *et al.* 2011; Montezano and Touyz 2012), which produces vasodilatory H_2O_2 (Schroder *et al.* 2012). As observed in the second study, chronic treatment with angII for 14 days may have resulted in greater production of Nox2-derived superoxide in WT *versus* angptl2 KD mice, in which Nox4-derived vasodilatory H_2O_2 may have gained importance. It remains to be elucidated whether or not angII could induce angptl2 expression and signaling through Rac1 in WT but not in angptl2 KD mice, which could further activate Nox1 and Nox2 to produce superoxide, ultimately leading to endothelial dysfunction.

Besides the integrin α5β1, TLR4 in ECs and monocytes has also been proposed to serve as a potential receptor for angptl2 (Oike and Tabata 2009). A main consequence of TLR4 signaling is pro-inflammatory cytokine induction (O'Neill *et al.* 2013). As such, TLR4 is known to recognize bacterial endotoxin lipopolysaccharide, leading to downstream activation of transcription factors, one of which is NFκB (O'Neill *et al.* 2013). This is particularly of interest in the context of endothelial dysfunction. Indeed, in a mouse model of obesity and diabetes, db/db mice with an additional mutation in TLR4 were protected from hypercholesterolemia, hyperglycemia and hypertension, and displayed preserved ACh-mediated vasorelaxation in the aorta and mesenteric artery, which was associated with normal eNOS phosphorylation levels (Liang *et al.* 2013). In line with this, TLR4 has been shown to contribute to early stages of atherogenesis in ApoE KO mice (Higashimori *et al.* 2011), and expression of TLR4 was also augmented in ECs and macrophages in human atherosclerotic lesions (Edfeldt *et al.* 2002). However, TLR4 is not considered to be a specific receptor for angptl2 and thus, we did not measure TLR4 expressions in

our studies. Their role in angptl2 regulation on endothelial function remains to be elucidated.

Taken together, the previously reported angptl2-α5β1-Rac1-NFκB (Tabata *et al.* 2009), as well as the hypothetical angptl2-TLR4 signaling pathways may provide insights for us to explain some of the observations regarding endothelial dysfunction induced by angptl2 and the beneficial effects of angptl2 knock-down.

4.2. Can angptl2 lower endothelial cell stress resistance?

Angptl2 may be pro-oxidative (Aoi *et al.* 2011; Aoi *et al.* 2014), which is also supported by our observation that NAC prevented angptl2-induced endothelial dysfunction and our preliminary data that recombinant angptl2 induced ROS production in ECs (Farhat *et al*. unpublished data), but on the other side of the coin, could angptl2 also interfere with the innate anti-oxidant defense mechanism in ECs?

With ROS being constantly generated over the course of life in the cells, as well as from the external environment, there is constant detoxification and elimination of these endogenous and exogenous oxidants by intricate anti-oxidant systems such as glutathione peroxidase, SOD, and catalase. In EC, these systems determine stress resistance against oxidative stress and NO function (Ungvari *et al.* 2010). A consequence of endothelial stress is a decline in both endothelial defense mechanisms and eNOS function, leading to a decrease in NO bioavailability (Brandes *et al.* 2005), as well as induction of compensatory vasodilatory pathways, as we observed previously in mice (Gendron and Thorin 2007; Gendron *et al.* 2010; Gendron *et al.* 2012). Pro-inflammatory and pro-oxidative angptl2 could, therefore, promote the cascade leading to endothelial stress.

In terms of the anti-oxidant defense systems, initial increased ROS has been reported to up-regulate the anti-oxidant defense mechanisms (Ungvari *et al.* 2010). Intriguingly, preliminary data from our laboratory demonstrates that acute addition of recombinant angptl2 in cultured EC for a short duration of 10 minutes up-regulates the nuclear protein expression of the transcription factor erythroid 2-related factor 2 (Nrf2), while 24-hour exposure significantly reduces it (Farhat *et al*. unpublished data). Nrf2 has emerged as a regulator of oxidative stress (Motohashi and Yamamoto

2004; Kensler *et al.* 2007; Ma and He 2012). Indeed, Nrf2 KO mice are significantly more susceptible to chemical toxicity and pathological conditions linked to oxidative stress (Kensler *et al.* 2007; Ma and He 2012). Under basal conditions, Nrf2 is suppressed by ubiquitination-proteasomal degradation mediated by Kelch-like erythroid cell-derived protein with cap 'n' collar homology-associated protein 1 (Keap1) (Taguchi *et al.* 2011). On the other hand, Nrf2 can be activated by shear stress (Hosoya *et al.* 2005; Dai *et al.* 2007) and dietary anti-oxidants (Thimmulappa *et al.* 2002) resulting in its stabilization and nuclear translocation. The activated Nrf2 can then induce anti-oxidant defense genes such as HO-1 (Chen *et al.* 2006). We found that chronic recombinant angptl2 treatment in ECs decreased HO-1 gene expression over 24 hours, which was associated with the lower nuclear expression of Nrf2 and increased expression of Keap1 at the same time-point (Farhat *et al*. unpublished data). In addition to its inhibitory effect on Nrf2, angptl2 could also affect other anti-oxidant defense systems, such as glutathione peroxidase, SOD, and catalase, but this remains to be demonstrated.

In the first study, we reported that with a 3-month HFD, there were attenuated vasodilations mediated by NO and EDHF in the femoral and mesenteric artery, respectively, isolated from the WT mice. In contrast, these vasodilatory pathways in the same vascular beds were preserved in the angptl2 KD mice, thus suggesting that low angptl2 levels may protect the endothelium and prevent the contribution of compensatory EDRFs in stressed vascular beds. This indirectly suggests that low angptl2 levels protect EC stress resistance. Does it preserve the anti-oxidant defense systems against increasing ROS and oxidative stress? This hypothesis can be supported by data gathered from the second study comparing endothelial function in cerebral arterioles, highly sensitive to oxidative stress, isolated from WT and angptl2 KD mice: in 6-month-old mice, NO production was significantly greater in cerebral arterioles of KD compared to WT mice, which produced greater levels of H_2O_2, as previously described by our laboratory (Drouin *et al.* 2007; Drouin and Thorin 2009). This observation may suggest enhanced stress resistance or lower oxidative stress in cerebral arterioles isolated from KD mice, leading to eNOS coupling and no production of endothelial eNOS-derived H_2O_2 in KD mice. Furthermore, angII

treatment for 14 days induced endothelial dysfunction in WT, but not in KD mice, again suggesting greater EC stress resistance against the deleterious effects of pro-inflammatory and pro-oxidative angII on endothelial function. This could most likely be explained by the lower oxidative stress in angptl2 KD mice after angII treatment, as addition of NAC restored vasodilation only in WT mice.

Taken together, the potential deleterious effects of angptl2 on the anti-oxidant defense system, therefore, may partially explain the preserved endothelial function in cerebral arterioles from angptl2 KD mice after 14 days, as well as the preservation of NO- and EDHF-mediated vasodilation in the femoral and mesenteric artery from KD mice after 3 months of HFD feeding. Analysis of Nrf2 nuclear expression, as well as HO-1 expression and other anti-oxidant defense systems in ECs isolated from these mice, will be necessary to support this hypothesis.

4.3. Could angptl2 also contribute to endothelial dysfunction *via* its role in AT1R recycling?

In the second study, low levels of angptl2 expression in angptl2 KD mice resulted in preservation of cerebral endothelial function when chronically challenged with angII. As explained earlier, this outcome may be in part due to lower oxidative stress in KD mice and greater EC stress resistance to angII. A less recognized role of angptl2, as introduced in sections 1.3.2.3 and 1.3.2.4., is its ability to promote recycling of the AT1R to the plasma membrane, prolonging its activity (Guo *et al.* 2001; Guo *et al.* 2003; Guo *et al.* 2006). Indeed, mice that overexpressed angptl2 in the kidney developed hypertension associated with over-activation of the intrarenal RAS (Guo *et al.* 2006). Could it be possible that the lack of angptl2 in KD mice contributed to preserved endothelial function after angII infusion *via* lower AT1R recycling?

In the vasculature, AT1R is predominantly expressed in VSMCs, but its expression can also be detected in ECs (Ramkhelawon *et al.* 2009; Saavedra 2012), and its endothelial expression has been associated with up-regulation of adhesion molecules such as VCAM-1 and P-selectin (Soehnlein *et al.* 2005). Importantly, its increased expression and activation, both in ECs (Ramkhelawon *et al.* 2009) and

VSMCs (Lyle and Griendling 2006), have been implicated in inflammation and increased ROS production, ultimately leading to endothelial dysfunction. In line with this, a major angII/AT1R downstream signaling target is the Nox enzymes (Nguyen Dinh Cat *et al.* 2013), and activity of Nox2 to produce $O_2^{\cdot -}$ appears to be more important in smaller resistance arteries (Griendling *et al.* 1994; Ushio-Fukai *et al.* 1996). In turn, ROS could regulate AT1R recycling, as reported recently (Nishida *et al.* 2011). In contrast, NO has been shown to reduce expression of endothelial AT1R (Ramkhelawon *et al.* 2009). Therefore, decreased NO bioavailability could, on top of impairing vascular function, also exacerbate effects of AT1R stimulation by angII, resulting in a feed-forward system. Accordingly, blockade of AT1R in the cerebral vasculature protected cerebral blood flow during stroke and decreased cerebral vascular inflammation (Saavedra 2011). Taken together, the potential role of angptl2 in mediating AT1R recycling intracellularly (Guo *et al.* 2001; Guo *et al.* 2003; Guo *et al.* 2006), and exacerbating angII effects could be one of the reasons why endothelial dysfunction developed in angII-treated WT mice, but not in angptl2 KD mice. Clearly, much work must be completed, such as determining AT1R protein expression and its turnover rate in vascular cells, in order to validate this hypothesis.

4.4. Could protection against high-fat diet-induced metabolic dysfunction in angptl2 KD mice be a consequence of blunted angiogenesis?

One of the initial consequence of excess caloric intake is the increased energy storage in adipocytes, which are encircled and nourished by a network of capillaries (Cao 2007). Along with the development of obesity, the vasculature surrounding the growing adipose tissue is now recognized to go through angiogenesis, which can also modulate adipogenesis (Rupnick *et al.* 2002; Fukumura *et al.* 2003; Cao 2007). Much like in pathological cancer settings, angiogenic vessels contribute to adipogenesis by supplying 1) oxygen and nutrients (Cao 2007), 2) growth factors and cytokines (Cao 2007), 3) circulating stem cells for differentiation (Crossno *et al.* 2006), and 4) infiltrating monocytes and neutrophils to adipocytes (Cao 2007; Powell 2007). In our first study, a HFD significantly increased body mass in WT mice, but to a lesser extent

in angptl2 KD mice, accompanied by smaller adipocyte size in both the mesenteric and epididymal white adipose tissues. In addition, we observed a worse lipid profile in WT compared to KD mice after a HFD. Since angptl2 can be derived from adipocytes (Tabata *et al.* 2009) and ECs (Farhat *et al.* 2013), could the overall protective effects of low angptl2 levels, as in the angptl2 KD mice, be a consequence of decreased angiogenesis in the adipose tissue? In fact, angptl2 has been initially reported to be a pro-angiogenic factor by inducing EC sprouting in HUVECs (Kim *et al*. 1999b). The pro-angiogenic features of angptl2 were confirmed (Farhat *et al*. 2014) and further supported by studies in hematopoietic stem cells (Broxmeyer *et al.* 2011) and zebrafish (Kubota *et al.* 2005). Therefore, there may possibly be slower blood vessel growth in the adipose tissues of angptl2 KD mice. In contrast to our hypothesis, the study by Tabata *et al*. using transgenic mice constitutively expressing angptl2 in the skin or adipose tissue developed vascular inflammation but not angiogenesis, as CD31, a marker of ECs (van Mourik *et al.* 1985), did not vary between WT and transgenic mice, and did not show improved hypoxia in adipose tissues after a HFD (Tabata *et al*. 2009). However, in contrast to our studies, adipocyte size did not seem to differ between the two strains of mice (Tabata *et al*. 2009). Undoubtedly, further investigations are necessary to determine whether angiogenic abilities in the adipose tissue vasculature were different between WT and KD mice, with or without the HFD treatment.

Related to angiogenesis and adipogenesis, expression levels of some adipokines such as leptin and adiponectin can also influence adipose mass (Friedman and Halaas 1998; Yamauchi *et al.* 2003). Interestingly, we observed that epididymal white adipose tissue in WT mice displayed lower adiponectin but greater leptin gene expressions after a HFD, while they did not change significantly after a HFD in angptl2 KD mice. The differential changes in gene expressions of these adipokines may perhaps explain the possible smaller adipocyte sizes observed in angptl2 KD mice. Adiponectin levels in the blood have been inversely correlated with BMI and have an overall negative effect in adipogenesis (Arita *et al.* 1999). Adiponectin is produced by adipocytes and has also been reported to inhibit EC proliferation, migration, and survival (Brakenhielm *et al.* 2004). On the other hand, leptin is

primarily produced by adipocytes and while its circulating levels positively correlate with size of fat depots (Harris 2014), it has also been reported to promote angiogenesis in HUVECs and porcine aortic ECs (Bouloumie *et al.* 1998). Importantly and related to the first study, after a HFD, 1) expression of angptl2 increased in WT mice, and 2) expression and plasma levels of leptin in KD mice were significantly lower than that in the WT mice, which may suggest a link between angptl2 and leptin in the context of angiogenesis. It is noteworthy to point out that expansion in adipose tissues, as seen in obesity, is associated with insulin resistance (Yki-Jarvinen 2005) and greater cholesterol synthesis and lower cholesterol absorption (Gylling and Miettinen 1997). Thus, the lack of angptl2 in KD mice could have partly contributed to the better lipid profile *via* decreased angiogenesis and adiposity, although it has been shown that angptl2 did not inhibit LPL (Miida and Hirayama 2010; Mattijssen and Kersten 2012). To date, no studies have directly shown an effect of angptl2 on lipid metabolism despite the fact that adipocytes could be the main source of angptl2 (Tabata *et al.* 2009). Only the recent studies from our laboratory has reported that chronic administration of recombinant angptl2 in pre-atherosclerotic and dyslipidemic mice further augmented the LDL and total cholesterol levels (Farhat *et al.* 2013), and that angptl2 KD mice fed a HFD displayed a better lipid profile (Yu *et al.* 2014). With the previous knowledge that other angptl proteins, such as angptls 3 and 4, as described in section 1.2.4.1., play a role in the regulation of lipid storage and breakdown (Hato *et al.* 2008), it would be worthwhile to determine if angptl2 has any direct effects on lipid metabolism.

Overall, there is evidence of angptl2 participation in promoting angiogenesis (Kim *et al.* 1999b; Kubota *et al.* 2005; Broxmeyer *et al.* 2011), which is the process required for the expansion of adipose tissues in the face of increased energy intake and obesity (Cao 2007). The findings of reduced adipocyte sizes and better lipid profile in angptl2 KD compared to WT mice challenged with a HFD, therefore, may be partially explained by the lower angiogenic properties in the adipose tissues from angptl2 KD mice.

4.5. Is there a functional consequence of the cleaved angptl2 protein on adipocytes or ECs?

In studying metastasis of osteosarcoma cells, Odagiri *et al*. found that the TLL1 protease could cleave angptl2 extracellularly at the linker region between the coiled-coil domain and fibrionogen-like domain into fragments, giving rise to a smaller angptl2 form at around 35 kD, which was then inactive to enhance tumour metastasis unlike the full form of angptl2 (Odagiri *et al.* 2014). This is not the first study demonstrating cleavage of an angptl protein, as previous papers have shown that both angptls 3 and 4 were cleaved into an N-terminal coiled-coil domain fragment and a C-terminal fibrinogen-like domain fragment (Ono *et al.* 2003; Ge *et al.* 2004). Moreover, the cleaved coiled-coil domain fragment alone was enough to mediate lipid metabolic effects (Ono *et al.* 2003; Ge *et al.* 2004). Interestingly, the tolloid-like gene was reported as a candidate gene in adipocyte differentiation (Burton and McGehee 2004), and consistent with this, a retroviral expression of TLL1 in 3T3-L1 pre-adipocytes inhibited adipocyte differentiation (Chao *et al.* 2008). Of note, angptl2 mRNA expression in differentiating adipocytes increased in a time-dependent manner *in vitro* (Tabata *et al*. 2009), which may reflect TLL1 activity and may thus indirectly suggest a potential biological function of the smaller angptl2 form. Furthermore, TLL1 was found expressed in embryonic ECs (Brunskill and Potter 2010), and its expression in the heart was crucial in normal heart development in mice (Clark *et al.* 1999). To date, the vascular existence and function of the cleaved angptl2 are unknown, and it remains to be elucidated whether this shorter angptl2 form exerts any biological effects in adipocytes or ECs.

4.6. Lowering levels of angptl2: is that the solution? But how?

With a growing body of evidence supporting the pro-inflammatory and pro-oxidative role of angptl2 (Kadomatsu *et al.* 2014), along with the current data presented in this thesis indicating angptl2 involvement in mediating endothelial dysfunction, it appears that lowering angptl2 expression levels or inhibiting its downstream signaling pathway could be beneficial for patients with chronic inflammatory disorders such as obesity or hypertension. Although the receptors

integrin α5β1 (Tabata *et al.* 2009), LILRB2 (Zheng *et al.* 2012), or TLR4 (Oike and Tabata 2009) have all been proposed to act as receptors for angptl2, it is still not clear that these are the actual receptors for angptl2, and in what specific cell and context are they expressed and function as the angptl2 receptor. For the time being, an antagonist for angptl2 is not yet available and it seems that lowering angptl2 levels is the only feasible solution in the clinical setting. Only two studies have reported plasma angptl2 lowering: 1) anti-diabetic treatment in patients using pioglitazone decreased plasma angptl2 (Tabata *et al*. 2009) and 2) lifestyle intervention combining physical training and nutritional counseling for 3 months successfully decreased body weight and circulating angptl2, and improved metabolic parameters in overweight, but otherwise healthy Japanese men (Muramoto *et al.* 2011).

The topics surrounding the benefits gained from physical exercise has attracted insurmountable attention in the field of cardiovascular research. From increased shear stress-induced NO production (Green *et al.* 2004) to restoration of bone marrow-derived circulating progenitor cells (Linke *et al.* 2008), physical exercise has been consistently demonstrated to exert beneficial impacts on vascular function. In rats, lower intrinsic aerobic exercise capacity could predict a high cardiovascular risk score, which was associated with lower NO production and endothelial dysfunction in the carotid artery (Wisloff *et al.* 2005). In human, endothelial dysfunction is a predictor for CVD progression and cardiovascular event rates, independent of other cardiovascular risk factors (Schachinger *et al.* 2000), while exercise could augment endothelial function through increasing NO production and limiting NO inactivation (Green *et al.* 2004; Higashi and Yoshizumi 2004). The demonstration that circulating angptl2 levels were elevated in patients with diabetes (Tabata *et al.* 2009; Doi *et al.* 2012), in concert with the recent finding that angptl2 levels could be lowered by lifestyle intervention in overweight but otherwise healthy subjects (Muramoto *et al.* 2011), suggest that physical exercise could also reduce circulating angptl2 in CAD patients and improve their endothelial function. Indeed, preliminary data from an ongoing project of our laboratory gathered from CAD patients (n=33) undergoing a 12-week exercise intervention program show that physical exercise lowers plasma angptl2 levels and improves pulmonary capacities reflected by greater VO_{2max}, which

negatively correlates with plasma angptl2 levels (Yu *et al*. 2014). Moreover, lower angptl2 levels are associated with better endothelial function measured in the forearm of these patients (Yu *et al*. 2014). Thus, physical training lowers circulating angptl2 levels and low angptl2 levels may be predictive of good pulmonary fitness and endothelial function.

How lowering angptl2 levels by physical exercise can contribute to greater endothelial function may perhaps be multi-factorial. First, as exercise reduces adiposity (Levin and Dunn-Meynell 2004; Johnson *et al.* 2009) and angptl2 can be produced and released from adipocytes (Tabata *et al.* 2009), exercise-induced adipose tissue mass reduction should reduce angptl2 production and circulating levels. Second, as angptl2 is pro-inflammatory and pro-oxidative (Kadomatsu *et al.* 2014), low circulating angptl2 levels can lead to lower ROS production in ECs, thus greater NO bioavailability and preserved functional vasodilatory pathways in various vascular beds, as we have observed in the two studies with angptl2 KD mice. Third, physical exercise is able to reduce EC senescence and oxidative stress (Gielen *et al.* 2010; Corbi *et al.* 2012), which may be linked to lower angptl2 levels as we have previously shown high expression of angptl2 in senescent ECs from active CAD smokers (Farhat *et al.* 2008). Fourth, physical exercise may increase anti-oxidant enzyme activity in ECs (Gielen *et al.* 2010). In parallel, our unpublished data suggests that chronic angptl2 stimulation in ECs may have deleterious effects in the stress resistance systems, particularly the Nrf2 regulatory pathway (Farhat *et al*. unpublished data). Therefore, physical exercise and low angptl2 levels may synergistically improve EC stress resistance. Fifth, physical exercise has been well established to have favorable effects on plasma cholesterol and LDL levels (Lira *et al.* 2012). From our own present findings, a reduction in cholesterol and LDL levels in HFD-treated angptl2 KD mice may be attributable to low angptl2 levels. This may, in turn, lead to lesser effects from oxidized LDL in ECs leading to its dysfunction. Sixth, long-term exercise training is linked to sustained heart rate reduction (Fujimoto *et al.* 2010), and a low resting heart rate is known to be cardio-protective (Cook *et al.* 2006; Fox *et al.* 2008). Interestingly, our young angptl2 KD mice displayed lower basal heart rate compared to WT littermates. Hence, there may be a link between the benefits of exercise training in

heart rate reduction and that of lower angptl2, leading to greater endothelial function. Lastly, exercise has been proposed to protect vascular function by stimulating the sympathetic nervous system and increasing release of epinephrine (Pott *et al.* 1996; Zouhal *et al.* 2008), which has been demonstrated to stimulate eNOS activity in cultured ECs (Kou and Michel 2007). In a recent study, serum angptl2 levels negatively correlated with epinephrine levels in metabolically healthy but obese women (Meng *et al.* 2013). Furthermore, epinephrine treatment in adipocytes lowered angptl2 gene expression (Meng *et al.* 2013). Therefore, there may be a possible link between exercise, increase in epinephrine, and reduction of angptl2 levels, which may synergistically contribute to the maintenance of vascular function.

In summary, findings from the recent Japanese study (Muramoto *et al.* 2011) along with our preliminary data suggesting a link between lower circulating angptl2 levels and improved endothelial function in trained CAD patients, provide evidence that a reduction in circulating angptl2 levels may be beneficial on vascular function. As there are no angptl2 antagonists available, the non-pharmacological approach of physical activity provides a way to lower circulating angptl2 levels.

4.7. Limitations of the studies

These are the first two studies that 1) characterized a newly generated angptl2 KD mouse model and 2) evaluated and compared endothelial function in both conduit and resistance arteries between KD and WT mice challenged with either a HFD or angII infusion. There are undoubtedly limitations in both studies presented in this thesis, which will be discussed here.

In the first study, we used the recombinant protein produced from the laboratory (Farhat *et al.* 2013; Farhat *et al.* 2014) to test the acute effects of angptl2 on endothelial function measured by ACh-induced vasodilation in isolated femoral artery of WT mice. We chose to use a concentration of 50 nM for this particular study despite the fact that at a higher concentration of 100 nM previously used to stimulate EC in culture or freshly isolated from mouse aorta, we reported dose-dependent ROS production (Farhat *et al*. unpublished data) and inflammatory effects of angptl2 (Farhat *et al.* 2013). In addition, the study by Tabata *et al*. showed maximal effects of

a recombinant angptl2 protein produced by their laboratory at the concentration of around 100 nM (Tabata *et al.* 2009). We believed that 50 nM was closer to the EC_{50} to stimulate ROS production in our *in vitro* settings. In human, physiological circulating angptl2 concentration has been reported to be 1.0 to 3.0 ng/ml (Farhat *et al*. 2013; Kim *et al*. 1999b; Tabata *et al*. 2009; Kadomatsu *et al*. 2011), and according to our latest ELISA angptl2 measurements in CAD patients, plasma angptl2 can be as high as 20 ng/ml (Yu *et al*. 2014). Therefore, the concentration of 50 nM that we used in our experiments is equivalent to around 100-fold of that in CAD patients, and represents a pharmacological dose. Thus, in the future, it will be crucial to also test the effects of angptl2 at lower ranges of concentration. For the time being, however, a major limiting factor is the quantity of purified angptl2 recombinant protein that is being produced by the laboratory, since it is an extremely time-consuming process with relatively low yield (Farhat *et al*. 2014).

In studying endothelial function in both studies, we isolated arteries from animals and discarded surrounding tissues in the preparation, which included the perivascular adipose tissues, and could be a limitation. Indeed, accumulating evidence shows that the perivascular adipose tissues in fact contribute to the production of adipokines (Rajsheker *et al.* 2010; Verhagen and Visseren 2011), which in turn could alter endothelial function (Gao *et al.* 2007; Payne *et al.* 2010; Lee *et al.* 2014). Notably, a recent study reported secretion of angptl2, which is also considered an adipokine (Tabata *et al*. 2009), from perivascular adipose tissue (Tian *et al.* 2013), which took part in the acceleration of vascular inflammation and neointimal hyperplasia after an endovascular injury (Tian *et al.* 2013). Thus, there are probably cross-talks between angptl2 secreted from the perivascular adipose tissue and the vascular endothelium, likely exerting its pro-inflammatory an pro-oxidative effects. In our preparations, exclusion of perivascular adipose tissue from WT mice could have led to underestimation of angptl2 impact on endothelial function that was observed in HFD- or angII-treated mice. Inclusion of perivascular adipose tissue from artery preparation could, therefore, most likely reflect a more physiological setting and give greater insights on angptl2 regulation of endothelial function.

Another major limitation of the studies is that in most of the data interpretation in our vascular reactivity studies, we attempted to dissect out contribution of each EDRF by using various inhibitors. By ways of deduction, we determined contribution of NO (using NOS inhibitor LNNA), PGI_2 (using COX inhibitor indomethacin), and EDHF (combining LNNA and indomethamin to reveal non-NO and non-PGI_2-mediated relaxation), to global endothelial function measured by ACh-mediated vasodilation. Nonetheless, it is imperative to point out that there are cross-talks between vasodilatory pathways, and compensation from the recruitment of other EDRFs when another one is being pharmacologically inhibited, as introduced (section 1.1.1.4.1.). It is therefore difficult to assess the exact relative contribution of each EDRF. On the other hand, we did not examine the effects of other EDCFs in our vascular reactivity studies besides TXA_2. As noted in the introduction (section 1.1.2.), EDCFs including TXA_2 and ET-1 play major roles in mediating endothelial dysfunction, in addition to decreased EDRF function or production. For example, as we used indomethacin in the first study to inhibit COX activities of the femoral artery, we did not observe any changes to endothelial function in both WT and angptl2 KD mice, suggesting that COX-derived TXA_2 did not majorly participate in our experimental settings. Nonetheless, more specific inhibitors, such as furegrelate to specifically inhibit TXA_2 synthase, should be used in order to determine participation of other EDCFs. In addition, I believe that if we treated these mice with a more extreme challenge, such as a longer time-period than 3 months for HFD feeding, or a combination of a HFD and a high fructose diet, we would begin to see the effects of EDCFs on endothelial dysfunction. For the current studies, the arteries are still able to compensate for the loss in NO bioavailability that we observed in the first study, as global endothelial function in WT mice fed a HFD did not have any significant alterations, but only the relative contribution of EDRFs differed compared with KD mice. Also, in the second study, cerebral endothelial dysfunction in angII-treated WT mice was reversible, as acute addition of NAC, indomethacin, or apocynin was able to reverse this dysfunction.

Despite these shortcomings, I believe that the present studies reveal an important role of angptl2 in mediating endothelial dysfunction in clinically relevant

pathological settings. Although these changes may seem minimal, such as recruitment of an alternative EDRF to vasodilate in various vascular beds, I believe that the impact of angptl2 would be amplified with increasing age and cardiovascular risks.

5. Conclusion and Perspectives

It is no longer questionable, since its identification 15 years ago (Kim *et al*. 1999b), that angptl2 is a multifaceted protein that participates in a plethora of physiological and pathophysiological processes. While optimal levels of angptl2 expression seem necessary in maintaining homeostasis in different cell systems, there is convincing data from ourselves and others demonstrating that the up-regulation of its expression resulting in excess angptl2 is implicated in various chronic inflammatory disorders. Despite the knowledge that inflammation plays a pivotal role in driving endothelial dysfunction, which is typically defined by a decrease in NO bioavailability, no studies have ever shown a direct link between angptl2 and endothelial dysfunction until very recently (Horio *et al*. 2014), but this aspect of the study was limited to NO-mediated vasorelaxation in the mouse aorta only.

The two studies included in this thesis focused on angptl2 contribution to endothelial dysfunction that extends beyond the prototypical NO as the main EDRF and also considered other vascular beds of varying vessel sizes. We are the first to show that 1) angptl2 acutely evokes endothelial dysfunction most likely by increasing ROS production, 2) the lack of angptl2 in mice potentially increases EC stress resistance, and 3) protects against endothelial dysfunction induced by either a HFD or angII. Although the question of how angptl2 exactly mediates its pro-inflammatory and pro-oxidative effects on endothelial function remains to be elucidated, this work nonetheless provides the first clues to this bigger puzzle.

In the future, it will be imperative to verify our findings in another mouse model, for instance an EC-specific KO or KD, as well as a transgenic mouse model overexpressing angptl2 globally or EC-specifically, to better distinguish the role of EC-derived angptl2 on regulating endothelial function and EC stress resistance. Other means to measure endothelial function should be used, such as the laser Doppler imaging technique, which allows for *in vivo* measurements. Besides ACh that was used in the current work, other stimuli of vasodilation should also be tested, such as shear stress, which represents a better physiological scenario.

As the important question of its receptor remains unresolved, antagonists of angptl2 are still not yet available. Fortunately, physical exercise may hold the key to

the reduction of circulating angptl2, as demonstrated in obese (Muramoto *et al.* 2011) and CAD (Yu *et al*. 2014) patients, which also correlated with improved pulmonary fitness after physical training. With CVD on the rise today, there is an overwhelming need for better prognostic indicators of endothelial dysfunction, an obligatory primary first step towards atherosclerosis. Based on current findings from our laboratory and others, angptl2 may reveal itself as a novel predictive biomarker in determining cardiovascular risks in the clinical setting, and lowering its expression in disease settings may be a promising therapeutic avenue.

REFERENCES

AbdAlla S, Lother H, Quitterer U (2000). AT1-receptor heterodimers show enhanced G-protein activation and altered receptor sequestration. *Nature*. **407**, 94-98.

Abu-Soud HM, Feldman PL, Clark P, Stuehr DJ (1994). Electron transfer in the nitric-oxide synthases. Characterization of L-arginine analogs that block heme iron reduction. *J Biol Chem*. **269**, 32318-32326.

Adachi H, Fujiwara Y, Kondo T, Nishikawa T, Ogawa R, Matsumura T, Ishii N, Nagai R, Miyata K, Tabata M, Motoshima H, Furukawa N, Tsuruzoe K, Kawashima J, Takeya M, Yamashita S, Koh GY, Nagy A, Suda T, Oike Y, Araki E (2009). Angptl 4 deficiency improves lipid metabolism, suppresses foam cell formation and protects against atherosclerosis. *Biochem Biophys Res Commun*. **379**, 806-811.

Adachi H, Kondo T, Koh GY, Nagy A, Oike Y, Araki E (2011). Angptl4 deficiency decreases serum triglyceride levels in low-density lipoprotein receptor knockout mice and streptozotocin-induced diabetic mice. *Biochem Biophys Res Commun*. **409**, 177-180.

Akhter S, Rahman MM, Lee HS, Kim HJ, Hong ST (2013). Dynamic roles of angiopoietin-like proteins 1, 2, 3, 4, 6 and 7 in the survival and enhancement of ex vivo expansion of bone-marrow hematopoietic stem cells. *Protein Cell*. **4**, 220-230.

Akimenko MA, Mari-Beffa M, Becerra J, Geraudie J (2003). Old questions, new tools, and some answers to the mystery of fin regeneration. *Dev Dyn*. **226**, 190-201.

Alfadda AA, Sallam RM (2012). Reactive oxygen species in health and disease. *J Biomed Biotechnol*. **2012**, 936486.

Ambasta RK, Kumar P, Griendling KK, Schmidt HH, Busse R, Brandes RP (2004). Direct interaction of the novel Nox proteins with p22phox is required for the formation of a functionally active NADPH oxidase. *J Biol Chem*. **279**, 45935-45941.

Amin AR, Attur M, Patel RN, Thakker GD, Marshall PJ, Rediske J, Stuchin SA, Patel IR, Abramson SB (1997). Superinduction of cyclooxygenase-2 activity in human osteoarthritis-affected cartilage. Influence of nitric oxide. *J Clin Invest*. **99**, 1231-1237.

Anderson GD, Hauser SD, McGarity KL, Bremer ME, Isakson PC, Gregory SA (1996). Selective inhibition of cyclooxygenase (COX)-2 reverses inflammation and expression of COX-2 and interleukin 6 in rat adjuvant arthritis. *J Clin Invest*. **97**, 2672-2679.

Ando H, Zhou J, Macova M, Imboden H, Saavedra JM (2004). Angiotensin II AT1 receptor blockade reverses pathological hypertrophy and inflammation in brain microvessels of spontaneously hypertensive rats. *Stroke*. **35**, 1726-1731.

Ando Y, Shimizugawa T, Takeshita S, Ono M, Shimamura M, Koishi R, Furukawa H (2003). A decreased expression of angiopoietin-like 3 is protective against atherosclerosis in apoE-deficient mice. *J Lipid Res*. **44**, 1216-1223.

Andreozzi F, Laratta E, Sciacqua A, Perticone F, Sesti G (2004). Angiotensin II impairs the insulin signaling pathway promoting production of nitric oxide by inducing phosphorylation of insulin receptor substrate-1 on Ser312 and Ser616 in human umbilical vein endothelial cells. *Circ Res*. **94**, 1211-1218.

Aoi J, Endo M, Kadomatsu T, Miyata K, Nakano M, Horiguchi H, Ogata A, Odagiri H, Yano M, Araki K, Jinnin M, Ito T, Hirakawa S, Ihn H, Oike Y (2011). Angiopoietin-like protein 2 is an important facilitator of inflammatory carcinogenesis and metastasis. *Cancer Res*. **71**, 7502-7512.

Aoi J, Endo M, Kadomatsu T, Miyata K, Ogata A, Horiguchi H, Odagiri H, Masuda T, Fukushima S, Jinnin M, Hirakawa S, Sawa T, Akaike T, Ihn H, Oike Y (2014). Angiopoietin-like Protein 2 Accelerates Carcinogenesis by Activating Chronic Inflammation and Oxidative Stress. *Mol Cancer Res*. **12**, 239-249.

Arbibe L, Mira JP, Teusch N, Kline L, Guha M, Mackman N, Godowski PJ, Ulevitch RJ, Knaus UG (2000). Toll-like receptor 2-mediated NF-kappa B activation requires a Rac1-dependent pathway. *Nat Immunol*. **1**, 533-540.

Arita Y, Kihara S, Ouchi N, Takahashi M, Maeda K, Miyagawa J, Hotta K, Shimomura I, Nakamura T, Miyaoka K, Kuriyama H, Nishida M, Yamashita S, Okubo K, Matsubara K, Muraguchi M, Ohmoto Y, Funahashi T, Matsuzawa Y (1999). Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. *Biochem Biophys Res Commun*. **257**, 79-83.

Aruoma OI, Halliwell B, Hoey BM, Butler J (1989). The antioxidant action of N-acetylcysteine: its reaction with hydrogen peroxide, hydroxyl radical, superoxide, and hypochlorous acid. *Free Radic Biol Med*. **6**, 593-597.

Atwal JK, Pinkston-Gosse J, Syken J, Stawicki S, Wu Y, Shatz C, Tessier-Lavigne M (2008). PirB is a functional receptor for myelin inhibitors of axonal regeneration. *Science*. **322**, 967-970.

Auch-Schwelk W, Katusic ZS, Vanhoutte PM (1990). Thromboxane A2 receptor antagonists inhibit endothelium-dependent contractions. *Hypertension*. **15**, 699-703.

Avogaro A, de Kreutzenberg SV, Federici M, Fadini GP (2013). The endothelium abridges insulin resistance to premature aging. *J Am Heart Assoc*. **2**, e000262.

Ayajiki K, Kindermann M, Hecker M, Fleming I, Busse R (1996). Intracellular pH and tyrosine phosphorylation but not calcium determine shear stress-induced nitric oxide production in native endothelial cells. *Circ Res*. **78**, 750-758.

Babior BM, Kipnes RS, Curnutte JT (1973). Biological defense mechanisms. The production by leukocytes of superoxide, a potential bactericidal agent. *J Clin Invest*. **52**, 741-744.

Backhed F, Ding H, Wang T, Hooper LV, Koh GY, Nagy A, Semenkovich CF, Gordon JI (2004). The gut microbiota as an environmental factor that regulates fat storage. *Proc Natl Acad Sci U S A*. **101**, 15718-15723.

Bagi Z (2009). Mechanisms of coronary microvascular adaptation to obesity. *Am J Physiol Regul Integr Comp Physiol*. **297**, R556-567.

Bai S, Thummel R, Godwin AR, Nagase H, Itoh Y, Li L, Evans R, McDermott J, Seiki M, Sarras MP, Jr. (2005). Matrix metalloproteinase expression and function during fin regeneration in zebrafish: analysis of MT1-MMP, MMP2 and TIMP2. *Matrix Biol*. **24**, 247-260.

Baker CS, Hall RJ, Evans TJ, Pomerance A, Maclouf J, Creminon C, Yacoub MH, Polak JM (1999). Cyclooxygenase-2 is widely expressed in atherosclerotic lesions affecting native and transplanted human coronary arteries and colocalizes with inducible nitric oxide synthase and nitrotyrosine particularly in macrophages. *Arterioscler Thromb Vasc Biol*. **19**, 646-655.
Balaban RS, Nemoto S, Finkel T (2005). Mitochondria, oxidants, and aging. *Cell*. **120**, 483-495.
Balla G, Jacob HS, Balla J, Rosenberg M, Nath K, Apple F, Eaton JW, Vercellotti GM (1992). Ferritin: a cytoprotective antioxidant strategem of endothelium. *J Biol Chem*. **267**, 18148-18153.
Bar-Sagi D, Hall A (2000). Ras and Rho GTPases: a family reunion. *Cell*. **103**, 227-238.
Bass J, Takahashi JS (2010). Circadian integration of metabolism and energetics. *Science*. **330**, 1349-1354.
Bath PM, Hassall DG, Gladwin AM, Palmer RM, Martin JF (1991). Nitric oxide and prostacyclin. Divergence of inhibitory effects on monocyte chemotaxis and adhesion to endothelium in vitro. *Arterioscler Thromb*. **11**, 254-260.
Baumbach GL, Sigmund CD, Faraci FM (2003). Cerebral arteriolar structure in mice overexpressing human renin and angiotensinogen. *Hypertension*. **41**, 50-55.
Beamer YB, Garner JT, Shelden CH (1973). Hypertrophied ligamentum flavum. Clinical and surgical significance. *Arch Surg*. **106**, 289-292.
Beckman JA, Creager MA, Libby P (2002). Diabetes and atherosclerosis: epidemiology, pathophysiology, and management. *JAMA*. **287**, 2570-2581.
Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA (1990). Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci U S A*. **87**, 1620-1624.
Beckman JS, Koppenol WH (1996). Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am J Physiol*. **271**, C1424-1437.
Bedard K, Krause KH (2007). The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiol Rev*. **87**, 245-313.
Beierwaltes WH (2002). Cyclooxygenase-2 products compensate for inhibition of nitric oxide regulation of renal perfusion. *Am J Physiol Renal Physiol*. **283**, F68-72.
Beigneux AP, Davies BS, Gin P, Weinstein MM, Farber E, Qiao X, Peale F, Bunting S, Walzem RL, Wong JS, Blaner WS, Ding ZM, Melford K, Wongsiriroj N, Shu X, de Sauvage F, Ryan RO, Fong LG, Bensadoun A, Young SG (2007). Glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1 plays a critical role in the lipolytic processing of chylomicrons. *Cell Metab*. **5**, 279-291.
Beny JL, von der Weid PY (1991). Hydrogen peroxide: an endogenous smooth muscle cell hyperpolarizing factor. *Biochem Biophys Res Commun*. **176**, 378-384.
Berridge MJ, Irvine RF (1984). Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature*. **312**, 315-321.

Beswick RA, Dorrance AM, Leite R, Webb RC (2001). NADH/NADPH oxidase and enhanced superoxide production in the mineralocorticoid hypertensive rat. *Hypertension*. **38**, 1107-1111.

Bharadwaj L, Prasad K (1995). Mediation of H2O2-induced vascular relaxation by endothelium-derived relaxing factor. *Mol Cell Biochem*. **149-150**, 267-270.

Bi M, Naczki C, Koritzinsky M, Fels D, Blais J, Hu N, Harding H, Novoa I, Varia M, Raleigh J, Scheuner D, Kaufman RJ, Bell J, Ron D, Wouters BG, Koumenis C (2005). ER stress-regulated translation increases tolerance to extreme hypoxia and promotes tumor growth. *EMBO J*. **24**, 3470-3481.

Blanco-Rivero J, Cachofeiro V, Lahera V, Aras-Lopez R, Marquez-Rodas I, Salaices M, Xavier FE, Ferrer M, Balfagon G (2005). Participation of prostacyclin in endothelial dysfunction induced by aldosterone in normotensive and hypertensive rats. *Hypertension*. **46**, 107-112.

Bohm F, Johansson BL, Hedin U, Alving K, Pernow J (2002). Enhanced vasoconstrictor effect of big endothelin-1 in patients with atherosclerosis: relation to conversion to endothelin-1. *Atherosclerosis*. **160**, 215-222.

Bolego C, Buccellati C, Radaelli T, Cetin I, Puglisi L, Folco G, Sala A (2006). eNOS, COX-2, and prostacyclin production are impaired in endothelial cells from diabetics. *Biochem Biophys Res Commun*. **339**, 188-190.

Bond M, Fabunmi RP, Baker AH, Newby AC (1998). Synergistic upregulation of metalloproteinase-9 by growth factors and inflammatory cytokines: an absolute requirement for transcription factor NF-kappa B. *FEBS Lett*. **435**, 29-34.

Bonetti PO, Lerman LO, Lerman A (2003). Endothelial dysfunction: a marker of atherosclerotic risk. *Arterioscler Thromb Vasc Biol*. **23**, 168-175.

Bos CL, Richel DJ, Ritsema T, Peppelenbosch MP, Versteeg HH (2004). Prostanoids and prostanoid receptors in signal transduction. *Int J Biochem Cell Biol*. **36**, 1187-1205.

Boulanger CM, Heymes C, Benessiano J, Geske RS, Levy BI, Vanhoutte PM (1998). Neuronal nitric oxide synthase is expressed in rat vascular smooth muscle cells: activation by angiotensin II in hypertension. *Circ Res*. **83**, 1271-1278.

Bouloumie A, Drexler HC, Lafontan M, Busse R (1998). Leptin, the product of Ob gene, promotes angiogenesis. *Circ Res*. **83**, 1059-1066.

Brakenhielm E, Veitonmaki N, Cao R, Kihara S, Matsuzawa Y, Zhivotovsky B, Funahashi T, Cao Y (2004). Adiponectin-induced antiangiogenesis and antitumor activity involve caspase-mediated endothelial cell apoptosis. *Proc Natl Acad Sci U S A*. **101**, 2476-2481.

Brandes RP, Fleming I, Busse R (2005). Endothelial aging. *Cardiovasc Res*. **66**, 286-294.

Brennan FM, McInnes IB (2008). Evidence that cytokines play a role in rheumatoid arthritis. *J Clin Invest*. **118**, 3537-3545.

Bresalier RS, Sandler RS, Quan H, Bolognese JA, Oxenius B, Horgan K, Lines C, Riddell R, Morton D, Lanas A, Konstam MA, Baron JA (2005). Cardiovascular events associated with rofecoxib in a colorectal adenoma chemoprevention trial. *N Engl J Med*. **352**, 1092-1102.

Brock TA, Alexander RW, Ekstein LS, Atkinson WJ, Gimbrone MA, Jr. (1985). Angiotensin increases cytosolic free calcium in cultured vascular smooth muscle cells. *Hypertension*. **7**, I105-109.

Brouet A, Sonveaux P, Dessy C, Balligand JL, Feron O (2001). Hsp90 ensures the transition from the early Ca2+-dependent to the late phosphorylation-dependent activation of the endothelial nitric-oxide synthase in vascular endothelial growth factor-exposed endothelial cells. *J Biol Chem*. **276**, 32663-32669.

Brown NJ, Agirbasli MA, Williams GH, Litchfield WR, Vaughan DE (1998). Effect of activation and inhibition of the renin-angiotensin system on plasma PAI-1. *Hypertension*. **32**, 965-971.

Broxmeyer HE, Srour EF, Cooper S, Wallace CT, Hangoc G, Youn BS (2011). Angiopoietin-like-2 and -3 act through their coiled-coil domains to enhance survival and replating capacity of human cord blood hematopoietic progenitors. *Blood Cells Mol Dis*. **48**, 25-29.

Brunskill EW, Potter SS (2010). Gene expression programs of mouse endothelial cells in kidney development and disease. *PLoS One*. **5**, e12034.

Bulut D, Liaghat S, Hanefeld C, Koll R, Miebach T, Mugge A (2003). Selective cyclo-oxygenase-2 inhibition with parecoxib acutely impairs endothelium-dependent vasodilatation in patients with essential hypertension. *J Hypertens*. **21**, 1663-1667.

Bunting S, Gryglewski R, Moncada S, Vane JR (1976). Arterial walls generate from prostaglandin endoperoxides a substance (prostaglandin X) which relaxes strips of mesenteric and coeliac ateries and inhibits platelet aggregation. *Prostaglandins*. **12**, 897-913.

Burleigh ME, Babaev VR, Yancey PG, Major AS, McCaleb JL, Oates JA, Morrow JD, Fazio S, Linton MF (2005). Cyclooxygenase-2 promotes early atherosclerotic lesion formation in ApoE-deficient and C57BL/6 mice. *J Mol Cell Cardiol*. **39**, 443-452.

Burridge K, Wennerberg K (2004). Rho and Rac take center stage. *Cell*. **116**, 167-179.

Burritt JB, Foubert TR, Baniulis D, Lord CI, Taylor RM, Mills JS, Baughan TD, Roos D, Parkos CA, Jesaitis AJ (2003). Functional epitope on human neutrophil flavocytochrome b558. *J Immunol*. **170**, 6082-6089.

Burton GR, McGehee RE, Jr. (2004). Identification of candidate genes involved in the regulation of adipocyte differentiation using microarray-based gene expression profiling. *Nutrition*. **20**, 109-114.

Busse R, Edwards G, Feletou M, Fleming I, Vanhoutte PM, Weston AH (2002). EDHF: bringing the concepts together. *Trends Pharmacol Sci*. **23**, 374-380.

Busse R, Fleming I (1995). Regulation and functional consequences of endothelial nitric oxide formation. *Ann Med*. **27**, 331-340.

Busse R, Mulsch A (1990). Induction of nitric oxide synthase by cytokines in vascular smooth muscle cells. *FEBS Lett*. **275**, 87-90.

Cai H (2005). Hydrogen peroxide regulation of endothelial function: origins, mechanisms, and consequences. *Cardiovasc Res*. **68**, 26-36.

Cai H, Harrison DG (2000). Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. *Circ Res*. **87**, 840-844.

Cai H, Li Z, Davis ME, Kanner W, Harrison DG, Dudley SC, Jr. (2003). Akt-dependent phosphorylation of serine 1179 and mitogen-activated protein kinase kinase/extracellular signal-regulated kinase 1/2 cooperatively mediate activation of the endothelial nitric-oxide synthase by hydrogen peroxide. *Mol Pharmacol*. **63**, 325-331.

Cai S, Khoo J, Mussa S, Alp NJ, Channon KM (2005). Endothelial nitric oxide synthase dysfunction in diabetic mice: importance of tetrahydrobiopterin in eNOS dimerisation. *Diabetologia*. **48**, 1933-1940.

Callen JP (2000). Dermatomyositis. *Lancet*. **355**, 53-57.

Camenisch G, Pisabarro MT, Sherman D, Kowalski J, Nagel M, Hass P, Xie MH, Gurney A, Bodary S, Liang XH, Clark K, Beresini M, Ferrara N, Gerber HP (2002). ANGPTL3 stimulates endothelial cell adhesion and migration via integrin alpha vbeta 3 and induces blood vessel formation in vivo. *J Biol Chem*. **277**, 17281-17290.

Cao Y (2007). Angiogenesis modulates adipogenesis and obesity. *J Clin Invest*. **117**, 2362-2368.

Capone C, Faraco G, Park L, Cao X, Davisson RL, Iadecola C (2010). The cerebrovascular dysfunction induced by slow pressor doses of angiotensin II precedes the development of hypertension. *Am J Physiol Heart Circ Physiol*. **300**, H397-407.

Carvajal JA, Germain AM, Huidobro-Toro JP, Weiner CP (2000). Molecular mechanism of cGMP-mediated smooth muscle relaxation. *J Cell Physiol*. **184**, 409-420.

Castelli W (1998). Lipoproteins and cardiovascular disease: biological basis and epidemiological studies. *Value Health*. **1**, 105-109.

Cavaghan MK, Ehrmann DA, Polonsky KS (2000). Interactions between insulin resistance and insulin secretion in the development of glucose intolerance. *J Clin Invest*. **106**, 329-333.

Cazes A, Galaup A, Chomel C, Bignon M, Brechot N, Le Jan S, Weber H, Corvol P, Muller L, Germain S, Monnot C (2006). Extracellular matrix-bound angiopoietin-like 4 inhibits endothelial cell adhesion, migration, and sprouting and alters actin cytoskeleton. *Circ Res*. **99**, 1207-1215.

Celermajer DS, Sorensen KE, Gooch VM, Spiegelhalter DJ, Miller OI, Sullivan ID, Lloyd JK, Deanfield JE (1992). Non-invasive detection of endothelial dysfunction in children and adults at risk of atherosclerosis. *Lancet*. **340**, 1111-1115.

Chan SL, Baumbach GL (2013). Nox2 deficiency prevents hypertension-induced vascular dysfunction and hypertrophy in cerebral arterioles. *Int J Hypertens*. **2013**, 793630.

Chao LC, Bensinger SJ, Villanueva CJ, Wroblewski K, Tontonoz P (2008). Inhibition of adipocyte differentiation by Nur77, Nurr1, and Nor1. *Mol Endocrinol*. **22**, 2596-2608.

Charpie JR, Schreur KD, Papadopoulos SM, Webb RC (1994). Endothelium dependency of contractile activity differs in infant and adult vertebral arteries. *J Clin Invest*. **93**, 1339-1343.
Chauhan SD, Hobbs AJ, Ahluwalia A (2004). C-type natriuretic peptide: new candidate for endothelium-derived hyperpolarising factor. *Int J Biochem Cell Biol*. **36**, 1878-1881.
Chauhan SD, Seggara G, Vo PA, Macallister RJ, Hobbs AJ, Ahluwalia A (2003). Protection against lipopolysaccharide-induced endothelial dysfunction in resistance and conduit vasculature of iNOS knockout mice. *FASEB J*. **17**, 773-775.
Chen G, Suzuki H, Weston AH (1988). Acetylcholine releases endothelium-derived hyperpolarizing factor and EDRF from rat blood vessels. *Br J Pharmacol*. **95**, 1165-1174.
Chen XL, Dodd G, Thomas S, Zhang X, Wasserman MA, Rovin BH, Kunsch C (2006). Activation of Nrf2/ARE pathway protects endothelial cells from oxidant injury and inhibits inflammatory gene expression. *Am J Physiol Heart Circ Physiol*. **290**, H1862-1870.
Cheng G, Diebold BA, Hughes Y, Lambeth JD (2006). Nox1-dependent reactive oxygen generation is regulated by Rac1. *J Biol Chem*. **281**, 17718-17726.
Chiu AT, Herblin WF, McCall DE, Ardecky RJ, Carini DJ, Duncia JV, Pease LJ, Wong PC, Wexler RR, Johnson AL, et al. (1989). Identification of angiotensin II receptor subtypes. *Biochem Biophys Res Commun*. **165**, 196-203.
Chomel C, Cazes A, Faye C, Bignon M, Gomez E, Ardidie-Robouant C, Barret A, Ricard-Blum S, Muller L, Germain S, Monnot C (2009). Interaction of the coiled-coil domain with glycosaminoglycans protects angiopoietin-like 4 from proteolysis and regulates its antiangiogenic activity. *FASEB J*. **23**, 940-949.
Chrissobolis S, Banfi B, Sobey CG, Faraci FM (2012). Role of Nox isoforms in angiotensin II-induced oxidative stress and endothelial dysfunction in brain. *J Appl Physiol (1985)*. **113**, 184-191.
Chrissobolis S, Miller AA, Drummond GR, Kemp-Harper BK, Sobey CG (2011). Oxidative stress and endothelial dysfunction in cerebrovascular disease. *Front Biosci (Landmark Ed)*. **16**, 1733-1745.
Chudek J, Wiecek A (2006). Adipose tissue, inflammation and endothelial dysfunction. *Pharmacol Rep*. **58 Suppl**, 81-88.
Cines DB, Pollak ES, Buck CA, Loscalzo J, Zimmerman GA, McEver RP, Pober JS, Wick TM, Konkle BA, Schwartz BS, Barnathan ES, McCrae KR, Hug BA, Schmidt AM, Stern DM (1998). Endothelial cells in physiology and in the pathophysiology of vascular disorders. *Blood*. **91**, 3527-3561.
Clark TG, Conway SJ, Scott IC, Labosky PA, Winnier G, Bundy J, Hogan BL, Greenspan DS (1999). The mammalian Tolloid-like 1 gene, Tll1, is necessary for normal septation and positioning of the heart. *Development*. **126**, 2631-2642.
Cohen JC, Boerwinkle E, Mosley TH, Jr., Hobbs HH (2006). Sequence variations in PCSK9, low LDL, and protection against coronary heart disease. *N Engl J Med*. **354**, 1264-1272.

Colotta F, Allavena P, Sica A, Garlanda C, Mantovani A (2009). Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability. *Carcinogenesis*. **30**, 1073-1081.

Conklin D, Gilbertson D, Taft DW, Maurer MF, Whitmore TE, Smith DL, Walker KM, Chen LH, Wattler S, Nehls M, Lewis KB (1999). Identification of a mammalian angiopoietin-related protein expressed specifically in liver. *Genomics*. **62**, 477-482.

Cook S, Togni M, Schaub MC, Wenaweser P, Hess OM (2006). High heart rate: a cardiovascular risk factor? *Eur Heart J*. **27**, 2387-2393.

Corbi G, Conti V, Russomanno G, Rengo G, Vitulli P, Ciccarelli AL, Filippelli A, Ferrara N (2012). Is physical activity able to modify oxidative damage in cardiovascular aging? *Oxid Med Cell Longev*. **2012**, 728547.

Corriu C, Feletou M, Edwards G, Weston AH, Vanhoutte PM (2001). Differential effects of prostacyclin and iloprost in the isolated carotid artery of the guinea-pig. *Eur J Pharmacol*. **426**, 89-94.

Corson MA, James NL, Latta SE, Nerem RM, Berk BC, Harrison DG (1996). Phosphorylation of endothelial nitric oxide synthase in response to fluid shear stress. *Circ Res*. **79**, 984-991.

Cosentino F, Eto M, De Paolis P, van der Loo B, Bachschmid M, Ullrich V, Kouroedov A, Delli Gatti C, Joch H, Volpe M, Luscher TF (2003). High glucose causes upregulation of cyclooxygenase-2 and alters prostanoid profile in human endothelial cells: role of protein kinase C and reactive oxygen species. *Circulation*. **107**, 1017-1023.

Cosentino F, Luscher TF (1998). Tetrahydrobiopterin and endothelial function. *Eur Heart J*. **19 Suppl G**, G3-8.

Costanzo A, Moretti F, Burgio VL, Bravi C, Guido F, Levrero M, Puri PL (2003). Endothelial activation by angiotensin II through NFkappaB and p38 pathways: Involvement of NFkappaB-inducible kinase (NIK), free oxygen radicals, and selective inhibition by aspirin. *J Cell Physiol*. **195**, 402-410.

Crawford Y, Kasman I, Yu L, Zhong C, Wu X, Modrusan Z, Kaminker J, Ferrara N (2009). PDGF-C mediates the angiogenic and tumorigenic properties of fibroblasts associated with tumors refractory to anti-VEGF treatment. *Cancer Cell*. **15**, 21-34.

Creager MA, Cooke JP, Mendelsohn ME, Gallagher SJ, Coleman SM, Loscalzo J, Dzau VJ (1990). Impaired vasodilation of forearm resistance vessels in hypercholesterolemic humans. *J Clin Invest*. **86**, 228-234.

Crossno JT, Jr., Majka SM, Grazia T, Gill RG, Klemm DJ (2006). Rosiglitazone promotes development of a novel adipocyte population from bone marrow-derived circulating progenitor cells. *J Clin Invest*. **116**, 3220-3228.

Csanyi G, Gajda M, Franczyk-Zarow M, Kostogrys R, Gwozdz P, Mateuszuk L, Sternak M, Wojcik L, Zalewska T, Walski M, Chlopicki S (2012). Functional alterations in endothelial NO, PGI(2) and EDHF pathways in aorta in ApoE/LDLR-/- mice. *Prostaglandins Other Lipid Mediat*. **98**, 107-115.

Csiszar A, Wang M, Lakatta EG, Ungvari Z (2008). Inflammation and endothelial dysfunction during aging: role of NF-kappaB. *J Appl Physiol (1985)*. **105**, 1333-1341.

Dai G, Vaughn S, Zhang Y, Wang ET, Garcia-Cardena G, Gimbrone MA, Jr. (2007). Biomechanical forces in atherosclerosis-resistant vascular regions regulate endothelial redox balance via phosphoinositol 3-kinase/Akt-dependent activation of Nrf2. *Circ Res*. **101**, 723-733.

Davies PF (1995). Flow-mediated endothelial mechanotransduction. *Physiol Rev*. **75**, 519-560.

Daviet L, Lehtonen JY, Tamura K, Griese DP, Horiuchi M, Dzau VJ (1999). Cloning and characterization of ATRAP, a novel protein that interacts with the angiotensin II type 1 receptor. *J Biol Chem*. **274**, 17058-17062.

Davignon J, Ganz P (2004). Role of endothelial dysfunction in atherosclerosis. *Circulation*. **109**, III27-32.

De Silva TM, Faraci FM (2013). Effects of angiotensin II on the cerebral circulation: role of oxidative stress. *Front Physiol*. **3**, 484.

de Winther MP, Kanters E, Kraal G, Hofker MH (2005). Nuclear factor kappaB signaling in atherogenesis. *Arterioscler Thromb Vasc Biol*. **25**, 904-914.

Deanfield JE, Halcox JP, Rabelink TJ (2007). Endothelial function and dysfunction: testing and clinical relevance. *Circulation*. **115**, 1285-1295.

Deng M, Lu Z, Zheng J, Wan X, Chen X, Hirayasu K, Sun H, Lam Y, Chen L, Wang Q, Song C, Huang N, Gao GF, Jiang Y, Arase H, Zhang CC (2014). A motif in LILRB2 critical for Angptl2 binding and activation. *Blood*.

Desai U, Lee EC, Chung K, Gao C, Gay J, Key B, Hansen G, Machajewski D, Platt KA, Sands AT, Schneider M, Van Sligtenhorst I, Suwanichkul A, Vogel P, Wilganowski N, Wingert J, Zambrowicz BP, Landes G, Powell DR (2007). Lipid-lowering effects of anti-angiopoietin-like 4 antibody recapitulate the lipid phenotype found in angiopoietin-like 4 knockout mice. *Proc Natl Acad Sci U S A*. **104**, 11766-11771.

Dhanabal M, LaRochelle WJ, Jeffers M, Herrmann J, Rastelli L, McDonald WF, Chillakuru RA, Yang M, Boldog FL, Padigaru M, McQueeney KD, Wu F, Minskoff SA, Shimkets RA, Lichenstein HS (2002). Angioarrestin: an antiangiogenic protein with tumor-inhibiting properties. *Cancer Res*. **62**, 3834-3841.

Didion SP, Faraci FM (2002). Effects of NADH and NADPH on superoxide levels and cerebral vascular tone. *Am J Physiol Heart Circ Physiol*. **282**, H688-695.

Dikalova A, Clempus R, Lassegue B, Cheng G, McCoy J, Dikalov S, San Martin A, Lyle A, Weber DS, Weiss D, Taylor WR, Schmidt HH, Owens GK, Lambeth JD, Griendling KK (2005). Nox1 overexpression potentiates angiotensin II-induced hypertension and vascular smooth muscle hypertrophy in transgenic mice. *Circulation*. **112**, 2668-2676.

Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, Zeiher AM (1999). Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature*. **399**, 601-605.

Dinauer MC, Orkin SH, Brown R, Jesaitis AJ, Parkos CA (1987). The glycoprotein encoded by the X-linked chronic granulomatous disease locus is a component of the neutrophil cytochrome b complex. *Nature*. **327**, 717-720.

Doblinger E, Hocherl K, Mederle K, Kattler V, Walter S, Hansen PB, Jensen B, Castrop H (2012). Angiotensin AT1 receptor-associated protein Arap1 in the kidney vasculature is suppressed by angiotensin II. *Am J Physiol Renal Physiol*. **302**, F1313-1324.

Doi Y, Ninomiya T, Hirakawa Y, Takahashi O, Mukai N, Hata J, Iwase M, Kitazono T, Oike Y, Kiyohara Y (2012). Angiopoietin-like protein 2 and risk of type 2 diabetes in a general Japanese population: the Hisayama study. *Diabetes Care*. **36**, 98-100.

Donato AJ, Lesniewski LA, Delp MD (2005). The effects of aging and exercise training on endothelin-1 vasoconstrictor responses in rat skeletal muscle arterioles. *Cardiovasc Res*. **66**, 393-401.

Doyle MP, Duling BR (1997). Acetylcholine induces conducted vasodilation by nitric oxide-dependent and -independent mechanisms. *Am J Physiol*. **272**, H1364-1371.

Drexler H (1997). Endothelial dysfunction: clinical implications. *Prog Cardiovasc Dis*. **39**, 287-324.

Drexler H (1999). Nitric oxide and coronary endothelial dysfunction in humans. *Cardiovasc Res*. **43**, 572-579.

Drexler H, Zeiher AM (1991). Endothelial function in human coronary arteries in vivo. Focus on hypercholesterolemia. *Hypertension*. **18**, II90-99.

Drouin A, Farhat N, Bolduc V, Thorin-Trescases N, Gillis MA, Villeneuve L, Nguyen A, Thorin E (2011). Up-regulation of thromboxane A(2) impairs cerebrovascular eNOS function in aging atherosclerotic mice. *Pflugers Arch*. **462**, 371-383.

Drouin A, Thorin E (2009). Flow-induced dilation is mediated by Akt-dependent activation of endothelial nitric oxide synthase-derived hydrogen peroxide in mouse cerebral arteries. *Stroke*. **40**, 1827-1833.

Drouin A, Thorin-Trescases N, Hamel E, Falck JR, Thorin E (2007). Endothelial nitric oxide synthase activation leads to dilatory H2O2 production in mouse cerebral arteries. *Cardiovasc Res*. **73**, 73-81.

Drummond GR, Sobey CG (2014). Endothelial NADPH oxidases: which NOX to target in vascular disease? *Trends Endocrinol Metab*.

Du K, Herzig S, Kulkarni RN, Montminy M (2003). TRB3: a tribbles homolog that inhibits Akt/PKB activation by insulin in liver. *Science*. **300**, 1574-1577.

Du XL, Edelstein D, Dimmeler S, Ju Q, Sui C, Brownlee M (2001). Hyperglycemia inhibits endothelial nitric oxide synthase activity by posttranslational modification at the Akt site. *J Clin Invest*. **108**, 1341-1348.

Dubois RN, Abramson SB, Crofford L, Gupta RA, Simon LS, Van De Putte LB, Lipsky PE (1998). Cyclooxygenase in biology and disease. *FASEB J*. **12**, 1063-1073.

Durand MJ, Gutterman DD (2013). Diversity in mechanisms of endothelium-dependent vasodilation in health and disease. *Microcirculation*. **20**, 239-247.

Durante W, Sen AK, Sunahara FA (1988). Impairment of endothelium-dependent relaxation in aortae from spontaneously diabetic rats. *Br J Pharmacol*. **94**, 463-468.

Dutta S, Rittinger K (2010). Regulation of NOXO1 activity through reversible interactions with p22 and NOXA1. *PLoS One*. **5**, e10478.

Ebert T, Bachmann A, Lossner U, Kratzsch J, Bluher M, Stumvoll M, Fasshauer M (2009). Serum levels of angiopoietin-related growth factor in diabetes mellitus and chronic hemodialysis. *Metabolism*. **58**, 547-551.

Edfeldt K, Swedenborg J, Hansson GK, Yan ZQ (2002). Expression of toll-like receptors in human atherosclerotic lesions: a possible pathway for plaque activation. *Circulation*. **105**, 1158-1161.

Edwards G, Dora KA, Gardener MJ, Garland CJ, Weston AH (1998). K+ is an endothelium-derived hyperpolarizing factor in rat arteries. *Nature*. **396**, 269-272.

Eguchi S, Frank GD, Mifune M, Inagami T (2003). Metalloprotease-dependent ErbB ligand shedding in mediating EGFR transactivation and vascular remodelling. *Biochem Soc Trans*. **31**, 1198-1202.

Eisenberg S (1984). High density lipoprotein metabolism. *J Lipid Res*. **25**, 1017-1058.

El-Gharbawy AH, Nadig VS, Kotchen JM, Grim CE, Sagar KB, Kaldunski M, Hamet P, Pausova Z, Gaudet D, Gossard F, Kotchen TA (2001). Arterial pressure, left ventricular mass, and aldosterone in essential hypertension. *Hypertension*. **37**, 845-850.

Endo M, Nakano M, Kadomatsu T, Fukuhara S, Kuroda H, Mikami S, Hato T, Aoi J, Horiguchi H, Miyata K, Odagiri H, Masuda T, Harada M, Horio H, Hishima T, Nomori H, Ito T, Yamamoto Y, Minami T, Okada S, Takahashi T, Mochizuki N, Iwase H, Oike Y (2012). Tumor cell-derived angiopoietin-like protein ANGPTL2 is a critical driver of metastasis. *Cancer Res*. **72**, 1784-1794.

Endo M, Yamamoto Y, Nakano M, Masuda T, Odagiri H, Horiguchi H, Miyata K, Kadomatsu T, Motokawa I, Okada S, Iwase H, Oike Y (2014). Serum ANGPTL2 levels reflect clinical features of breast cancer patients: implications for the pathogenesis of breast cancer metastasis. *Int J Biol Markers*, 0.

Ernst JA, Li H, Kim HS, Nakamura GR, Yansura DG, Vandlen RL (2005). Isolation and characterization of the B-cell marker CD20. *Biochemistry*. **44**, 15150-15158.

Essalmani R, Susan-Resiga D, Chamberland A, Asselin MC, Canuel M, Constam D, Creemers JW, Day R, Gauthier D, Prat A, Seidah NG (2013). Furin is the primary in vivo convertase of angiopoietin-like 3 and endothelial lipase in hepatocytes. *J Biol Chem*. **288**, 26410-26418.

Faraci FM (2005). Oxidative stress: the curse that underlies cerebral vascular dysfunction? *Stroke*. **36**, 186-188.

Faraci FM (2006). Reactive oxygen species: influence on cerebral vascular tone. *J Appl Physiol (1985)*. **100**, 739-743.

Faraci FM (2011). Protecting against vascular disease in brain. *Am J Physiol Heart Circ Physiol*. **300**, H1566-1582.

Faraci FM, Heistad DD (1998). Regulation of the cerebral circulation: role of endothelium and potassium channels. *Physiol Rev*. **78**, 53-97.
Faraci FM, Lentz SR (2004). Hyperhomocysteinemia, oxidative stress, and cerebral vascular dysfunction. *Stroke*. **35**, 345-347.
Faraci FM, Modrick ML, Lynch CM, Didion LA, Fegan PE, Didion SP (2006). Selective cerebral vascular dysfunction in Mn-SOD-deficient mice. *J Appl Physiol (1985)*. **100**, 2089-2093.
Faraci FM, Sobey CG (1998). Role of potassium channels in regulation of cerebral vascular tone. *J Cereb Blood Flow Metab*. **18**, 1047-1063.
Farhat N, Mamarbachi AM, Thorin E, Allen BG (2014). Cloning, expression and purification of functionally active human angiopoietin-like protein 2. *Springerplus*. **3**, 337.
Farhat N, Thorin-Trescases N, Mamarbachi M, Villeneuve L, Yu C, Martel C, Duquette N, Gayda M, Nigam A, Juneau M, Allen BG, Thorin E (2013). Angiopoietin-like 2 promotes atherogenesis in mice. *J Am Heart Assoc*. **2**, e000201.
Farhat N, Thorin-Trescases N, Voghel G, Villeneuve L, Mamarbachi M, Perrault LP, Carrier M, Thorin E (2008). Stress-induced senescence predominates in endothelial cells isolated from atherosclerotic chronic smokers. *Can J Physiol Pharmacol*. **86**, 761-769.
Feletou M, Huang Y, Vanhoutte PM (2011). Endothelium-mediated control of vascular tone: COX-1 and COX-2 products. *Br J Pharmacol*. **164**, 894-912.
Feletou M, Vanhoutte PM (1988). Endothelium-dependent hyperpolarization of canine coronary smooth muscle. *Br J Pharmacol*. **93**, 515-524.
Feletou M, Vanhoutte PM (2006). Endothelial dysfunction: a multifaceted disorder (The Wiggers Award Lecture). *Am J Physiol Heart Circ Physiol*. **291**, H985-1002.
Feletou M, Vanhoutte PM (2007). Endothelium-dependent hyperpolarizations: past beliefs and present facts. *Ann Med*. **39**, 495-516.
Ferrario CM (2006). Role of angiotensin II in cardiovascular disease therapeutic implications of more than a century of research. *J Renin Angiotensin Aldosterone Syst*. **7**, 3-14.
Fichtlscherer S, Breuer S, Zeiher AM (2004). Prognostic value of systemic endothelial dysfunction in patients with acute coronary syndromes: further evidence for the existence of the "vulnerable" patient. *Circulation*. **110**, 1926-1932.
Firestein GS (2003). Evolving concepts of rheumatoid arthritis. *Nature*. **423**, 356-361.
Fleming I (2000). Cytochrome P450 2C is an EDHF synthase in coronary arteries. *Trends Cardiovasc Med*. **10**, 166-170.
Flier JS (2004). Obesity wars: molecular progress confronts an expanding epidemic. *Cell*. **116**, 337-350.
Florez A, de Haro J, Martinez E, Varela C, Bleda S, Acin F (2009). Selective cyclooxygenase-2 inhibition reduces endothelial dysfunction and improves inflammatory status in patients with intermittent claudication. *Rev Esp Cardiol (Engl Ed)*. **62**, 851-857.

Floyd RA, Carney JM (1992). Free radical damage to protein and DNA: mechanisms involved and relevant observations on brain undergoing oxidative stress. *Ann Neurol*. **32 Suppl**, S22-27.

Folkman J (1971). Tumor angiogenesis: therapeutic implications. *N Engl J Med*. **285**, 1182-1186.

Folsom AR, Peacock JM, Demerath E, Boerwinkle E (2008). Variation in ANGPTL4 and risk of coronary heart disease: the Atherosclerosis Risk in Communities Study. *Metabolism*. **57**, 1591-1596.

Forman BM, Chen J, Evans RM (1997). Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors alpha and delta. *Proc Natl Acad Sci U S A*. **94**, 4312-4317.

Forstermann U, Closs EI, Pollock JS, Nakane M, Schwarz P, Gath I, Kleinert H (1994). Nitric oxide synthase isozymes. Characterization, purification, molecular cloning, and functions. *Hypertension*. **23**, 1121-1131.

Forstermann U, Munzel T (2006). Endothelial nitric oxide synthase in vascular disease: from marvel to menace. *Circulation*. **113**, 1708-1714.

Forstermann U, Nakane M, Tracey WR, Pollock JS (1993). Isoforms of nitric oxide synthase: functions in the cardiovascular system. *Eur Heart J*. **14 Suppl I**, 10-15.

Fox K, Ford I, Steg PG, Tendera M, Robertson M, Ferrari R (2008). Heart rate as a prognostic risk factor in patients with coronary artery disease and left-ventricular systolic dysfunction (BEAUTIFUL): a subgroup analysis of a randomised controlled trial. *Lancet*. **372**, 817-821.

Fraile ML, Conde MV, Sanz L, Moreno MJ, Marco EJ, Lopez de Pablo AL (1994). Different influence of superoxide anions and hydrogen peroxide on endothelial function of isolated cat cerebral and pulmonary arteries. *Gen Pharmacol*. **25**, 1197-1205.

Francis SH, Corbin JD (1994). Progress in understanding the mechanism and function of cyclic GMP-dependent protein kinase. *Adv Pharmacol*. **26**, 115-170.

Franssen R, Monajemi H, Stroes ES, Kastelein JJ (2008). Obesity and dyslipidemia. *Endocrinol Metab Clin North Am*. **37**, 623-633, viii.

Friedman JM, Halaas JL (1998). Leptin and the regulation of body weight in mammals. *Nature*. **395**, 763-770.

Fu Z, Berhane F, Fite A, Seyoum B, Abou-Samra AB, Zhang R (2014). Elevated circulating lipasin/betatrophin in human type 2 diabetes and obesity. *Sci Rep*. **4**, 5013.

Fujii K, Heistad DD, Faraci FM (1991). Flow-mediated dilatation of the basilar artery in vivo. *Circ Res*. **69**, 697-705.

Fujimoto K, Koishi R, Shimizugawa T, Ando Y (2006). Angptl3-null mice show low plasma lipid concentrations by enhanced lipoprotein lipase activity. *Exp Anim*. **55**, 27-34.

Fujimoto N, Prasad A, Hastings JL, Arbab-Zadeh A, Bhella PS, Shibata S, Palmer D, Levine BD (2010). Cardiovascular effects of 1 year of progressive and vigorous exercise training in previously sedentary individuals older than 65 years of age. *Circulation*. **122**, 1797-1805.

Fukai T, Siegfried MR, Ushio-Fukai M, Cheng Y, Kojda G, Harrison DG (2000). Regulation of the vascular extracellular superoxide dismutase by nitric oxide and exercise training. *J Clin Invest*. **105**, 1631-1639.
Fukumura D, Ushiyama A, Duda DG, Xu L, Tam J, Krishna V, Chatterjee K, Garkavtsev I, Jain RK (2003). Paracrine regulation of angiogenesis and adipocyte differentiation during in vivo adipogenesis. *Circ Res*. **93**, e88-97.
Fulton D, Mahboubi K, McGiff JC, Quilley J (1995). Cytochrome P450-dependent effects of bradykinin in the rat heart. *Br J Pharmacol*. **114**, 99-102.
Furchgott RF, Zawadzki JV (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*. **288**, 373-376.
Gao L, Ge C, Fang T, Zhao F, Chen T, Yao M, Li J, Li H (2014). ANGPTL2 Promotes Tumor Metastasis in Hepatocellular Carcinoma. *J Gastroenterol Hepatol*.
Gao YJ, Lu C, Su LY, Sharma AM, Lee RM (2007). Modulation of vascular function by perivascular adipose tissue: the role of endothelium and hydrogen peroxide. *Br J Pharmacol*. **151**, 323-331.
Garland CJ, Plane F, Kemp BK, Cocks TM (1995). Endothelium-dependent hyperpolarization: a role in the control of vascular tone. *Trends Pharmacol Sci*. **16**, 23-30.
Gavazzi G, Banfi B, Deffert C, Fiette L, Schappi M, Herrmann F, Krause KH (2006). Decreased blood pressure in NOX1-deficient mice. *FEBS Lett*. **580**, 497-504.
Gavazzi G, Deffert C, Trocme C, Schappi M, Herrmann FR, Krause KH (2007). NOX1 deficiency protects from aortic dissection in response to angiotensin II. *Hypertension*. **50**, 189-196.
Ge H, Cha JY, Gopal H, Harp C, Yu X, Repa JJ, Li C (2005). Differential regulation and properties of angiopoietin-like proteins 3 and 4. *J Lipid Res*. **46**, 1484-1490.
Ge H, Yang G, Huang L, Motola DL, Pourbahrami T, Li C (2004). Oligomerization and regulated proteolytic processing of angiopoietin-like protein 4. *J Biol Chem*. **279**, 2038-2045.
Gendron ME, Theoret JF, Mamarbachi AM, Drouin A, Nguyen A, Bolduc V, Thorin-Trescases N, Merhi Y, Thorin E (2010). Late chronic catechin antioxidant treatment is deleterious to the endothelial function in aging mice with established atherosclerosis. *Am J Physiol Heart Circ Physiol*. **298**, H2062-2070.
Gendron ME, Thorin E (2007). A change in the redox environment and thromboxane A2 production precede endothelial dysfunction in mice. *Am J Physiol Heart Circ Physiol*. **293**, H2508-2515.
Gendron ME, Thorin-Trescases N, Mamarbachi AM, Villeneuve L, Theoret JF, Mehri Y, Thorin E (2012). Time-dependent beneficial effect of chronic polyphenol treatment with catechin on endothelial dysfunction in aging mice. *Dose Response*. **10**, 108-119.
Georgiadi A, Wang Y, Stienstra R, Tjeerdema N, Janssen A, Stalenhoef A, van der Vliet JA, de Roos A, Tamsma JT, Smit JW, Tan NS, Muller M, Rensen PC, Kersten S (2013). Overexpression of angiopoietin-like protein 4 protects

against atherosclerosis development. *Arterioscler Thromb Vasc Biol*. **33**, 1529-1537.
Giaid A, Yanagisawa M, Langleben D, Michel RP, Levy R, Shennib H, Kimura S, Masaki T, Duguid WP, Stewart DJ (1993). Expression of endothelin-1 in the lungs of patients with pulmonary hypertension. *N Engl J Med*. **328**, 1732-1739.
Gielen S, Schuler G, Adams V (2010). Cardiovascular effects of exercise training: molecular mechanisms. *Circulation*. **122**, 1221-1238.
Girouard H, Park L, Anrather J, Zhou P, Iadecola C (2006). Angiotensin II attenuates endothelium-dependent responses in the cerebral microcirculation through nox-2-derived radicals. *Arterioscler Thromb Vasc Biol*. **26**, 826-832.
Girouard H, Park L, Anrather J, Zhou P, Iadecola C (2007). Cerebrovascular nitrosative stress mediates neurovascular and endothelial dysfunction induced by angiotensin II. *Arterioscler Thromb Vasc Biol*. **27**, 303-309.
Gluais P, Lonchampt M, Morrow JD, Vanhoutte PM, Feletou M (2005). Acetylcholine-induced endothelium-dependent contractions in the SHR aorta: the Janus face of prostacyclin. *Br J Pharmacol*. **146**, 834-845.
Go AS, Mozaffarian D, Roger VL, Benjamin EJ, Berry JD, Borden WB, Bravata DM, Dai S, Ford ES, Fox CS, Franco S, Fullerton HJ, Gillespie C, Hailpern SM, Heit JA, Howard VJ, Huffman MD, Kissela BM, Kittner SJ, Lackland DT, Lichtman JH, Lisabeth LD, Magid D, Marcus GM, Marelli A, Matchar DB, McGuire DK, Mohler ER, Moy CS, Mussolino ME, Nichol G, Paynter NP, Schreiner PJ, Sorlie PD, Stein J, Turan TN, Virani SS, Wong ND, Woo D, Turner MB (2013). Heart disease and stroke statistics--2013 update: a report from the American Heart Association. *Circulation*. **127**, e6-e245.
Godecke A, Ziegler M, Ding Z, Schrader J (2002). Endothelial dysfunction of coronary resistance vessels in apoE-/- mice involves NO but not prostacyclin-dependent mechanisms. *Cardiovasc Res*. **53**, 253-262.
Goldberg IJ, Eckel RH, Abumrad NA (2009). Regulation of fatty acid uptake into tissues: lipoprotein lipase- and CD36-mediated pathways. *J Lipid Res*. **50 Suppl**, S86-90.
Goldstein DS (1981). Plasma norepinephrine in essential hypertension. A study of the studies. *Hypertension*. **3**, 48-52.
Gonzalez FJ (2005). Role of cytochromes P450 in chemical toxicity and oxidative stress: studies with CYP2E1. *Mutat Res*. **569**, 101-110.
Goodwin DC, Gunther MR, Hsi LC, Crews BC, Eling TE, Mason RP, Marnett LJ (1998). Nitric oxide trapping of tyrosyl radicals generated during prostaglandin endoperoxide synthase turnover. Detection of the radical derivative of tyrosine 385. *J Biol Chem*. **273**, 8903-8909.
Goto K, Kansui Y, Oniki H, Ohtsubo T, Matsumura K, Kitazono T (2012). Upregulation of endothelium-derived hyperpolarizing factor compensates for the loss of nitric oxide in mesenteric arteries of Dahl salt-sensitive hypertensive rats. *Hypertens Res*. **35**, 849-854.
Green DJ, Maiorana A, O'Driscoll G, Taylor R (2004). Effect of exercise training on endothelium-derived nitric oxide function in humans. *J Physiol*. **561**, 1-25.

Griendling KK, Minieri CA, Ollerenshaw JD, Alexander RW (1994). Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. *Circ Res*. **74**, 1141-1148.

Griendling KK, Rittenhouse SE, Brock TA, Ekstein LS, Gimbrone MA, Jr., Alexander RW (1986). Sustained diacylglycerol formation from inositol phospholipids in angiotensin II-stimulated vascular smooth muscle cells. *J Biol Chem*. **261**, 5901-5906.

Griendling KK, Ushio-Fukai M, Lassegue B, Alexander RW (1997). Angiotensin II signaling in vascular smooth muscle. New concepts. *Hypertension*. **29**, 366-373.

Griffoni C, Spisni E, Strillacci A, Toni M, Bachschmid MM, Tomasi V (2007). Selective inhibition of prostacyclin synthase activity by rofecoxib. *J Cell Mol Med*. **11**, 327-338.

Groemping Y, Lapouge K, Smerdon SJ, Rittinger K (2003). Molecular basis of phosphorylation-induced activation of the NADPH oxidase. *Cell*. **113**, 343-355.

Groemping Y, Rittinger K (2005). Activation and assembly of the NADPH oxidase: a structural perspective. *Biochem J*. **386**, 401-416.

Groves P, Kurz S, Just H, Drexler H (1995). Role of endogenous bradykinin in human coronary vasomotor control. *Circulation*. **92**, 3424-3430.

Grundy SM, Brewer HB, Jr., Cleeman JI, Smith SC, Jr., Lenfant C (2004). Definition of metabolic syndrome: Report of the National Heart, Lung, and Blood Institute/American Heart Association conference on scientific issues related to definition. *Circulation*. **109**, 433-438.

Grunfeld S, Hamilton CA, Mesaros S, McClain SW, Dominiczak AF, Bohr DF, Malinski T (1995). Role of superoxide in the depressed nitric oxide production by the endothelium of genetically hypertensive rats. *Hypertension*. **26**, 854-857.

Gryglewski RJ, Palmer RM, Moncada S (1986). Superoxide anion is involved in the breakdown of endothelium-derived vascular relaxing factor. *Nature*. **320**, 454-456.

Guilherme A, Virbasius JV, Puri V, Czech MP (2008). Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes. *Nat Rev Mol Cell Biol*. **9**, 367-377.

Gunnett CA, Heistad DD, Faraci FM (2003). Gene-targeted mice reveal a critical role for inducible nitric oxide synthase in vascular dysfunction during diabetes. *Stroke*. **34**, 2970-2974.

Guo DF, Chenier I, Lavoie JL, Chan JS, Hamet P, Tremblay J, Chen XM, Wang DH, Inagami T (2006). Development of hypertension and kidney hypertrophy in transgenic mice overexpressing ARAP1 gene in the kidney. *Hypertension*. **48**, 453-459.

Guo DF, Chenier I, Tardif V, Orlov SN, Inagami T (2003). Type 1 angiotensin II receptor-associated protein ARAP1 binds and recycles the receptor to the plasma membrane. *Biochem Biophys Res Commun*. **310**, 1254-1265.

Guo DF, Sun YL, Hamet P, Inagami T (2001). The angiotensin II type 1 receptor and receptor-associated proteins. *Cell Res*. **11**, 165-180.

Gylling H, Miettinen TA (1997). Cholesterol absorption, synthesis, and LDL metabolism in NIDDM. *Diabetes Care*. **20**, 90-95.

Haas JA, Lockhart JC, Larson TS, Henrikson T, Knox FG (1994). Natriuretic response to renal interstitial hydrostatic pressure during angiotensin II blockade. *Am J Physiol*. **266**, F117-119.

Hadi HA, Suwaidi JA (2007). Endothelial dysfunction in diabetes mellitus. *Vasc Health Risk Manag*. **3**, 853-876.

Hara S, Miyata A, Yokoyama C, Inoue H, Brugger R, Lottspeich F, Ullrich V, Tanabe T (1994). Isolation and molecular cloning of prostacyclin synthase from bovine endothelial cells. *J Biol Chem*. **269**, 19897-19903.

Harman D (1956). Aging: a theory based on free radical and radiation chemistry. *J Gerontol*. **11**, 298-300.

Harris MB, Ju H, Venema VJ, Liang H, Zou R, Michell BJ, Chen ZP, Kemp BE, Venema RC (2001). Reciprocal phosphorylation and regulation of endothelial nitric-oxide synthase in response to bradykinin stimulation. *J Biol Chem*. **276**, 16587-16591.

Harris RB (2014). Direct and indirect effects of leptin on adipocyte metabolism. *Biochim Biophys Acta*. **1842**, 414-423.

Harrison DG, Widder J, Grumbach I, Chen W, Weber M, Searles C (2006). Endothelial mechanotransduction, nitric oxide and vascular inflammation. *J Intern Med*. **259**, 351-363.

Hato T, Tabata M, Oike Y (2008). The role of angiopoietin-like proteins in angiogenesis and metabolism. *Trends Cardiovasc Med*. **18**, 6-14.

Hatsuda S, Shoji T, Shinohara K, Kimoto E, Mori K, Fukumoto S, Koyama H, Emoto M, Nishizawa Y (2007). Association between plasma angiopoietin-like protein 3 and arterial wall thickness in healthy subjects. *J Vasc Res*. **44**, 61-66.

Haynes WG, Strachan FE, Webb DJ (1995). Endothelin ETA and ETB receptors cause vasoconstriction of human resistance and capacitance vessels in vivo. *Circulation*. **92**, 357-363.

Heeneman S, Haendeler J, Saito Y, Ishida M, Berk BC (2000). Angiotensin II induces transactivation of two different populations of the platelet-derived growth factor beta receptor. Key role for the p66 adaptor protein Shc. *J Biol Chem*. **275**, 15926-15932.

Heitzer T, Brockhoff C, Mayer B, Warnholtz A, Mollnau H, Henne S, Meinertz T, Munzel T (2000). Tetrahydrobiopterin improves endothelium-dependent vasodilation in chronic smokers : evidence for a dysfunctional nitric oxide synthase. *Circ Res*. **86**, E36-41.

Heller R, Unbehaun A, Schellenberg B, Mayer B, Werner-Felmayer G, Werner ER (2001). L-ascorbic acid potentiates endothelial nitric oxide synthesis via a chemical stabilization of tetrahydrobiopterin. *J Biol Chem*. **276**, 40-47.

Henderson LM, Banting G, Chappell JB (1995). The arachidonate-activable, NADPH oxidase-associated H+ channel. Evidence that gp91-phox functions as an essential part of the channel. *J Biol Chem*. **270**, 5909-5916.

Henrion D, Kubis N, Levy BI (2001). Physiological and pathophysiological functions of the AT(2) subtype receptor of angiotensin II: from large arteries to the microcirculation. *Hypertension*. **38**, 1150-1157.

Herrick S, Blanc-Brude O, Gray A, Laurent G (1999). Fibrinogen. *Int J Biochem Cell Biol*. **31**, 741-746.

Hibbs JB, Jr., Taintor RR, Vavrin Z, Rachlin EM (1988). Nitric oxide: a cytotoxic activated macrophage effector molecule. *Biochem Biophys Res Commun*. **157**, 87-94.

Hickey KA, Rubanyi G, Paul RJ, Highsmith RF (1985). Characterization of a coronary vasoconstrictor produced by cultured endothelial cells. *Am J Physiol*. **248**, C550-556.

Higashi Y, Yoshizumi M (2004). Exercise and endothelial function: role of endothelium-derived nitric oxide and oxidative stress in healthy subjects and hypertensive patients. *Pharmacol Ther*. **102**, 87-96.

Higashimori M, Tatro JB, Moore KJ, Mendelsohn ME, Galper JB, Beasley D (2011). Role of toll-like receptor 4 in intimal foam cell accumulation in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol*. **31**, 50-57.

Higuchi S, Ohtsu H, Suzuki H, Shirai H, Frank GD, Eguchi S (2007). Angiotensin II signal transduction through the AT1 receptor: novel insights into mechanisms and pathophysiology. *Clin Sci (Lond)*. **112**, 417-428.

Horio E, Kadomatsu T, Miyata K, Arai Y, Hosokawa K, Doi Y, Ninomiya T, Horiguchi H, Endo M, Tabata M, Tazume H, Tian Z, Takahashi O, Terada K, Takeya M, Hao H, Hirose N, Minami T, Suda T, Kiyohara Y, Ogawa H, Kaikita K, Oike Y (2014). Role of Endothelial Cell-Derived Angptl2 in Vascular Inflammation Leading to Endothelial Dysfunction and Atherosclerosis Progression. *Arterioscler Thromb Vasc Biol*. **34**, 790-800.

Hosoya T, Maruyama A, Kang MI, Kawatani Y, Shibata T, Uchida K, Warabi E, Noguchi N, Itoh K, Yamamoto M (2005). Differential responses of the Nrf2-Keap1 system to laminar and oscillatory shear stresses in endothelial cells. *J Biol Chem*. **280**, 27244-27250.

Hotamisligil GS, Arner P, Caro JF, Atkinson RL, Spiegelman BM (1995). Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. *J Clin Invest*. **95**, 2409-2415.

Hsu YH, Chen JJ, Chang NC, Chen CH, Liu JC, Chen TH, Jeng CJ, Chao HH, Cheng TH (2004). Role of reactive oxygen species-sensitive extracellular signal-regulated kinase pathway in angiotensin II-induced endothelin-1 gene expression in vascular endothelial cells. *J Vasc Res*. **41**, 64-74.

Hsueh WA, Law RE (1999). Insulin signaling in the arterial wall. *Am J Cardiol*. **84**, 21J-24J.

Huang A, Vita JA, Venema RC, Keaney JF, Jr. (2000). Ascorbic acid enhances endothelial nitric-oxide synthase activity by increasing intracellular tetrahydrobiopterin. *J Biol Chem*. **275**, 17399-17406.

Iacobellis G, Gao YJ, Sharma AM (2008). Do cardiac and perivascular adipose tissue play a role in atherosclerosis? *Curr Diab Rep*. **8**, 20-24.

Iadecola C (1992). Does nitric oxide mediate the increases in cerebral blood flow elicited by hypercapnia? *Proc Natl Acad Sci U S A*. **89**, 3913-3916.

Ibrahim MM (2006). RAS inhibition in hypertension. *J Hum Hypertens*. **20**, 101-108.

Ichihara A, Imig JD, Inscho EW, Navar LG (1998). Cyclooxygenase-2 participates in tubular flow-dependent afferent arteriolar tone: interaction with neuronal NOS. *Am J Physiol*. **275**, F605-612.

Iesaki T, Gupte SA, Kaminski PM, Wolin MS (1999). Inhibition of guanylate cyclase stimulation by NO and bovine arterial relaxation to peroxynitrite and H2O2. *Am J Physiol*. **277**, H978-985.

Ignarro LJ (1989). Endothelium-derived nitric oxide: actions and properties. *FASEB J*. **3**, 31-36.

Iida Y, Katusic ZS (2000). Mechanisms of cerebral arterial relaxations to hydrogen peroxide. *Stroke*. **31**, 2224-2230.

Ikushima H, Miyazono K (2010). TGFbeta signalling: a complex web in cancer progression. *Nat Rev Cancer*. **10**, 415-424.

Inaba T, Matsuda M, Shimamura M, Takei N, Terasaka N, Ando Y, Yasumo H, Koishi R, Makishima M, Shimomura I (2003). Angiopoietin-like protein 3 mediates hypertriglyceridemia induced by the liver X receptor. *J Biol Chem*. **278**, 21344-21351.

Inagami T, Guo DF, Kitami Y (1994). Molecular biology of angiotensin II receptors: an overview. *J Hypertens Suppl*. **12**, S83-94.

Inoue A, Yanagisawa M, Kimura S, Kasuya Y, Miyauchi T, Goto K, Masaki T (1989). The human endothelin family: three structurally and pharmacologically distinct isopeptides predicted by three separate genes. *Proc Natl Acad Sci U S A*. **86**, 2863-2867.

Inukai K, Nakashima Y, Watanabe M, Kurihara S, Awata T, Katagiri H, Oka Y, Katayama S (2004). ANGPTL3 is increased in both insulin-deficient and -resistant diabetic states. *Biochem Biophys Res Commun*. **317**, 1075-1079.

Ito T, Kato T, Iwama Y, Muramatsu M, Shimizu K, Asano H, Okumura K, Hashimoto H, Satake T (1991). Prostaglandin H2 as an endothelium-derived contracting factor and its interaction with endothelium-derived nitric oxide. *J Hypertens*. **9**, 729-736.

Ito Y, Oike Y, Yasunaga K, Hamada K, Miyata K, Matsumoto S, Sugano S, Tanihara H, Masuho Y, Suda T (2003). Inhibition of angiogenesis and vascular leakiness by angiopoietin-related protein 4. *Cancer Res*. **63**, 6651-6657.

Iwasa S, Fan J, Shimokama T, Nagata M, Watanabe T (1999). Increased immunoreactivity of endothelin-1 and endothelin B receptor in human atherosclerotic lesions. A possible role in atherogenesis. *Atherosclerosis*. **146**, 93-100.

Jeong YS, Kim D, Lee YS, Kim HJ, Han JY, Im SS, Chong HK, Kwon JK, Cho YH, Kim WK, Osborne TF, Horton JD, Jun HS, Ahn YH, Ahn SM, Cha JY (2011). Integrated expression profiling and genome-wide analysis of ChREBP targets reveals the dual role for ChREBP in glucose-regulated gene expression. *PLoS One*. **6**, e22544.

Jiang DJ, Hu GY, Jiang JL, Xiang HL, Deng HW, Li YJ (2003). Relationship between protective effect of xanthone on endothelial cells and endogenous nitric oxide synthase inhibitors. *Bioorg Med Chem*. **11**, 5171-5177.

Jin W, Wang X, Millar JS, Quertermous T, Rothblat GH, Glick JM, Rader DJ (2007). Hepatic proprotein convertases modulate HDL metabolism. *Cell Metab*. **6**, 129-136.

Johnson NA, Sachinwalla T, Walton DW, Smith K, Armstrong A, Thompson MW, George J (2009). Aerobic exercise training reduces hepatic and visceral lipids in obese individuals without weight loss. *Hepatology*. **50**, 1105-1112.

Jonker JT, Smit JW, Hammer S, Snel M, van der Meer RW, Lamb HJ, Mattijssen F, Mudde K, Jazet IM, Dekkers OM, de Roos A, Romijn JA, Kersten S, Rensen PC (2013). Dietary modulation of plasma angiopoietin-like protein 4 concentrations in healthy volunteers and in patients with type 2 diabetes. *Am J Clin Nutr*. **97**, 255-260.

Jubelin BC, Gierman JL (1996). Erythrocytes may synthesize their own nitric oxide. *Am J Hypertens.* **9**, 1214-1219.

Jung O, Schreiber JG, Geiger H, Pedrazzini T, Busse R, Brandes RP (2004). gp91phox-containing NADPH oxidase mediates endothelial dysfunction in renovascular hypertension. *Circulation*. **109**, 1795-1801.

Kaapa P, Viinikka L, Ylikorkala O (1982). Plasma prostacyclin from birth to adolescence. *Arch Dis Child*. **57**, 459-461.

Kadomatsu T, Endo M, Miyata K, Oike Y (2014). Diverse roles of ANGPTL2 in physiology and pathophysiology. *Trends Endocrinol Metab*. **25**, 245-254.

Kadomatsu T, Tabata M, Oike Y (2011). Angiopoietin-like proteins: emerging targets for treatment of obesity and related metabolic diseases. *FEBS J*. **278**, 559-564.

Kadomatsu T, Uragami S, Akashi M, Tsuchiya Y, Nakajima H, Nakashima Y, Endo M, Miyata K, Terada K, Todo T, Node K, Oike Y (2013). A molecular clock regulates angiopoietin-like protein 2 expression. *PLoS One*. **8**, e57921.

Kai H, Griendling KK, Lassegue B, Ollerenshaw JD, Runge MS, Alexander RW (1994). Agonist-induced phosphorylation of the vascular type 1 angiotensin II receptor. *Hypertension*. **24**, 523-527.

Kanda H, Tateya S, Tamori Y, Kotani K, Hiasa K, Kitazawa R, Kitazawa S, Miyachi H, Maeda S, Egashira K, Kasuga M (2006). MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. *J Clin Invest*. **116**, 1494-1505.

Kang RY, Freire-Moar J, Sigal E, Chu CQ (1996). Expression of cyclooxygenase-2 in human and an animal model of rheumatoid arthritis. *Br J Rheumatol*. **35**, 711-718.

Katano H, Yamada K (2013). Upregulation of ANGPTL4 messenger RNA and protein in severely calcified carotid plaques. *J Stroke Cerebrovasc Dis*. **23**, 933-947.

Katoh Y, Katoh M (2006). Comparative integromics on Angiopoietin family members. *Int J Mol Med*. **17**, 1145-1149.

Katusic ZS (1996). Superoxide anion and endothelial regulation of arterial tone. *Free Radic Biol Med*. **20**, 443-448.

Katusic ZS, Vanhoutte PM (1989). Superoxide anion is an endothelium-derived contracting factor. *Am J Physiol*. **257**, H33-37.

Katzmarzyk PT, Janssen I (2004). The economic costs associated with physical inactivity and obesity in Canada: an update. *Can J Appl Physiol*. **29**, 90-115.

Kawahara T, Ritsick D, Cheng G, Lambeth JD (2005). Point mutations in the proline-rich region of p22phox are dominant inhibitors of Nox1- and Nox2-dependent reactive oxygen generation. *J Biol Chem*. **280**, 31859-31869.

Kazama K, Anrather J, Zhou P, Girouard H, Frys K, Milner TA, Iadecola C (2004). Angiotensin II impairs neurovascular coupling in neocortex through NADPH oxidase-derived radicals. *Circ Res*. **95**, 1019-1026.

Kelly RA, Balligand JL, Smith TW (1996). Nitric oxide and cardiac function. *Circ Res*. **79**, 363-380.

Kelm M, Schrader J (1990). Control of coronary vascular tone by nitric oxide. *Circ Res*. **66**, 1561-1575.

Kensler TW, Wakabayashi N, Biswal S (2007). Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. *Annu Rev Pharmacol Toxicol*. **47**, 89-116.

Kersten S, Lichtenstein L, Steenbergen E, Mudde K, Hendriks HF, Hesselink MK, Schrauwen P, Muller M (2009). Caloric restriction and exercise increase plasma ANGPTL4 levels in humans via elevated free fatty acids. *Arterioscler Thromb Vasc Biol*. **29**, 969-974.

Kersten S, Mandard S, Tan NS, Escher P, Metzger D, Chambon P, Gonzalez FJ, Desvergne B, Wahli W (2000). Characterization of the fasting-induced adipose factor FIAF, a novel peroxisome proliferator-activated receptor target gene. *J Biol Chem*. **275**, 28488-28493.

Kessler P, Bauersachs J, Busse R, Schini-Kerth VB (1997). Inhibition of inducible nitric oxide synthase restores endothelium-dependent relaxations in proinflammatory mediator-induced blood vessels. *Arterioscler Thromb Vasc Biol*. **17**, 1746-1755.

Kikuchi R, Tsuda H, Kozaki K, Kanai Y, Kasamatsu T, Sengoku K, Hirohashi S, Inazawa J, Imoto I (2008). Frequent inactivation of a putative tumor suppressor, angiopoietin-like protein 2, in ovarian cancer. *Cancer Res*. **68**, 5067-5075.

Kim HK, Youn BS, Shin MS, Namkoong C, Park KH, Baik JH, Kim JB, Park JY, Lee KU, Kim YB, Kim MS (2010). Hypothalamic Angptl4/Fiaf is a novel regulator of food intake and body weight. *Diabetes*. **59**, 2772-2780.

Kim I, Kim HG, Kim H, Kim HH, Park SK, Uhm CS, Lee ZH, Koh GY (2000). Hepatic expression, synthesis and secretion of a novel fibrinogen/angiopoietin-related protein that prevents endothelial-cell apoptosis. *Biochem J*. **346 Pt 3**, 603-610.

Kim I, Kwak HJ, Ahn JE, So JN, Liu M, Koh KN, Koh GY (1999a). Molecular cloning and characterization of a novel angiopoietin family protein, angiopoietin-3. *FEBS Lett*. **443**, 353-356.

Kim I, Moon SO, Koh KN, Kim H, Uhm CS, Kwak HJ, Kim NG, Koh GY (1999b). Molecular cloning, expression, and characterization of angiopoietin-related protein. angiopoietin-related protein induces endothelial cell sprouting. *J Biol Chem*. **274**, 26523-26528.

Kim JA, Montagnani M, Koh KK, Quon MJ (2006). Reciprocal relationships between insulin resistance and endothelial dysfunction: molecular and pathophysiological mechanisms. *Circulation*. **113**, 1888-1904.

Kim JA, Wei Y, Sowers JR (2008). Role of mitochondrial dysfunction in insulin resistance. *Circ Res*. **102**, 401-414.

Kim SF, Huri DA, Snyder SH (2005). Inducible nitric oxide synthase binds, S-nitrosylates, and activates cyclooxygenase-2. *Science*. **310**, 1966-1970.

Kitazawa M, Nagano M, Masumoto KH, Shigeyoshi Y, Natsume T, Hashimoto S (2011). Angiopoietin-like 2, a circadian gene, improves type 2 diabetes through potentiation of insulin sensitivity in mice adipocytes. *Endocrinology*. **152**, 2558-2567.

Kitazawa M, Ohizumi Y, Oike Y, Hishinuma T, Hashimoto S (2007). Angiopoietin-related growth factor suppresses gluconeogenesis through the Akt/forkhead box class O1-dependent pathway in hepatocytes. *J Pharmacol Exp Ther*. **323**, 787-793.

Klaunig JE, Kamendulis LM (2004). The role of oxidative stress in carcinogenesis. *Annu Rev Pharmacol Toxicol*. **44**, 239-267.

Klaunig JE, Wang Z, Pu X, Zhou S (2011). Oxidative stress and oxidative damage in chemical carcinogenesis. *Toxicol Appl Pharmacol*. **254**, 86-99.

Klein S, de Fougerolles AR, Blaikie P, Khan L, Pepe A, Green CD, Koteliansky V, Giancotti FG (2002). Alpha 5 beta 1 integrin activates an NF-kappa B-dependent program of gene expression important for angiogenesis and inflammation. *Mol Cell Biol*. **22**, 5912-5922.

Klip A, Ramlal T, Bilan PJ, Marette A, Liu Z, Mitsumoto Y (1993). What signals are involved in the stimulation of glucose transport by insulin in muscle cells? *Cell Signal*. **5**, 519-529.

Koishi R, Ando Y, Ono M, Shimamura M, Yasumo H, Fujiwara T, Horikoshi H, Furukawa H (2002). Angptl3 regulates lipid metabolism in mice. *Nat Genet*. **30**, 151-157.

Koller A, Huang A, Sun D, Kaley G (1995). Exercise training augments flow-dependent dilation in rat skeletal muscle arterioles. Role of endothelial nitric oxide and prostaglandins. *Circ Res*. **76**, 544-550.

Kolovou GD, Kostakou PM, Anagnostopoulou KK (2011). Familial hypercholesterolemia and triglyceride metabolism. *Int J Cardiol*. **147**, 349-358.

Komori K, Vanhoutte PM (1990). Endothelium-derived hyperpolarizing factor. *Blood Vessels*. **27**, 238-245.

Kontos HA, Wei EP, Povlishock JT, Christman CW (1984). Oxygen radicals mediate the cerebral arteriolar dilation from arachidonate and bradykinin in cats. *Circ Res*. **55**, 295-303.

Korstanje R, Eriksson P, Samnegard A, Olsson PG, Forsman-Semb K, Sen S, Churchill GA, Rollins J, Harris S, Hamsten A, Paigen B (2004). Locating Ath8, a locus for murine atherosclerosis susceptibility and testing several of its candidate genes in mice and humans. *Atherosclerosis*. **177**, 443-450.

Koster A, Chao YB, Mosior M, Ford A, Gonzalez-DeWhitt PA, Hale JE, Li D, Qiu Y, Fraser CC, Yang DD, Heuer JG, Jaskunas SR, Eacho P (2005). Transgenic angiopoietin-like (angptl)4 overexpression and targeted disruption of angptl4 and angptl3: regulation of triglyceride metabolism. *Endocrinology*. **146**, 4943-4950.

Kostic MM, Schrader J (1992). Role of nitric oxide in reactive hyperemia of the guinea pig heart. *Circ Res*. **70**, 208-212.
Kotsis V, Stabouli S, Papakatsika S, Rizos Z, Parati G (2010). Mechanisms of obesity-induced hypertension. *Hypertens Res*. **33**, 386-393.
Kou R, Michel T (2007). Epinephrine regulation of the endothelial nitric-oxide synthase: roles of RAC1 and beta3-adrenergic receptors in endothelial NO signaling. *J Biol Chem*. **282**, 32719-32729.
Kubota Y, Oike Y, Satoh S, Tabata Y, Niikura Y, Morisada T, Akao M, Urano T, Ito Y, Miyamoto T, Nagai N, Koh GY, Watanabe S, Suda T (2005). Cooperative interaction of Angiopoietin-like proteins 1 and 2 in zebrafish vascular development. *Proc Natl Acad Sci U S A*. **102**, 13502-13507.
Kuo L, Chilian WM, Davis MJ (1991). Interaction of pressure- and flow-induced responses in porcine coronary resistance vessels. *Am J Physiol*. **261**, H1706-1715.
Kuwajima M, Foster DW, McGarry JD (1988). Regulation of lipoprotein lipase in different rat tissues. *Metabolism*. **37**, 597-601.
Laakso M, Edelman SV, Brechtel G, Baron AD (1990). Decreased effect of insulin to stimulate skeletal muscle blood flow in obese man. A novel mechanism for insulin resistance. *J Clin Invest*. **85**, 1844-1852.
Laakso M, Edelman SV, Brechtel G, Baron AD (1992). Impaired insulin-mediated skeletal muscle blood flow in patients with NIDDM. *Diabetes*. **41**, 1076-1083.
Lambeth JD (2004). NOX enzymes and the biology of reactive oxygen. *Nat Rev Immunol*. **4**, 181-189.
Lamontagne D, Konig A, Bassenge E, Busse R (1992). Prostacyclin and nitric oxide contribute to the vasodilator action of acetylcholine and bradykinin in the intact rabbit coronary bed. *J Cardiovasc Pharmacol*. **20**, 652-657.
Lamping KG, Nuno DW, Shesely EG, Maeda N, Faraci FM (2000). Vasodilator mechanisms in the coronary circulation of endothelial nitric oxide synthase-deficient mice. *Am J Physiol Heart Circ Physiol*. **279**, H1906-1912.
Landmesser U, Dikalov S, Price SR, McCann L, Fukai T, Holland SM, Mitch WE, Harrison DG (2003). Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. *J Clin Invest*. **111**, 1201-1209.
Landmesser U, Spiekermann S, Dikalov S, Tatge H, Wilke R, Kohler C, Harrison DG, Hornig B, Drexler H (2002). Vascular oxidative stress and endothelial dysfunction in patients with chronic heart failure: role of xanthine-oxidase and extracellular superoxide dismutase. *Circulation*. **106**, 3073-3078.
Larsen E, Palabrica T, Sajer S, Gilbert GE, Wagner DD, Furie BC, Furie B (1990). PADGEM-dependent adhesion of platelets to monocytes and neutrophils is mediated by a lineage-specific carbohydrate, LNF III (CD15). *Cell*. **63**, 467-474.
Lassegue B, Alexander RW, Nickenig G, Clark M, Murphy TJ, Griendling KK (1995). Angiotensin II down-regulates the vascular smooth muscle AT1 receptor by transcriptional and post-transcriptional mechanisms: evidence for homologous and heterologous regulation. *Mol Pharmacol*. **48**, 601-609.

Lassegue B, Clempus RE (2003). Vascular NAD(P)H oxidases: specific features, expression, and regulation. *Am J Physiol Regul Integr Comp Physiol*. **285**, R277-297.

Lawes CM, Bennett DA, Feigin VL, Rodgers A (2004). Blood pressure and stroke: an overview of published reviews. *Stroke*. **35**, 776-785.

Lawes CM, Vander Hoorn S, Law MR, Elliott P, MacMahon S, Rodgers A (2006). Blood pressure and the global burden of disease 2000. Part II: estimates of attributable burden. *J Hypertens*. **24**, 423-430.

Le Jan S, Amy C, Cazes A, Monnot C, Lamande N, Favier J, Philippe J, Sibony M, Gasc JM, Corvol P, Germain S (2003). Angiopoietin-like 4 is a proangiogenic factor produced during ischemia and in conventional renal cell carcinoma. *Am J Pathol*. **162**, 1521-1528.

Lee HJ, Kim JH, Martinus RD, Park SH (2013). Angiopoietin-like protein 2, a chronic inflammatory mediator, is a new target induced by TGF-beta1 through a Smad3-dependent mechanism. *Biochem Biophys Res Commun*.

Lee MH, Chen SJ, Tsao CM, Wu CC (2014). Perivascular Adipose Tissue Inhibits Endothelial Function of Rat Aortas via Caveolin-1. *PLoS One*. **9**, e99947.

Leffler CW, Busija DW, Armstead WM, Mirro R (1990). H2O2 effects on cerebral prostanoids and pial arteriolar diameter in piglets. *Am J Physiol*. **258**, H1382-1387.

Legry V, Bokor S, Cottel D, Beghin L, Catasta G, Nagy E, Gonzalez-Gross M, Spinneker A, Stehle P, Molnar D, Moreno LA, Amouyel P, Dallongeville J, Meirhaeghe A (2009). Associations between common genetic polymorphisms in angiopoietin-like proteins 3 and 4 and lipid metabolism and adiposity in European adolescents and adults. *J Clin Endocrinol Metab*. **94**, 5070-5077.

Lemarie CA, Schiffrin EL (2010). The angiotensin II type 2 receptor in cardiovascular disease. *J Renin Angiotensin Aldosterone Syst*. **11**, 19-31.

Levin BE, Dunn-Meynell AA (2004). Chronic exercise lowers the defended body weight gain and adiposity in diet-induced obese rats. *Am J Physiol Regul Integr Comp Physiol*. **286**, R771-778.

Levy JV (1980). Prostacyclin-induced contraction of isolated aortic strips from normal and spontaneously hypertensive rats (SHR). *Prostaglandins*. **19**, 517-525.

Ley K, Laudanna C, Cybulsky MI, Nourshargh S (2007). Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat Rev Immunol*. **7**, 678-689.

Li Q, Gong W, Yang Z, Lu B, Yang Y, Zhao W, Hu R (2013). Serum Angptl2 levels are independently associated with albuminuria in type 2 diabetes. *Diabetes Res Clin Pract*. **100**, 385-390.

Li RC, Cindrova-Davies T, Skepper JN, Sellers LA (2004). Prostacyclin induces apoptosis of vascular smooth muscle cells by a cAMP-mediated inhibition of extracellular signal-regulated kinase activity and can counteract the mitogenic activity of endothelin-1 or basic fibroblast growth factor. *Circ Res*. **94**, 759-767.

Liang CF, Liu JT, Wang Y, Xu A, Vanhoutte PM (2013). Toll-like receptor 4 mutation protects obese mice against endothelial dysfunction by decreasing

NADPH oxidase isoforms 1 and 4. *Arterioscler Thromb Vasc Biol*. **33**, 777-784.
Libby P, Ridker PM, Maseri A (2002). Inflammation and atherosclerosis. *Circulation*. **105**, 1135-1143.
Lichtenstein L, Berbee JF, van Dijk SJ, van Dijk KW, Bensadoun A, Kema IP, Voshol PJ, Muller M, Rensen PC, Kersten S (2007). Angptl4 upregulates cholesterol synthesis in liver via inhibition of LPL- and HL-dependent hepatic cholesterol uptake. *Arterioscler Thromb Vasc Biol*. **27**, 2420-2427.
Lin J, Freeman MR (2003). Transactivation of ErbB1 and ErbB2 receptors by angiotensin II in normal human prostate stromal cells. *Prostate*. **54**, 1-7.
Linke A, Erbs S, Hambrecht R (2008). Effects of exercise training upon endothelial function in patients with cardiovascular disease. *Front Biosci*. **13**, 424-432.
Lira FS, Carnevali LC, Jr., Zanchi NE, Santos RV, Lavoie JM, Seelaender M (2012). Exercise intensity modulation of hepatic lipid metabolism. *J Nutr Metab*. **2012**, 809576.
Lisanti MP, Scherer PE, Vidugiriene J, Tang Z, Hermanowski-Vosatka A, Tu YH, Cook RF, Sargiacomo M (1994). Characterization of caveolin-rich membrane domains isolated from an endothelial-rich source: implications for human disease. *J Cell Biol*. **126**, 111-126.
Liu B, Luo W, Zhang Y, Li H, Zhang J, Tan XR, Zhou Y (2012). Concomitant activation of functionally opposing prostacyclin and thromboxane prostanoid receptors by cyclo-oxygenase-1-mediated prostacyclin synthesis in mouse arteries. *Exp Physiol*. **97**, 895-904.
Liu Y, Bubolz AH, Mendoza S, Zhang DX, Gutterman DD (2011). H2O2 is the transferrable factor mediating flow-induced dilation in human coronary arterioles. *Circ Res*. **108**, 566-573.
Lockette W, Otsuka Y, Carretero O (1986). The loss of endothelium-dependent vascular relaxation in hypertension. *Hypertension*. **8**, II61-66.
Lohmann SM, Vaandrager AB, Smolenski A, Walter U, De Jonge HR (1997). Distinct and specific functions of cGMP-dependent protein kinases. *Trends Biochem Sci*. **22**, 307-312.
Lopez-Ilasaca M, Liu X, Tamura K, Dzau VJ (2003). The angiotensin II type I receptor-associated protein, ATRAP, is a transmembrane protein and a modulator of angiotensin II signaling. *Mol Biol Cell*. **14**, 5038-5050.
Loukogeorgakis SP, van den Berg MJ, Sofat R, Nitsch D, Charakida M, Haiyee B, de Groot E, MacAllister RJ, Kuijpers TW, Deanfield JE (2010). Role of NADPH oxidase in endothelial ischemia/reperfusion injury in humans. *Circulation*. **121**, 2310-2316.
Lowenstein CJ, Dinerman JL, Snyder SH (1994). Nitric oxide: a physiologic messenger. *Ann Intern Med*. **120**, 227-237.
Lu TT, Repa JJ, Mangelsdorf DJ (2001). Orphan nuclear receptors as eLiXiRs and FiXeRs of sterol metabolism. *J Biol Chem*. **276**, 37735-37738.
Luksha L, Agewall S, Kublickiene K (2009). Endothelium-derived hyperpolarizing factor in vascular physiology and cardiovascular disease. *Atherosclerosis*. **202**, 330-344.

Lumeng CN, Saltiel AR (2011). Inflammatory links between obesity and metabolic disease. *J Clin Invest*. **121**, 2111-2117.
Luscher TF, Boulanger CM, Dohi Y, Yang ZH (1992). Endothelium-derived contracting factors. *Hypertension*. **19**, 117-130.
Luscher TF, Vanhoutte PM (1986). Endothelium-dependent contractions to acetylcholine in the aorta of the spontaneously hypertensive rat. *Hypertension*. **8**, 344-348.
Luscher TF, Yang ZH, Diederich D, Buhler FR (1989). Endothelium-derived vasoactive substances: potential role in hypertension, atherosclerosis, and vascular occlusion. *J Cardiovasc Pharmacol*. **14 Suppl 6**, S63-69.
Lyle AN, Griendling KK (2006). Modulation of vascular smooth muscle signaling by reactive oxygen species. *Physiology (Bethesda)*. **21**, 269-280.
Ma Q, He X (2012). Molecular basis of electrophilic and oxidative defense: promises and perils of Nrf2. *Pharmacol Rev*. **64**, 1055-1081.
MacLeod J (1943). The role of oxygen in the metabolism and motility of human spermatozoa. *American Journal of Physiology*. **138**, 0512-0518.
Maeda S, Tanabe T, Miyauchi T, Otsuki T, Sugawara J, Iemitsu M, Kuno S, Ajisaka R, Yamaguchi I, Matsuda M (2003). Aerobic exercise training reduces plasma endothelin-1 concentration in older women. *J Appl Physiol (1985)*. **95**, 336-341.
Maines MD (1997). The heme oxygenase system: a regulator of second messenger gases. *Annu Rev Pharmacol Toxicol*. **37**, 517-554.
Mancini M, Toker A (2009). NFAT proteins: emerging roles in cancer progression. *Nat Rev Cancer*. **9**, 810-820.
Mandard S, Zandbergen F, Tan NS, Escher P, Patsouris D, Koenig W, Kleemann R, Bakker A, Veenman F, Wahli W, Muller M, Kersten S (2004). The direct peroxisome proliferator-activated receptor target fasting-induced adipose factor (FIAF/PGAR/ANGPTL4) is present in blood plasma as a truncated protein that is increased by fenofibrate treatment. *J Biol Chem*. **279**, 34411-34420.
Mandard S, Zandbergen F, van Straten E, Wahli W, Kuipers F, Muller M, Kersten S (2006). The fasting-induced adipose factor/angiopoietin-like protein 4 is physically associated with lipoproteins and governs plasma lipid levels and adiposity. *J Biol Chem*. **281**, 934-944.
Marklund SL, Westman NG, Lundgren E, Roos G (1982). Copper- and zinc-containing superoxide dismutase, manganese-containing superoxide dismutase, catalase, and glutathione peroxidase in normal and neoplastic human cell lines and normal human tissues. *Cancer Res*. **42**, 1955-1961.
Marnett LJ (2000). Cyclooxygenase mechanisms. *Curr Opin Chem Biol*. **4**, 545-552.
Marrero MB, Schieffer B, Paxton WG, Heerdt L, Berk BC, Delafontaine P, Bernstein KE (1995). Direct stimulation of Jak/STAT pathway by the angiotensin II AT1 receptor. *Nature*. **375**, 247-250.
Massague J (2008). TGFbeta in Cancer. *Cell*. **134**, 215-230.
Matoba T, Shimokawa H, Nakashima M, Hirakawa Y, Mukai Y, Hirano K, Kanaide H, Takeshita A (2000). Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in mice. *J Clin Invest*. **106**, 1521-1530.

Matsuno K, Yamada H, Iwata K, Jin D, Katsuyama M, Matsuki M, Takai S, Yamanishi K, Miyazaki M, Matsubara H, Yabe-Nishimura C (2005). Nox1 is involved in angiotensin II-mediated hypertension: a study in Nox1-deficient mice. *Circulation*. **112**, 2677-2685.

Matsuzawa A, Saegusa K, Noguchi T, Sadamitsu C, Nishitoh H, Nagai S, Koyasu S, Matsumoto K, Takeda K, Ichijo H (2005). ROS-dependent activation of the TRAF6-ASK1-p38 pathway is selectively required for TLR4-mediated innate immunity. *Nat Immunol*. **6**, 587-592.

Matthews JR, Botting CH, Panico M, Morris HR, Hay RT (1996). Inhibition of NF-kappaB DNA binding by nitric oxide. *Nucleic Acids Res*. **24**, 2236-2242.

Mattijssen F, Kersten S (2012). Regulation of triglyceride metabolism by Angiopoietin-like proteins. *Biochim Biophys Acta*. **1821**, 782-789.

Mayhan WG (1992). Role of prostaglandin H2-thromboxane A2 in responses of cerebral arterioles during chronic hypertension. *Am J Physiol*. **262**, H539-543.

McCoy MG, Sun GS, Marchadier D, Maugeais C, Glick JM, Rader DJ (2002). Characterization of the lipolytic activity of endothelial lipase. *J Lipid Res*. **43**, 921-929.

McGuire JJ, Ding H, Triggle CR (2001). Endothelium-derived relaxing factors: a focus on endothelium-derived hyperpolarizing factor(s). *Can J Physiol Pharmacol*. **79**, 443-470.

Mederle K, Schweda F, Kattler V, Doblinger E, Miyata K, Hocherl K, Oike Y, Castrop H (2013). The angiotensin II AT1 receptor-associated protein Arap1 is involved in sepsis-induced hypotension. *Crit Care*. **17**, R130.

Medzhitov R (2008). Origin and physiological roles of inflammation. *Nature*. **454**, 428-435.

Mehta PK, Griendling KK (2007). Angiotensin II cell signaling: physiological and pathological effects in the cardiovascular system. *Am J Physiol Cell Physiol*. **292**, C82-97.

Meijer RI, Serne EH, Smulders YM, van Hinsbergh VW, Yudkin JS, Eringa EC (2011). Perivascular adipose tissue and its role in type 2 diabetes and cardiovascular disease. *Curr Diab Rep*. **11**, 211-217.

Meng QX, Wen L, Chen XY, Zhong HJ (2013). Association of serum angiopoietin-like protein 2 and epinephrine levels in metabolically healthy but obese individuals: and evidence. *Exp Ther Med*. **5**, 1631-1636.

Mervaala EM, Cheng ZJ, Tikkanen I, Lapatto R, Nurminen K, Vapaatalo H, Muller DN, Fiebeler A, Ganten U, Ganten D, Luft FC (2001). Endothelial dysfunction and xanthine oxidoreductase activity in rats with human renin and angiotensinogen genes. *Hypertension*. **37**, 414-418.

Miida T, Hirayama S (2010). Impacts of angiopoietin-like proteins on lipoprotein metabolism and cardiovascular events. *Curr Opin Lipidol*. **21**, 70-75.

Millatt LJ, Abdel-Rahman EM, Siragy HM (1999). Angiotensin II and nitric oxide: a question of balance. *Regul Pept*. **81**, 1-10.

Miller AA, Drummond GR, Schmidt HH, Sobey CG (2005). NADPH oxidase activity and function are profoundly greater in cerebral versus systemic arteries. *Circ Res*. **97**, 1055-1062.

Miller M, Stone NJ, Ballantyne C, Bittner V, Criqui MH, Ginsberg HN, Goldberg AC, Howard WJ, Jacobson MS, Kris-Etherton PM, Lennie TA, Levi M, Mazzone T, Pennathur S (2011). Triglycerides and cardiovascular disease: a scientific statement from the American Heart Association. *Circulation*. **123**, 2292-2333.

Miller WL, Redfield MM, Burnett JC, Jr. (1989). Integrated cardiac, renal, and endocrine actions of endothelin. *J Clin Invest*. **83**, 317-320.

Minor RL, Jr., Myers PR, Guerra R, Jr., Bates JN, Harrison DG (1990). Diet-induced atherosclerosis increases the release of nitrogen oxides from rabbit aorta. *J Clin Invest*. **86**, 2109-2116.

Mirzaei K, Hossein-Nezhad A, Chamari M, Shahbazi S (2011). Evidence of a role of ANGPTL6 in resting metabolic rate and its potential application in treatment of obesity. *Minerva Endocrinol*. **36**, 13-21.

Mitko K, Ulbrich SE, Wenigerkind H, Sinowatz F, Blum H, Wolf E, Bauersachs S (2008). Dynamic changes in messenger RNA profiles of bovine endometrium during the oestrous cycle. *Reproduction*. **135**, 225-240.

Miura H, Gutterman DD (1998). Human coronary arteriolar dilation to arachidonic acid depends on cytochrome P-450 monooxygenase and Ca2+-activated K+ channels. *Circ Res*. **83**, 501-507.

Miura H, Liu Y, Gutterman DD (1999). Human coronary arteriolar dilation to bradykinin depends on membrane hyperpolarization: contribution of nitric oxide and Ca2+-activated K+ channels. *Circulation*. **99**, 3132-3138.

Mizutani N, Ozaki N, Seino Y, Fukami A, Sakamoto E, Fukuyama T, Sugimura Y, Nagasaki H, Arima H, Oiso Y (2012). Reduction of insulin signaling upregulates angiopoietin-like protein 4 through elevated free fatty acids in diabetic mice. *Exp Clin Endocrinol Diabetes*. **120**, 139-144.

Moncada S (1997). Nitric oxide in the vasculature: physiology and pathophysiology. *Ann N Y Acad Sci*. **811**, 60-67; discussion 67-69.

Moncada S, Erusalimsky JD (2002). Does nitric oxide modulate mitochondrial energy generation and apoptosis? *Nat Rev Mol Cell Biol*. **3**, 214-220.

Moncada S, Gryglewski R, Bunting S, Vane JR (1976). An enzyme isolated from arteries transforms prostaglandin endoperoxides to an unstable substance that inhibits platelet aggregation. *Nature*. **263**, 663-665.

Moncada S, Vane JR (1978). Pharmacology and endogenous roles of prostaglandin endoperoxides, thromboxane A2, and prostacyclin. *Pharmacol Rev*. **30**, 293-331.

Montezano AC, Burger D, Ceravolo GS, Yusuf H, Montero M, Touyz RM (2011). Novel Nox homologues in the vasculature: focusing on Nox4 and Nox5. *Clin Sci (Lond)*. **120**, 131-141.

Montezano AC, Touyz RM (2012). Oxidative stress, Noxs, and hypertension: experimental evidence and clinical controversies. *Ann Med*. **44 Suppl 1**, S2-16.

Morikawa K, Shimokawa H, Matoba T, Kubota H, Akaike T, Talukder MA, Hatanaka M, Fujiki T, Maeda H, Takahashi S, Takeshita A (2003). Pivotal role of Cu,Zn-superoxide dismutase in endothelium-dependent hyperpolarization. *J Clin Invest*. **112**, 1871-1879.

Morrell NW, Upton PD, Kotecha S, Huntley A, Yacoub MH, Polak JM, Wharton J (1999). Angiotensin II activates MAPK and stimulates growth of human

pulmonary artery smooth muscle via AT1 receptors. *Am J Physiol*. **277**, L440-448.

Morrow JD, Hill KE, Burk RF, Nammour TM, Badr KF, Roberts LJ, 2nd (1990). A series of prostaglandin F2-like compounds are produced in vivo in humans by a non-cyclooxygenase, free radical-catalyzed mechanism. *Proc Natl Acad Sci U S A*. **87**, 9383-9387.

Motohashi H, Yamamoto M (2004). Nrf2-Keap1 defines a physiologically important stress response mechanism. *Trends Mol Med*. **10**, 549-557.

Mueller CF, Laude K, McNally JS, Harrison DG (2005). ATVB in focus: redox mechanisms in blood vessels. *Arterioscler Thromb Vasc Biol*. **25**, 274-278.

Mugge A, Elwell JH, Peterson TE, Hofmeyer TG, Heistad DD, Harrison DG (1991). Chronic treatment with polyethylene-glycolated superoxide dismutase partially restores endothelium-dependent vascular relaxations in cholesterol-fed rabbits. *Circ Res*. **69**, 1293-1300.

Mukoyama M, Nakajima M, Horiuchi M, Sasamura H, Pratt RE, Dzau VJ (1993). Expression cloning of type 2 angiotensin II receptor reveals a unique class of seven-transmembrane receptors. *J Biol Chem*. **268**, 24539-24542.

Muramoto A, Tsushita K, Kato A, Ozaki N, Tabata M, Endo M, Oike Y, Oiso Y (2011). Angiopoietin-like protein 2 sensitively responds to weight reduction induced by lifestyle intervention on overweight Japanese men. *Nutr Diabetes*. **1**, e20.

Murphy C, Kanaganayagam GS, Jiang B, Chowienczyk PJ, Zbinden R, Saha M, Rahman S, Shah AM, Marber MS, Kearney MT (2007). Vascular dysfunction and reduced circulating endothelial progenitor cells in young healthy UK South Asian men. *Arterioscler Thromb Vasc Biol*. **27**, 936-942.

Murphy MP (2009). How mitochondria produce reactive oxygen species. *Biochem J*. **417**, 1-13.

Murphy TJ, Alexander RW, Griendling KK, Runge MS, Bernstein KE (1991). Isolation of a cDNA encoding the vascular type-1 angiotensin II receptor. *Nature*. **351**, 233-236.

Musunuru K, Pirruccello JP, Do R, Peloso GM, Guiducci C, Sougnez C, Garimella KV, Fisher S, Abreu J, Barry AJ, Fennell T, Banks E, Ambrogio L, Cibulskis K, Kernytsky A, Gonzalez E, Rudzicz N, Engert JC, DePristo MA, Daly MJ, Cohen JC, Hobbs HH, Altshuler D, Schonfeld G, Gabriel SB, Yue P, Kathiresan S (2010). Exome sequencing, ANGPTL3 mutations, and familial combined hypolipidemia. *N Engl J Med*. **363**, 2220-2227.

Nagareddy PR, Xia Z, McNeill JH, MacLeod KM (2005). Increased expression of iNOS is associated with endothelial dysfunction and impaired pressor responsiveness in streptozotocin-induced diabetes. *Am J Physiol Heart Circ Physiol*. **289**, H2144-2152.

Nakae J, Biggs WH, 3rd, Kitamura T, Cavenee WK, Wright CV, Arden KC, Accili D (2002). Regulation of insulin action and pancreatic beta-cell function by mutated alleles of the gene encoding forkhead transcription factor Foxo1. *Nat Genet*. **32**, 245-253.

Nakamura T, Okada T, Endo M, Kadomatsu T, Taniwaki T, Sei A, Odagiri H, Masuda T, Fujimoto T, Oike Y, Mizuta H (2014). Angiopoietin-like protein 2 induced

by mechanical stress accelerates degeneration and hypertrophy of the ligamentum flavum in lumbar spinal canal stenosis. *PLoS One*. **9**, e85542.

Namkung J, Koh SB, Kong ID, Choi JW, Yeh BI (2011). Serum levels of angiopoietin-related growth factor are increased in metabolic syndrome. *Metabolism*. **60**, 564-568.

Neels JG, Olefsky JM (2006). Inflamed fat: what starts the fire? *J Clin Invest*. **116**, 33-35.

Negishi M, Sugimoto Y, Ichikawa A (1995). Molecular mechanisms of diverse actions of prostanoid receptors. *Biochim Biophys Acta*. **1259**, 109-119.

Nelson MT, Patlak JB, Worley JF, Standen NB (1990). Calcium channels, potassium channels, and voltage dependence of arterial smooth muscle tone. *Am J Physiol*. **259**, C3-18.

Nettleton JA, Volcik KA, Hoogeveen RC, Boerwinkle E (2009). Carbohydrate intake modifies associations between ANGPTL4[E40K] genotype and HDL-cholesterol concentrations in White men from the Atherosclerosis Risk in Communities (ARIC) study. *Atherosclerosis*. **203**, 214-220.

Nguyen Dinh Cat A, Montezano AC, Burger D, Touyz RM (2013). Angiotensin II, NADPH oxidase, and redox signaling in the vasculature. *Antioxid Redox Signal*. **19**, 1110-1120.

Niki D, Katsu K, Yokouchi Y (2009). Ontogeny of angiopoietin-like protein 1, 2, 3, 4, 5, and 7 genes during chick embryonic development. *Dev Growth Differ*. **51**, 821-832.

Nishida M, Kitajima N, Saiki S, Nakaya M, Kurose H (2011). Regulation of Angiotensin II receptor signaling by cysteine modification of NF-kappaB. *Nitric Oxide*. **25**, 112-117.

Nishikawa T, Edelstein D, Du XL, Yamagishi S, Matsumura T, Kaneda Y, Yorek MA, Beebe D, Oates PJ, Hammes HP, Giardino I, Brownlee M (2000). Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. *Nature*. **404**, 787-790.

Nistala R, Wei Y, Sowers JR, Whaley-Connell A (2009). Renin-angiotensin-aldosterone system-mediated redox effects in chronic kidney disease. *Transl Res*. **153**, 102-113.

Nitenberg A, Valensi P, Sachs R, Dali M, Aptecar E, Attali JR (1993). Impairment of coronary vascular reserve and ACh-induced coronary vasodilation in diabetic patients with angiographically normal coronary arteries and normal left ventricular systolic function. *Diabetes*. **42**, 1017-1025.

Niwa K, Haensel C, Ross ME, Iadecola C (2001). Cyclooxygenase-1 participates in selected vasodilator responses of the cerebral circulation. *Circ Res*. **88**, 600-608.

Nunoi H, Rotrosen D, Gallin JI, Malech HL (1988). Two forms of autosomal chronic granulomatous disease lack distinct neutrophil cytosol factors. *Science*. **242**, 1298-1301.

O'Neill LA, Golenbock D, Bowie AG (2013). The history of Toll-like receptors - redefining innate immunity. *Nat Rev Immunol*. **13**, 453-460.

Ocana E, Bohorquez JC, Perez-Requena J, Brieva JA, Rodriguez C (2003). Characterisation of T and B lymphocytes infiltrating abdominal aortic aneurysms. *Atherosclerosis*. **170**, 39-48.
Odagiri H, Kadomatsu T, Endo M, Masuda T, Morioka MS, Fukuhara S, Miyamoto T, Kobayashi E, Miyata K, Aoi J, Horiguchi H, Nishimura N, Terada K, Yakushiji T, Manabe I, Mochizuki N, Mizuta H, Oike Y (2014). The Secreted Protein ANGPTL2 Promotes Metastasis of Osteosarcoma Cells Through Integrin alpha5beta1, p38 MAPK, and Matrix Metalloproteinases. *Sci Signal*. **7**, ra7.
Ogata A, Endo M, Aoi J, Takahashi O, Kadomatsu T, Miyata K, Tian Z, Jinnin M, Fukushima S, Ihn H, Oike Y (2012). The role of angiopoietin-like protein 2 in pathogenesis of dermatomyositis. *Biochem Biophys Res Commun*. **418**, 494-499.
Oike Y, Akao M, Yasunaga K, Yamauchi T, Morisada T, Ito Y, Urano T, Kimura Y, Kubota Y, Maekawa H, Miyamoto T, Miyata K, Matsumoto S, Sakai J, Nakagata N, Takeya M, Koseki H, Ogawa Y, Kadowaki T, Suda T (2005). Angiopoietin-related growth factor antagonizes obesity and insulin resistance. *Nat Med*. **11**, 400-408.
Oike Y, Ito Y, Maekawa H, Morisada T, Kubota Y, Akao M, Urano T, Yasunaga K, Suda T (2004a). Angiopoietin-related growth factor (AGF) promotes angiogenesis. *Blood*. **103**, 3760-3765.
Oike Y, Tabata M (2009). Angiopoietin-like proteins--potential therapeutic targets for metabolic syndrome and cardiovascular disease. *Circ J*. **73**, 2192-2197.
Oike Y, Yasunaga K, Ito Y, Matsumoto S, Maekawa H, Morisada T, Arai F, Nakagata N, Takeya M, Masuho Y, Suda T (2003). Angiopoietin-related growth factor (AGF) promotes epidermal proliferation, remodeling, and regeneration. *Proc Natl Acad Sci U S A*. **100**, 9494-9499.
Oike Y, Yasunaga K, Suda T (2004b). Angiopoietin-related/angiopoietin-like proteins regulate angiogenesis. *Int J Hematol*. **80**, 21-28.
Oka S, Kamata H, Kamata K, Yagisawa H, Hirata H (2000). N-acetylcysteine suppresses TNF-induced NF-kappaB activation through inhibition of IkappaB kinases. *FEBS Lett*. **472**, 196-202.
Okada T, Tsukano H, Endo M, Tabata M, Miyata K, Kadomatsu T, Miyashita K, Semba K, Nakamura E, Tsukano M, Mizuta H, Oike Y (2010). Synoviocyte-derived angiopoietin-like protein 2 contributes to synovial chronic inflammation in rheumatoid arthritis. *Am J Pathol*. **176**, 2309-2319.
Okazaki H, Hirakawa S, Shudou M, Nakaoka Y, Shirakata Y, Miyata K, Oike Y, Hashimoto K, Sayama K (2012). Targeted overexpression of Angptl6/angiopoietin-related growth factor in the skin promotes angiogenesis and lymphatic vessel enlargement in response to ultraviolet B. *J Dermatol*. **39**, 366-374.
Ono M, Shimizugawa T, Shimamura M, Yoshida K, Noji-Sakikawa C, Ando Y, Koishi R, Furukawa H (2003). Protein region important for regulation of lipid metabolism in angiopoietin-like 3 (ANGPTL3): ANGPTL3 is cleaved and activated in vivo. *J Biol Chem*. **278**, 41804-41809.

Ouchi N, Parker JL, Lugus JJ, Walsh K (2011). Adipokines in inflammation and metabolic disease. *Nat Rev Immunol*. **11**, 85-97.

Oudot A, Martin C, Busseuil D, Vergely C, Demaison L, Rochette L (2006). NADPH oxidases are in part responsible for increased cardiovascular superoxide production during aging. *Free Radic Biol Med*. **40**, 2214-2222.

Pacher P, Beckman JS, Liaudet L (2007). Nitric oxide and peroxynitrite in health and disease. *Physiol Rev*. **87**, 315-424.

Paclet MH, Henderson LM, Campion Y, Morel F, Dagher MC (2004). Localization of Nox2 N-terminus using polyclonal antipeptide antibodies. *Biochem J*. **382**, 981-986.

Palmer RM, Ashton DS, Moncada S (1988). Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature.* **333**, 664-666.

Palmer RM, Ferrige AG, Moncada S (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*. **327**, 524-526.

Paravicini TM, Chrissobolis S, Drummond GR, Sobey CG (2004). Increased NADPH-oxidase activity and Nox4 expression during chronic hypertension is associated with enhanced cerebral vasodilatation to NADPH in vivo. *Stroke*. **35**, 584-589.

Paravicini TM, Touyz RM (2008). NADPH oxidases, reactive oxygen species, and hypertension: clinical implications and therapeutic possibilities. *Diabetes Care*. **31 Suppl 2**, S170-180.

Pardanaud L, Yassine F, Dieterlen-Lievre F (1989). Relationship between vasculogenesis, angiogenesis and haemopoiesis during avian ontogeny. *Development*. **105**, 473-485.

Parkos CA, Dinauer MC, Walker LE, Allen RA, Jesaitis AJ, Orkin SH (1988). Primary structure and unique expression of the 22-kilodalton light chain of human neutrophil cytochrome b. *Proc Natl Acad Sci U S A*. **85**, 3319-3323.

Payne GA, Borbouse L, Kumar S, Neeb Z, Alloosh M, Sturek M, Tune JD (2010). Epicardial perivascular adipose-derived leptin exacerbates coronary endothelial dysfunction in metabolic syndrome via a protein kinase C-beta pathway. *Arterioscler Thromb Vasc Biol*. **30**, 1711-1717.

Peng HB, Libby P, Liao JK (1995). Induction and stabilization of I kappa B alpha by nitric oxide mediates inhibition of NF-kappa B. *J Biol Chem*. **270**, 14214-14219.

Pernow J, Shemyakin A, Bohm F (2012). New perspectives on endothelin-1 in atherosclerosis and diabetes mellitus. *Life Sci*. **91**, 507-516.

Perona R, Montaner S, Saniger L, Sanchez-Perez I, Bravo R, Lacal JC (1997). Activation of the nuclear factor-kappaB by Rho, CDC42, and Rac-1 proteins. *Genes Dev*. **11**, 463-475.

Phillips SA, Bian JT, Church EC, Das EK, Vidovich M, Gutterman DD (2009). Hydrogen peroxide prevents impaired endothelium-dependent dilation following acute exertion in chronic exercising but not in sedentary subjects. *Circulation*, S1013.

Pisciotta L, Favari E, Magnolo L, Simonelli S, Adorni MP, Sallo R, Fancello T, Zavaroni I, Ardigo D, Bernini F, Calabresi L, Franceschini G, Tarugi P, Calandra S, Bertolini S (2012). Characterization of three kindreds with familial

combined hypolipidemia caused by loss-of-function mutations of ANGPTL3. *Circ Cardiovasc Genet*. **5**, 42-50.

Potenza MA, Addabbo F, Montagnani M (2009). Vascular actions of insulin with implications for endothelial dysfunction. *Am J Physiol Endocrinol Metab*. **297**, E568-577.

Pott F, Jensen K, Hansen H, Christensen NJ, Lassen NA, Secher NH (1996). Middle cerebral artery blood velocity and plasma catecholamines during exercise. *Acta Physiol Scand*. **158**, 349-356.

Pou S, Pou WS, Bredt DS, Snyder SH, Rosen GM (1992). Generation of superoxide by purified brain nitric oxide synthase. *J Biol Chem*. **267**, 24173-24176.

Powell K (2007). Obesity: the two faces of fat. *Nature*. **447**, 525-527.

Pritchard KA, Jr., Groszek L, Smalley DM, Sessa WC, Wu M, Villalon P, Wolin MS, Stemerman MB (1995). Native low-density lipoprotein increases endothelial cell nitric oxide synthase generation of superoxide anion. *Circ Res*. **77**, 510-518.

Puddu P, Puddu GM, Zaca F, Muscari A (2000). Endothelial dysfunction in hypertension. *Acta Cardiol*. **55**, 221-232.

Quagliarini F, Wang Y, Kozlitina J, Grishin NV, Hyde R, Boerwinkle E, Valenzuela DM, Murphy AJ, Cohen JC, Hobbs HH (2012). Atypical angiopoietin-like protein that regulates ANGPTL3. *Proc Natl Acad Sci U S A*. **109**, 19751-19756.

Radomski MW, Moncada S (1993). The biological and pharmacological role of nitric oxide in platelet function. *Adv Exp Med Biol*. **344**, 251-264.

Rajsheker S, Manka D, Blomkalns AL, Chatterjee TK, Stoll LL, Weintraub NL (2010). Crosstalk between perivascular adipose tissue and blood vessels. *Curr Opin Pharmacol*. **10**, 191-196.

Ramkhelawon B, Vilar J, Rivas D, Mees B, de Crom R, Tedgui A, Lehoux S (2009). Shear stress regulates angiotensin type 1 receptor expression in endothelial cells. *Circ Res*. **105**, 869-875.

Rao GN, Runge MS, Alexander RW (1995). Hydrogen peroxide activation of cytosolic phospholipase A2 in vascular smooth muscle cells. *Biochim Biophys Acta*. **1265**, 67-72.

Rapoport RM, Williams SP (1996). Role of prostaglandins in acetylcholine-induced contraction of aorta from spontaneously hypertensive and Wistar-Kyoto rats. *Hypertension*. **28**, 64-75.

Rask-Madsen C, King GL (2007). Mechanisms of Disease: endothelial dysfunction in insulin resistance and diabetes. *Nat Clin Pract Endocrinol Metab*. **3**, 46-56.

Ray PD, Huang BW, Tsuji Y (2012). Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. *Cell Signal*. **24**, 981-990.

Recalcati S, Taramelli D, Conte D, Cairo G (1998). Nitric oxide-mediated induction of ferritin synthesis in J774 macrophages by inflammatory cytokines: role of selective iron regulatory protein-2 downregulation. *Blood*. **91**, 1059-1066.

Reimold AM, Kim J, Finberg R, Glimcher LH (2001). Decreased immediate inflammatory gene induction in activating transcription factor-2 mutant mice. *Int Immunol*. **13**, 241-248.

Ren G, Kim JY, Smas CM (2012). Identification of RIFL, a novel adipocyte-enriched insulin target gene with a role in lipid metabolism. *Am J Physiol Endocrinol Metab*. **303**, E334-351.

Richardson MR, Robbins EP, Vemula S, Critser PJ, Whittington C, Voytik-Harbin SL, Yoder MC (2014). Angiopoietin-like protein 2 regulates endothelial colony forming cell vasculogenesis. *Angiogenesis*.

Rodriguez-Manas L, El-Assar M, Vallejo S, Lopez-Doriga P, Solis J, Petidier R, Montes M, Nevado J, Castro M, Gomez-Guerrero C, Peiro C, Sanchez-Ferrer CF (2009). Endothelial dysfunction in aged humans is related with oxidative stress and vascular inflammation. *Aging Cell*. **8**, 226-238.

Romeo S, Pennacchio LA, Fu Y, Boerwinkle E, Tybjaerg-Hansen A, Hobbs HH, Cohen JC (2007). Population-based resequencing of ANGPTL4 uncovers variations that reduce triglycerides and increase HDL. *Nat Genet*. **39**, 513-516.

Romeo S, Yin W, Kozlitina J, Pennacchio LA, Boerwinkle E, Hobbs HH, Cohen JC (2009). Rare loss-of-function mutations in ANGPTL family members contribute to plasma triglyceride levels in humans. *J Clin Invest*. **119**, 70-79.

Rosenblum WI (1987). Hydroxyl radical mediates the endothelium-dependent relaxation produced by bradykinin in mouse cerebral arterioles. *Circ Res*. **61**, 601-603.

Rotrosen D, Leto TL (1990). Phosphorylation of neutrophil 47-kDa cytosolic oxidase factor. Translocation to membrane is associated with distinct phosphorylation events. *J Biol Chem*. **265**, 19910-19915.

Rousset S, Alves-Guerra MC, Mozo J, Miroux B, Cassard-Doulcier AM, Bouillaud F, Ricquier D (2004). The biology of mitochondrial uncoupling proteins. *Diabetes*. **53 Suppl 1**, S130-135.

Royer-Pokora B, Kunkel LM, Monaco AP, Goff SC, Newburger PE, Baehner RL, Cole FS, Curnutte JT, Orkin SH (1986). Cloning the gene for an inherited human disorder--chronic granulomatous disease--on the basis of its chromosomal location. *Nature*. **322**, 32-38.

Rubanyi GM, Romero JC, Vanhoutte PM (1986). Flow-induced release of endothelium-derived relaxing factor. *Am J Physiol*. **250**, H1145-1149.

Rubanyi GM, Vanhoutte PM (1987). Nature of endothelium-derived relaxing factor: are there two relaxing mediators? *Circ Res*. **61**, II61-67.

Ruge T, Sukonina V, Kroupa O, Makoveichuk E, Lundgren M, Svensson MK, Olivecrona G, Eriksson JW (2012). Effects of hyperinsulinemia on lipoprotein lipase, angiopoietin-like protein 4, and glycosylphosphatidylinositol-anchored high-density lipoprotein binding protein 1 in subjects with and without type 2 diabetes mellitus. *Metabolism*. **61**, 652-660.

Rummery NM, Hill CE (2004). Vascular gap junctions and implications for hypertension. *Clin Exp Pharmacol Physiol*. **31**, 659-667.

Rupnick MA, Panigrahy D, Zhang CY, Dallabrida SM, Lowell BB, Langer R, Folkman MJ (2002). Adipose tissue mass can be regulated through the vasculature. *Proc Natl Acad Sci U S A*. **99**, 10730-10735.

Saavedra JM (2011). Angiotensin II AT(1) receptor blockers ameliorate inflammatory stress: a beneficial effect for the treatment of brain disorders. *Cell Mol Neurobiol*. **32**, 667-681.

Saavedra JM (2012). Angiotensin II AT(1) receptor blockers as treatments for inflammatory brain disorders. *Clin Sci (Lond)*. **123**, 567-590.

Sairyo K, Biyani A, Goel VK, Leaman DW, Booth R, Jr., Thomas J, Ebraheim NA, Cowgill IA, Mohan SE (2007). Lumbar ligamentum flavum hypertrophy is due to accumulation of inflammation-related scar tissue. *Spine (Phila Pa 1976)*. **32**, E340-347.

Saltiel AR, Kahn CR (2001). Insulin signalling and the regulation of glucose and lipid metabolism. *Nature*. **414**, 799-806.

Samora JB, Frisbee JC, Boegehold MA (2008). Hydrogen peroxide emerges as a regulator of tone in skeletal muscle arterioles during junenvile growth. *Mircocirculation*. **15**, 151-161.

Sanan DA, Newland DL, Tao R, Marcovina S, Wang J, Mooser V, Hammer RE, Hobbs HH (1998). Low density lipoprotein receptor-negative mice expressing human apolipoprotein B-100 develop complex atherosclerotic lesions on a chow diet: no accentuation by apolipoprotein(a). *Proc Natl Acad Sci U S A*. **95**, 4544-4549.

Sanlioglu S, Williams CM, Samavati L, Butler NS, Wang G, McCray PB, Jr., Ritchie TC, Hunninghake GW, Zandi E, Engelhardt JF (2001). Lipopolysaccharide induces Rac1-dependent reactive oxygen species formation and coordinates tumor necrosis factor-alpha secretion through IKK regulation of NF-kappa B. *J Biol Chem*. **276**, 30188-30198.

Sasaki H, Suzuki A, Shitara M, Hikosaka Y, Okuda K, Moriyama S, Yano M, Fujii Y (2012). Angiopoietin-like protein ANGPTL2 gene expression is correlated with lymph node metastasis in lung cancer. *Oncol Lett*. **4**, 1325-1328.

Sasaki K, Yamano Y, Bardhan S, Iwai N, Murray JJ, Hasegawa M, Matsuda Y, Inagami T (1991). Cloning and expression of a complementary DNA encoding a bovine adrenal angiotensin II type-1 receptor. *Nature*. **351**, 230-233.

Sasamura H, Dzau VJ, Pratt RE (1994). Desensitization of angiotensin receptor function. *Kidney Int*. **46**, 1499-1501.

Sase K, Michel T (1997). Expression and regulation of endothelial nitric oxide synthase. *Trends Cardiovasc Med*. **7**, 28-37.

Sbarra AJ, Karnovsky ML (1959). Biochemical Basis of Phagocytosis .1. Metabolic Changes during the Ingestion of Particles by Polymorphonuclear Leukocytes. *Journal of Biological Chemistry*. **234**, 1355-1362.

Schachinger V, Britten MB, Zeiher AM (2000). Prognostic impact of coronary vasodilator dysfunction on adverse long-term outcome of coronary heart disease. *Circulation*. **101**, 1899-1906.

Schermerhorn M (2009). A 66-year-old man with an abdominal aortic aneurysm: review of screening and treatment. *JAMA*. **302**, 2015-2022.

Schiffrin EL (1999). State-of-the-Art lecture. Role of endothelin-1 in hypertension. *Hypertension*. **34**, 876-881.

Schmidt TS, Alp NJ (2007). Mechanisms for the role of tetrahydrobiopterin in endothelial function and vascular disease. *Clin Sci (Lond)*. **113**, 47-63.

Schrage WG, Eisenach JH, Joyner MJ (2007). Ageing reduces nitric-oxide- and prostaglandin-mediated vasodilatation in exercising humans. *J Physiol*. **579**, 227-236.

Schroder K, Zhang M, Benkhoff S, Mieth A, Pliquett R, Kosowski J, Kruse C, Luedike P, Michaelis UR, Weissmann N, Dimmeler S, Shah AM, Brandes RP (2012). Nox4 is a protective reactive oxygen species generating vascular NADPH oxidase. *Circ Res*. **110**, 1217-1225.

Segal AW, West I, Wientjes F, Nugent JH, Chavan AJ, Haley B, Garcia RC, Rosen H, Scrace G (1992). Cytochrome b-245 is a flavocytochrome containing FAD and the NADPH-binding site of the microbicidal oxidase of phagocytes. *Biochem J*. **284 (Pt 3)**, 781-788.

Seibert K, Masferrer JL (1994). Role of inducible cyclooxygenase (COX-2) in inflammation. *Receptor*. **4**, 17-23.

Seiler C, Hess OM, Buechi M, Suter TM, Krayenbuehl HP (1993). Influence of serum cholesterol and other coronary risk factors on vasomotion of angiographically normal coronary arteries. *Circulation*. **88**, 2139-2148.

Seo B, Oemar BS, Siebenmann R, von Segesser L, Luscher TF (1994). Both ETA and ETB receptors mediate contraction to endothelin-1 in human blood vessels. *Circulation*. **89**, 1203-1208.

Sessa WC (1994). The nitric oxide synthase family of proteins. *J Vasc Res*. **31**, 131-143.

Shen GX (2010). Oxidative stress and diabetic cardiovascular disorders: roles of mitochondria and NADPH oxidase. *Can J Physiol Pharmacol*. **88**, 241-248.

Shenker A, Goldsmith P, Unson CG, Spiegel AM (1991). The G protein coupled to the thromboxane A2 receptor in human platelets is a member of the novel Gq family. *J Biol Chem*. **266**, 9309-9313.

Shi Y, Feletou M, Ku DD, Man RY, Vanhoutte PM (2007). The calcium ionophore A23187 induces endothelium-dependent contractions in femoral arteries from rats with streptozotocin-induced diabetes. *Br J Pharmacol*. **150**, 624-632.

Shi Y, Vanhoutte PM (2009). Reactive oxygen-derived free radicals are key to the endothelial dysfunction of diabetes. *J Diabetes*. **1**, 151-162.

Shimamura M, Matsuda M, Ando Y, Koishi R, Yasumo H, Furukawa H, Shimomura I (2004). Leptin and insulin down-regulate angiopoietin-like protein 3, a plasma triglyceride-increasing factor. *Biochem Biophys Res Commun*. **322**, 1080-1085.

Shimamura M, Matsuda M, Kobayashi S, Ando Y, Ono M, Koishi R, Furukawa H, Makishima M, Shimomura I (2003). Angiopoietin-like protein 3, a hepatic secretory factor, activates lipolysis in adipocytes. *Biochem Biophys Res Commun*. **301**, 604-609.

Shimamura M, Matsuda M, Yasumo H, Okazaki M, Fujimoto K, Kono K, Shimizugawa T, Ando Y, Koishi R, Kohama T, Sakai N, Kotani K, Komuro R, Ishida T, Hirata K, Yamashita S, Furukawa H, Shimomura I (2007). Angiopoietin-like protein3 regulates plasma HDL cholesterol through suppression of endothelial lipase. *Arterioscler Thromb Vasc Biol*. **27**, 366-372.

Shimizugawa T, Ono M, Shimamura M, Yoshida K, Ando Y, Koishi R, Ueda K, Inaba T, Minekura H, Kohama T, Furukawa H (2002). ANGPTL3 decreases very low density lipoprotein triglyceride clearance by inhibition of lipoprotein lipase. *J Biol Chem*. **277**, 33742-33748.

Shimokawa H, Flavahan NA, Vanhoutte PM (1991). Loss of endothelial pertussis toxin-sensitive G protein function in atherosclerotic porcine coronary arteries. *Circulation*. **83**, 652-660.

Shimokawa H, Yasutake H, Fujii K, Owada MK, Nakaike R, Fukumoto Y, Takayanagi T, Nagao T, Egashira K, Fujishima M, Takeshita A (1996). The importance of the hyperpolarizing mechanism increases as the vessel size decreases in endothelium-dependent relaxations in rat mesenteric circulation. *J Cardiovasc Pharmacol*. **28**, 703-711.

Shinozaki K, Hirayama A, Nishio Y, Yoshida Y, Ohtani T, Okamura T, Massada M, Kikkawa R, Kodama K, Kashiwagi A (2001). Coronary endothelial dysfunction in the insulin-resistant state is linked to abnormal pteridine metabolism and vascular oxidative stress. *J Am Coll Cardiol*. **38,** 1821-1828.

Shirahase H, Usui H, Kurahashi K, Fujiwara M, Fukui K (1988). Endothelium-dependent contraction induced by nicotine in isolated canine basilar artery--possible involvement of a thromboxane A2 (TXA2) like substance. *Life Sci*. **42**, 437-445.

Shiroishi M, Tsumoto K, Amano K, Shirakihara Y, Colonna M, Braud VM, Allan DS, Makadzange A, Rowland-Jones S, Willcox B, Jones EY, van der Merwe PA, Kumagai I, Maenaka K (2003). Human inhibitory receptors Ig-like transcript 2 (ILT2) and ILT4 compete with CD8 for MHC class I binding and bind preferentially to HLA-G. *Proc Natl Acad Sci U S A*. **100**, 8856-8861.

Shoji T, Hatsuda S, Tsuchikura S, Kimoto E, Kakiya R, Tahara H, Koyama H, Emoto M, Tabata T, Nishizawa Y (2009). Plasma angiopoietin-like protein 3 (ANGPTL3) concentration is associated with uremic dyslipidemia. *Atherosclerosis*. **207**, 579-584.

Sibai B, Dekker G, Kupferminc M (2005). Pre-eclampsia. *Lancet*. **365**, 785-799.

Simon LS (1996). Actions and toxicity of nonsteroidal anti-inflammatory drugs. *Curr Opin Rheumatol*. **8**, 169-175.

Sindler AL, Delp MD, Reyes R, Wu G, Muller-Delp JM (2009). Effects of ageing and exercise training on eNOS uncoupling in skeletal muscle resistance arterioles. *J Physiol*. **587**, 3885-3897.

Skeggs LT, Jr., Kahn JR, Shumway NP (1956a). The preparation and function of the hypertensin-converting enzyme. *J Exp Med*. **103**, 295-299.

Skeggs LT, Jr., Kahn JR, Shumway NP (1956b). The purification of hypertensin II. *J Exp Med*. **103**, 301-307.

Skeggs LT, Jr., Marsh WH, Kahn JR, Shumway NP (1954). The purification of hypertensin I. *J Exp Med*. **100**, 363-370.

Sobenin IA, Tertov VV, Orekhov AN (1996). Atherogenic modified LDL in diabetes. *Diabetes*. **45 Suppl 3**, S35-39.

Smart-Halajko MC, Robciuc MR, Cooper JA, Jauhiainen M, Kumari M, Kivimaki M, Khaw KT, Boekholdt SM, Wareham NJ, Gaunt TR, Day IN, Braund PS, Nelson CP, Hall AS, Samani NJ, Humphries SE, Ehnholm C, Talmud PJ (2010). The relationship between plasma angiopoietin-like protein 4 levels, angiopoietin-like protein 4 genotype, and coronary heart disease risk. *Arterioscler Thromb Vasc Biol*. **30**, 2277-2282.

Sobey CG, Heistad DD, Faraci FM (1997). Mechanisms of bradykinin-induced cerebral vasodilatation in rats. Evidence that reactive oxygen species activate K+ channels. *Stroke*. **28**, 2290-2294; discussion 2295.

Soehnlein O, Schmeisser A, Cicha I, Reiss C, Ulbrich H, Lindbom L, Daniel WG, Garlichs CD (2005). ACE inhibition lowers angiotensin-II-induced monocyte adhesion to HUVEC by reduction of p65 translocation and AT 1 expression. *J Vasc Res*. **42**, 399-407.

Spencer M, Yao-Borengasser A, Unal R, Rasouli N, Gurley CM, Zhu B, Peterson CA, Kern PA (2010). Adipose tissue macrophages in insulin-resistant subjects are associated with collagen VI and fibrosis and demonstrate alternative activation. *Am J Physiol Endocrinol Metab*. **299**, E1016-1027.

Spiecker M, Peng HB, Liao JK (1997). Inhibition of endothelial vascular cell adhesion molecule-1 expression by nitric oxide involves the induction and nuclear translocation of IkappaBalpha. *J Biol Chem*. **272**, 30969-30974.

Spisni E, Manica F, Tomasi V (1992). Involvement of prostanoids in the regulation of angiogenesis by polypeptide growth factors. *Prostaglandins Leukot Essent Fatty Acids*. **47**, 111-115.

Staiger H, Haas C, Machann J, Werner R, Weisser M, Schick F, Machicao F, Stefan N, Fritsche A, Haring HU (2009). Muscle-derived angiopoietin-like protein 4 is induced by fatty acids via peroxisome proliferator-activated receptor (PPAR)-delta and is of metabolic relevance in humans. *Diabetes*. **58**, 579-589.

Steinberg HO, Chaker H, Leaming R, Johnson A, Brechtel G, Baron AD (1996). Obesity/insulin resistance is associated with endothelial dysfunction. Implications for the syndrome of insulin resistance. *J Clin Invest*. **97**, 2601-2610.

Stejskal D, Karpisek M, Reutova H, Humenanska V, Petzel M, Kusnierova P, Vareka I, Varekova R, Stejskal P (2008). Angiopoietin-like protein 4: development, analytical characterization, and clinical testing of a new ELISA. *Gen Physiol Biophys*. **27**, 59-63.

Stepan H, Ebert T, Schrey S, Reisenbuchler C, Stein S, Lossner U, Bluher M, Stumvoll M, Kratzsch J, Faber R, Fasshauer M (2009). Serum levels of angiopoietin-related growth factor are increased in preeclampsia. *Am J Hypertens*. **22**, 314-318.

Stocker R, Yamamoto Y, McDonagh AF, Glazer AN, Ames BN (1987). Bilirubin is an antioxidant of possible physiological importance. *Science*. **235**, 1043-1046.

Stork AP, Cocks TM (1994). Pharmacological reactivity of human epicardial coronary arteries: characterization of relaxation responses to endothelium-derived relaxing factor. *Br J Pharmacol*. **113**, 1099-1104.

Stroes E, Kastelein J, Cosentino F, Erkelens W, Wever R, Koomans H, Luscher T, Rabelink T (1997). Tetrahydrobiopterin restores endothelial function in hypercholesterolemia. *J Clin Invest*. **99**, 41-46.

Stump CS, Short KR, Bigelow ML, Schimke JM, Nair KS (2003). Effect of insulin on human skeletal muscle mitochondrial ATP production, protein synthesis, and mRNA transcripts. *Proc Natl Acad Sci U S A*. **100**, 7996-8001.

Su D, Coudriet GM, Hyun Kim D, Lu Y, Perdomo G, Qu S, Slusher S, Tse HM, Piganelli J, Giannoukakis N, Zhang J, Dong HH (2009). FoxO1 links insulin

resistance to proinflammatory cytokine IL-1beta production in macrophages. *Diabetes*. **58**, 2624-2633.
Suh YA, Arnold RS, Lassegue B, Shi J, Xu X, Sorescu D, Chung AB, Griendling KK, Lambeth JD (1999). Cell transformation by the superoxide-generating oxidase Mox1. *Nature*. **401**, 79-82.
Sukonina V, Lookene A, Olivecrona T, Olivecrona G (2006). Angiopoietin-like protein 4 converts lipoprotein lipase to inactive monomers and modulates lipase activity in adipose tissue. *Proc Natl Acad Sci U S A*. **103**, 17450-17455.
Sulciner DJ, Irani K, Yu ZX, Ferrans VJ, Goldschmidt-Clermont P, Finkel T (1996). rac1 regulates a cytokine-stimulated, redox-dependent pathway necessary for NF-kappaB activation. *Mol Cell Biol*. **16**, 7115-7121.
Sun H, Zheng JM, Chen S, Zeng CH, Liu ZH, Li LS (2007). Enhanced expression of ANGPTL2 in the microvascular lesions of diabetic glomerulopathy. *Nephron Exp Nephrol*. **105**, e117-123.
Sun K, Kusminski CM, Scherer PE (2011). Adipose tissue remodeling and obesity. *J Clin Invest*. **121**, 2094-2101.
Sun SY (2010). N-acetylcysteine, reactive oxygen species and beyond. *Cancer Biol Ther*. **9**, 109-110.
Suzuki H, DeLano FA, Parks DA, Jamshidi N, Granger DN, Ishii H, Suematsu M, Zweifach BW, Schmid-Schonbein GW (1998). Xanthine oxidase activity associated with arterial blood pressure in spontaneously hypertensive rats. *Proc Natl Acad Sci U S A*. **95**, 4754-4759.
Tabata M, Kadomatsu T, Fukuhara S, Miyata K, Ito Y, Endo M, Urano T, Zhu HJ, Tsukano H, Tazume H, Kaikita K, Miyashita K, Iwawaki T, Shimabukuro M, Sakaguchi K, Ito T, Nakagata N, Yamada T, Katagiri H, Kasuga M, Ando Y, Ogawa H, Mochizuki N, Itoh H, Suda T, Oike Y (2009). Angiopoietin-like protein 2 promotes chronic adipose tissue inflammation and obesity-related systemic insulin resistance. *Cell Metab*. **10**, 178-188.
Taddei S, Vanhoutte PM (1993). Endothelium-dependent contractions to endothelin in the rat aorta are mediated by thromboxane A2. *J Cardiovasc Pharmacol*. **22 Suppl 8**, S328-331.
Taddei S, Virdis A, Mattei P, Ghiadoni L, Fasolo CB, Sudano I, Salvetti A (1997). Hypertension causes premature aging of endothelial function in humans. *Hypertension*. **29**, 736-743.
Taguchi K, Motohashi H, Yamamoto M (2011). Molecular mechanisms of the Keap1-Nrf2 pathway in stress response and cancer evolution. *Genes Cells*. **16**, 123-140.
Takac I, Schroder K, Brandes RP (2011). The Nox family of NADPH oxidases: friend or foe of the vascular system? *Curr Hypertens Rep*. **14**, 70-78.
Takahashi JS, Hong HK, Ko CH, McDearmon EL (2008). The genetics of mammalian circadian order and disorder: implications for physiology and disease. *Nat Rev Genet*. **9**, 764-775.
Takaoka M, Nagata D, Kihara S, Shimomura I, Kimura Y, Tabata Y, Saito Y, Nagai R, Sata M (2009). Periadventitial adipose tissue plays a critical role in vascular remodeling. *Circ Res*. **105**, 906-911.

Talmud PJ, Smart M, Presswood E, Cooper JA, Nicaud V, Drenos F, Palmen J, Marmot MG, Boekholdt SM, Wareham NJ, Khaw KT, Kumari M, Humphries SE (2008). ANGPTL4 E40K and T266M: effects on plasma triglyceride and HDL levels, postprandial responses, and CHD risk. *Arterioscler Thromb Vasc Biol*. **28**, 2319-2325.

Tang EH, Vanhoutte PM (2008). Gene expression changes of prostanoid synthases in endothelial cells and prostanoid receptors in vascular smooth muscle cells caused by aging and hypertension. *Physiol Genomics*. **32**, 409-418.

Tazume H, Miyata K, Tian Z, Endo M, Horiguchi H, Takahashi O, Horio E, Tsukano H, Kadomatsu T, Nakashima Y, Kunitomo R, Kaneko Y, Moriyama S, Sakaguchi H, Okamoto K, Hara M, Yoshinaga T, Yoshimura K, Aoki H, Araki K, Hao H, Kawasuji M, Oike Y (2012). Macrophage-derived angiopoietin-like protein 2 accelerates development of abdominal aortic aneurysm. *Arterioscler Thromb Vasc Biol*. **32**, 1400-1409.

Teahan C, Rowe P, Parker P, Totty N, Segal AW (1987). The X-linked chronic granulomatous disease gene codes for the beta-chain of cytochrome b-245. *Nature*. **327**, 720-721.

Teicher BA (2012). Searching for molecular targets in sarcoma. *Biochem Pharmacol*. **84**, 1-10.

Thimmulappa RK, Mai KH, Srisuma S, Kensler TW, Yamamoto M, Biswal S (2002). Identification of Nrf2-regulated genes induced by the chemopreventive agent sulforaphane by oligonucleotide microarray. *Cancer Res*. **62**, 5196-5203.

Thomson L, Trujillo M, Telleri R, Radi R (1995). Kinetics of cytochrome c2+ oxidation by peroxynitrite: implications for superoxide measurements in nitric oxide-producing biological systems. *Arch Biochem Biophys*. **319**, 491-497.

Thorin E, Thorin-Trescases N (2009). Vascular endothelial ageing, heartbeat after heartbeat. *Cardiovasc Res*. **84**, 24-32.

Tian Z, Miyata K, Tazume H, Sakaguchi H, Kadomatsu T, Horio E, Takahashi O, Komohara Y, Araki K, Hirata Y, Tabata M, Takanashi S, Takeya M, Hao H, Shimabukuro M, Sata M, Kawasuji M, Oike Y (2013). Perivascular adipose tissue-secreted angiopoietin-like protein 2 (Angptl2) accelerates neointimal hyperplasia after endovascular injury. *J Mol Cell Cardiol*. **57**, 1-12.

Tosaka M, Hashiba Y, Saito N, Imai H, Shimizu T, Sasaki T (2002). Contractile responses to reactive oxygen species in the canine basilar artery in vitro: selective inhibitory effect of MCI-186, a new hydroxyl radical scavenger. *Acta Neurochir (Wien)*. **144**, 1305-1310; discussion 1310.

Touyz RM (2003). The role of angiotensin II in regulating vascular structural and functional changes in hypertension. *Curr Hypertens Rep*. **5**, 155-164.

Toyono T, Usui T, Yokoo S, Kimakura M, Nakagawa S, Yamagami S, Miyata K, Oike Y, Amano S (2013). Angiopoietin-like protein 2 is a potent hemangiogenic and lymphangiogenic factor in corneal inflammation. *Invest Ophthalmol Vis Sci*. **54**, 4278-4285.

Traupe T, Lang M, Goettsch W, Munter K, Morawietz H, Vetter W, Barton M (2002). Obesity increases prostanoid-mediated vasoconstriction and vascular thromboxane receptor gene expression. *J Hypertens*. **20**, 2239-2245.

Tripathy D, Mohanty P, Dhindsa S, Syed T, Ghanim H, Aljada A, Dandona P (2003). Elevation of free fatty acids induces inflammation and impairs vascular reactivity in healthy subjects. *Diabetes*. **52**, 2882-2887.

Tsai AL, Palmer G, Kulmacz RJ (1992). Prostaglandin H synthase. Kinetics of tyrosyl radical formation and of cyclooxygenase catalysis. *J Biol Chem*. **267**, 17753-17759.

Tsigkos S, Koutsilieris M, Papapetropoulos A (2003). Angiopoietins in angiogenesis and beyond. *Expert Opin Investig Drugs*. **12**, 933-941.

Tsukahara H, Ende H, Magazine HI, Bahou WF, Goligorsky MS (1994). Molecular and functional characterization of the non-isopeptide-selective ETB receptor in endothelial cells. Receptor coupling to nitric oxide synthase. *J Biol Chem*. **269**, 21778-21785.

Tsurumi Y, Tamura K, Tanaka Y, Koide Y, Sakai M, Yabana M, Noda Y, Hashimoto T, Kihara M, Hirawa N, Toya Y, Kiuchi Y, Iwai M, Horiuchi M, Umemura S (2006). Interacting molecule of AT1 receptor, ATRAP, is colocalized with AT1 receptor in the mouse renal tubules. *Kidney Int*. **69**, 488-494.

Turrens JF (2003). Mitochondrial formation of reactive oxygen species. *J Physiol*. **552**, 335-344.

Tuuri AL, Jauhiainen MS, Ehnholm CP, Tikkanen MJ, Nicholls MG, Kaaja RJ (2013). Elevated serum angiopoietin-like protein 6 in women with subsequent pregnancy-induced hypertension: a preliminary study. *Hypertens Pregnancy*. **32**, 203-213.

Ukai H, Ueda HR (2010). Systems biology of mammalian circadian clocks. *Annu Rev Physiol*. **72**, 579-603.

Ulrich S, Hingorani AD, Martin J, Vallance P (2000). What is the optimal age for starting lipid lowering treatment? A mathematical model. *BMJ*. **320**, 1134-1140.

Ungvari Z, Kaley G, de Cabo R, Sonntag WE, Csiszar A (2010). Mechanisms of vascular aging: new perspectives. *J Gerontol A Biol Sci Med Sci*. **65**, 1028-1041.

Upmacis RK, Deeb RS, Hajjar DP (2006). Oxidative alterations of cyclooxygenase during atherogenesis. *Prostaglandins Other Lipid Mediat*. **80**, 1-14.

Urakami-Harasawa L, Shimokawa H, Nakashima M, Egashira K, Takeshita A (1997). Importance of endothelium-derived hyperpolarizing factor in human arteries. *J Clin Invest*. **100**, 2793-2799.

Urano T, Ito Y, Akao M, Sawa T, Miyata K, Tabata M, Morisada T, Hato T, Yano M, Kadomatsu T, Yasunaga K, Shibata R, Murohara T, Akaike T, Tanihara H, Suda T, Oike Y (2008). Angiopoietin-related growth factor enhances blood flow via activation of the ERK1/2-eNOS-NO pathway in a mouse hind-limb ischemia model. *Arterioscler Thromb Vasc Biol*. **28**, 827-834.

Ushio-Fukai M, Zafari AM, Fukui T, Ishizaka N, Griendling KK (1996). p22phox is a critical component of the superoxide-generating NADH/NADPH oxidase system and regulates angiotensin II-induced hypertrophy in vascular smooth muscle cells. *J Biol Chem*. **271**, 23317-23321.

Usui T, Ninomiya T, Nagata M, Takahashi O, Doi Y, Hata J, Fukuhara M, Kitazono T, Oike Y, Kiyohara Y (2013). Angiopoietin-like protein 2 is associated with

chronic kidney disease in a general Japanese population: the Hisayama Study. *Circ J*. **77**, 2311-2317.
Utsugi M, Dobashi K, Ishizuka T, Kawata T, Hisada T, Shimizu Y, Ono A, Mori M (2006). Rac1 negatively regulates lipopolysaccharide-induced IL-23 p19 expression in human macrophages and dendritic cells and NF-kappaB p65 trans activation plays a novel role. *J Immunol*. **177**, 4550-4557.
Valentin F, Field MC, Tippins JR (2004). The mechanism of oxidative stress stabilization of the thromboxane receptor in COS-7 cells. *J Biol Chem*. **279**, 8316-8324.
Valenzuela DM, Griffiths JA, Rojas J, Aldrich TH, Jones PF, Zhou H, McClain J, Copeland NG, Gilbert DJ, Jenkins NA, Huang T, Papadopoulos N, Maisonpierre PC, Davis S, Yancopoulos GD (1999). Angiopoietins 3 and 4: diverging gene counterparts in mice and humans. *Proc Natl Acad Sci U S A*. **96**, 1904-1909.
van Dam H, Castellazzi M (2001). Distinct roles of Jun : Fos and Jun : ATF dimers in oncogenesis. *Oncogene*. **20**, 2453-2464.
van Dongen JJ, Krissansen GW, Wolvers-Tettero IL, Comans-Bitter WM, Adriaansen HJ, Hooijkaas H, van Wering ER, Terhorst C (1988). Cytoplasmic expression of the CD3 antigen as a diagnostic marker for immature T-cell malignancies. *Blood*. **71**, 603-612.
van Mourik JA, Leeksma OC, Reinders JH, de Groot PG, Zandbergen-Spaargaren J (1985). Vascular endothelial cells synthesize a plasma membrane protein indistinguishable from the platelet membrane glycoprotein IIa. *J Biol Chem*. **260**, 11300-11306.
van Raalte DH, Brands M, Serlie MJ, Mudde K, Stienstra R, Sauerwein HP, Kersten S, Diamant M (2012). Angiopoietin-like protein 4 is differentially regulated by glucocorticoids and insulin in vitro and in vivo in healthy humans. *Exp Clin Endocrinol Diabetes*. **120**, 598-603.
van Vlijmen BJ, van den Maagdenberg AM, Gijbels MJ, van der Boom H, HogenEsch H, Frants RR, Hofker MH, Havekes LM (1994). Diet-induced hyperlipoproteinemia and atherosclerosis in apolipoprotein E3-Leiden transgenic mice. *J Clin Invest*. **93**, 1403-1410.
Vane JR, Bakhle YS, Botting RM (1998). Cyclooxygenases 1 and 2. *Annu Rev Pharmacol Toxicol*. **38**, 97-120.
Vasquez-Vivar J, Kalyanaraman B, Martasek P, Hogg N, Masters BS, Karoui H, Tordo P, Pritchard KA, Jr. (1998). Superoxide generation by endothelial nitric oxide synthase: the influence of cofactors. *Proc Natl Acad Sci U S A*. **95**, 9220-9225.
Verhagen SN, Visseren FL (2011). Perivascular adipose tissue as a cause of atherosclerosis. *Atherosclerosis*. **214**, 3-10.
Violi F, Sanguigni V, Carnevale R, Plebani A, Rossi P, Finocchi A, Pignata C, De Mattia D, Martire B, Pietrogrande MC, Martino S, Gambineri E, Soresina AR, Pignatelli P, Martino F, Basili S, Loffredo L (2009). Hereditary deficiency of gp91(phox) is associated with enhanced arterial dilatation: results of a multicenter study. *Circulation*. **120**, 1616-1622.

Virdis A, Colucci R, Fornai M, Polini A, Daghini E, Duranti E, Ghisu N, Versari D, Dardano A, Blandizzi C, Taddei S, Del Tacca M, Monzani F (2009). Inducible nitric oxide synthase is involved in endothelial dysfunction of mesenteric small arteries from hypothyroid rats. *Endocrinology*. **150**, 1033-1042.
Virdis A, Neves MF, Amiri F, Touyz RM, Schiffrin EL (2004). Role of NAD(P)H oxidase on vascular alterations in angiotensin II-infused mice. *J Hypertens*. **22**, 535-542.
Volpp BD, Nauseef WM, Clark RA (1988). Two cytosolic neutrophil oxidase components absent in autosomal chronic granulomatous disease. *Science*. **242**, 1295-1297.
Walker CG, Zariwala MG, Holness MJ, Sugden MC (2007). Diet, obesity and diabetes: a current update. *Clin Sci (Lond)*. **112**, 93-111.
Wang H, Eckel RH (2009). Lipoprotein lipase: from gene to obesity. *Am J Physiol Endocrinol Metab*. **297**, E271-288.
Wang Y, Chen H, Li H, Zhang J, Gao Y (2013a). Effect of angiopoietin-like protein 4 on rat pulmonary microvascular endothelial cells exposed to LPS. *Int J Mol Med*. **32**, 568-576.
Wang Y, Marsden PA (1995). Nitric oxide synthases: gene structure and regulation. *Adv Pharmacol*. **34**, 71-90.
Wang Y, Quagliarini F, Gusarova V, Gromada J, Valenzuela DM, Cohen JC, Hobbs HH (2013b). Mice lacking ANGPTL8 (Betatrophin) manifest disrupted triglyceride metabolism without impaired glucose homeostasis. *Proc Natl Acad Sci U S A*. **110**, 16109-16114.
Wei EP, Kontos HA (1990). H2O2 and endothelium-dependent cerebral arteriolar dilation. Implications for the identity of endothelium-derived relaxing factor generated by acetylcholine. *Hypertension*. **16**, 162-169.
Wei EP, Kontos HA, Beckman JS (1996). Mechanisms of cerebral vasodilation by superoxide, hydrogen peroxide, and peroxynitrite. *Am J Physiol*. **271**, H1262-1266.
Weitzberg E, Ahlborg G, Lundberg JM (1993). Differences in vascular effects and removal of endothelin-1 in human lung, brain, and skeletal muscle. *Clin Physiol*. **13**, 653-662.
Wever RM, van Dam T, van Rijn HJ, de Groot F, Rabelink TJ (1997). Tetrahydrobiopterin regulates superoxide and nitric oxide generation by recombinant endothelial nitric oxide synthase. *Biochem Biophys Res Commun*. **237**, 340-344.
Wheatcroft SB, Kearney MT, Shah AM, Grieve DJ, Williams IL, Miell JP, Crossey PA (2003). Vascular endothelial function and blood pressure homeostasis in mice overexpressing IGF binding protein-1. *Diabetes*. **52**, 2075-2082.
Wheatcroft SB, Shah AM, Li JM, Duncan E, Noronha BT, Crossey PA, Kearney MT (2004). Preserved glucoregulation but attenuation of the vascular actions of insulin in mice heterozygous for knockout of the insulin receptor. *Diabetes*. **53**, 2645-2652.
White RP, Deane C, Vallance P, Markus HS (1998). Nitric oxide synthase inhibition in humans reduces cerebral blood flow but not the hyperemic response to hypercapnia. *Stroke*. **29**, 467-472.

Whitebread S, Mele M, Kamber B, de Gasparo M (1989). Preliminary biochemical characterization of two angiotensin II receptor subtypes. *Biochem Biophys Res Commun*. **163**, 284-291.

Wientjes FB, Hsuan JJ, Totty NF, Segal AW (1993). p40phox, a third cytosolic component of the activation complex of the NADPH oxidase to contain src homology 3 domains. *Biochem J*. **296 (Pt 3)**, 557-561.

Wilcox JN, Subramanian RR, Sundell CL, Tracey WR, Pollock JS, Harrison DG, Marsden PA (1997). Expression of multiple isoforms of nitric oxide synthase in normal and atherosclerotic vessels. *Arterioscler Thromb Vasc Biol*. **17**, 2479-2488.

Williams SP, Dorn GW, 2nd, Rapoport RM (1994). Prostaglandin I2 mediates contraction and relaxation of vascular smooth muscle. *Am J Physiol*. **267**, H796-803.

Wind S, Beuerlein K, Armitage ME, Taye A, Kumar AH, Janowitz D, Neff C, Shah AM, Wingler K, Schmidt HH (2010). Oxidative stress and endothelial dysfunction in aortas of aged spontaneously hypertensive rats by NOX1/2 is reversed by NADPH oxidase inhibition. *Hypertension*. **56**, 490-497.

Wingler K, Hermans JJ, Schiffers P, Moens A, Paul M, Schmidt HH (2011). NOX1, 2, 4, 5: counting out oxidative stress. *Br J Pharmacol*. **164**, 866-883.

Winquist RJ, Bunting PB, Baskin EP, Wallace AA (1984). Decreased endothelium-dependent relaxation in New Zealand genetic hypertensive rats. *J Hypertens*. **2**, 541-545.

Wise H, Jones RL (1996). Focus on prostacyclin and its novel mimetics. *Trends Pharmacol Sci*. **17**, 17-21.

Wisloff U, Najjar SM, Ellingsen O, Haram PM, Swoap S, Al-Share Q, Fernstrom M, Rezaei K, Lee SJ, Koch LG, Britton SL (2005). Cardiovascular risk factors emerge after artificial selection for low aerobic capacity. *Science*. **307**, 418-420.

Wolfle SE, de Wit C (2005). Intact endothelium-dependent dilation and conducted responses in resistance vessels of hypercholesterolemic mice in vivo. *J Vasc Res*. **42**, 475-482.

Woodman CR, Price EM, Laughlin MH (2003). Selected Contribution: Aging impairs nitric oxide and prostacyclin mediation of endothelium-dependent dilation in soleus feed arteries. *J Appl Physiol (1985)*. **95**, 2164-2170.

Wu KK, Liou JY (2005). Cellular and molecular biology of prostacyclin synthase. *Biochem Biophys Res Commun*. **338**, 45-52.

Xia Y, Roman LJ, Masters BS, Zweier JL (1998). Inducible nitric-oxide synthase generates superoxide from the reductase domain. *J Biol Chem*. **273**, 22635-22639.

Xiao HB, Sun ZL, Lu XY, Li DZ, Xu JP, Hu YP (2008). Beneficial effect of 3,4,5,6-tetrahydroxyxanthone on dyslipidemia in apolipoprotein E-deficient mice. *Can J Physiol Pharmacol*. **86**, 815-826.

Xu A, Lam MC, Chan KW, Wang Y, Zhang J, Hoo RL, Xu JY, Chen B, Chow WS, Tso AW, Lam KS (2005). Angiopoietin-like protein 4 decreases blood glucose and improves glucose tolerance but induces hyperlipidemia and hepatic steatosis in mice. *Proc Natl Acad Sci U S A*. **102**, 6086-6091.

Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, Sole J, Nichols A, Ross JS, Tartaglia LA, Chen H (2003). Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J Clin Invest*. **112**, 1821-1830.

Xu J, Zou MH (2009). Molecular insights and therapeutic targets for diabetic endothelial dysfunction. *Circulation*. **120**, 1266-1286.

Yamauchi T, Kamon J, Ito Y, Tsuchida A, Yokomizo T, Kita S, Sugiyama T, Miyagishi M, Hara K, Tsunoda M, Murakami K, Ohteki T, Uchida S, Takekawa S, Waki H, Tsuno NH, Shibata Y, Terauchi Y, Froguel P, Tobe K, Koyasu S, Taira K, Kitamura T, Shimizu T, Nagai R, Kadowaki T (2003). Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. *Nature*. **423**, 762-769.

Yan C, Kim D, Aizawa T, Berk BC (2003). Functional interplay between angiotensin II and nitric oxide: cyclic GMP as a key mediator. *Arterioscler Thromb Vasc Biol*. **23**, 26-36.

Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, Yazaki Y, Goto K, Masaki T (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature*. **332**, 411-415.

Yang D, Feletou M, Boulanger CM, Wu HF, Levens N, Zhang JN, Vanhoutte PM (2002). Oxygen-derived free radicals mediate endothelium-dependent contractions to acetylcholine in aortas from spontaneously hypertensive rats. *Br J Pharmacol*. **136**, 104-110.

Yang ST, Mayhan WG, Faraci FM, Heistad DD (1991). Mechanisms of impaired endothelium-dependent cerebral vasodilatation in response to bradykinin in hypertensive rats. *Stroke*. **22**, 1177-1182.

Yang W, Ando J, Korenaga R, Toyo-oka T, Kamiya A (1994). Exogenous nitric oxide inhibits proliferation of cultured vascular endothelial cells. *Biochem Biophys Res Commun*. **203**, 1160-1167.

Yang YM, Huang A, Kaley G, Sun D (2009). eNOS uncoupling and endothelial dysfunction in aged vessels. *Am J Physiol Heart Circ Physiol*. **297**, H1829-1836.

Yau MH, Wang Y, Lam KS, Zhang J, Wu D, Xu A (2009). A highly conserved motif within the NH2-terminal coiled-coil domain of angiopoietin-like protein 4 confers its inhibitory effects on lipoprotein lipase by disrupting the enzyme dimerization. *J Biol Chem*. **284**, 11942-11952.

Yi P, Park JS, Melton DA (2013). Betatrophin: a hormone that controls pancreatic beta cell proliferation. *Cell*. **153**, 747-758.

Yki-Jarvinen H (2005). Fat in the liver and insulin resistance. *Ann Med*. **37**, 347-356.

Yoon JC, Chickering TW, Rosen ED, Dussault B, Qin Y, Soukas A, Friedman JM, Holmes WE, Spiegelman BM (2000). Peroxisome proliferator-activated receptor gamma target gene encoding a novel angiopoietin-related protein associated with adipose differentiation. *Mol Cell Biol*. **20**, 5343-5349.

Yoshida K, Shimizugawa T, Ono M, Furukawa H (2002). Angiopoietin-like protein 4 is a potent hyperlipidemia-inducing factor in mice and inhibitor of lipoprotein lipase. *J Lipid Res*. **43**, 1770-1772.

Yu C, Hayami D, Gayda M, Larouche JF, Lapierre G, Henri C, Lambert J, Juneau M, Lalonge J, Thorin-Trescases N, Arsenault A, Thorin E, Nigam A (2014). Physical exercise reduces angiopoietin-like 2 circulating levels only in CAD patients with endothelial dysfunction. *Circulation.* **130**(Suppl.2), A17925.

Yu C, Luo X, Farhat N, Daneault C, Duquette N, Martel C, Lambert J, Thorin-Trescases N, Rosiers CD, Thorin E (2014). Lack of Angiopoietin-Like-2 Expression Limits the Metabolic Stress Induced by a High-Fat Diet and Maintains Endothelial Function in Mice. *J Am Heart Assoc*. **3**.

Zafarullah M, Li WQ, Sylvester J, Ahmad M (2003). Molecular mechanisms of N-acetylcysteine actions. *Cell Mol Life Sci*. **60**, 6-20.

Zamzami N, Marchetti P, Castedo M, Decaudin D, Macho A, Hirsch T, Susin SA, Petit PX, Mignotte B, Kroemer G (1995). Sequential reduction of mitochondrial transmembrane potential and generation of reactive oxygen species in early programmed cell death. *J Exp Med*. **182**, 367-377.

Zembowicz A, Hatchett RJ, Jakubowski AM, Gryglewski RJ (1993). Involvement of nitric oxide in the endothelium-dependent relaxation induced by hydrogen peroxide in the rabbit aorta. *Br J Pharmacol*. **110**, 151-158.

Zeng G, Quon MJ (1996). Insulin-stimulated production of nitric oxide is inhibited by wortmannin. Direct measurement in vascular endothelial cells. *J Clin Invest*. **98**, 894-898.

Zeng L, Dai J, Ying K, Zhao E, Jin W, Ye Y, Xu J, Xie Y, Mao Y (2003). Identification of a novel human angiopoietin-like gene expressed mainly in heart. *J Hum Genet*. **48**, 159-162.

Zhang CC, Kaba M, Ge G, Xie K, Tong W, Hug C, Lodish HF (2006a). Angiopoietin-like proteins stimulate ex vivo expansion of hematopoietic stem cells. *Nat Med*. **12**, 240-245.

Zhang R (2012). Lipasin, a novel nutritionally-regulated liver-enriched factor that regulates serum triglyceride levels. *Biochem Biophys Res Commun*. **424**, 786-792.

Zhang X, Hintze TH (2001). cAMP signal transduction cascade, a novel pathway for the regulation of endothelial nitric oxide production in coronary blood vessels. *Arterioscler Thromb Vasc Biol*. **21**, 797-803.

Zhang Y, Hu X, Tian R, Wei W, Hu W, Chen X, Han W, Chen H, Gong Y (2006b). Angiopoietin-related growth factor (AGF) supports adhesion, spreading, and migration of keratinocytes, fibroblasts, and endothelial cells through interaction with RGD-binding integrins. *Biochem Biophys Res Commun*. **347**, 100-108.

Zheng J, Umikawa M, Cui C, Li J, Chen X, Zhang C, Huynh H, Kang X, Silvany R, Wan X, Ye J, Canto AP, Chen SH, Wang HY, Ward ES, Zhang CC (2012). Inhibitory receptors bind ANGPTLs and support blood stem cells and leukaemia development. *Nature*. **485**, 656-660.

Zheng JY, Zou JJ, Wang WZ, Feng XY, Shi YY, Zhao Y, Jin G, Liu ZM (2011). Tumor necrosis factor-alpha increases angiopoietin-like protein 2 gene expression by activating Foxo1 in 3T3-L1 adipocytes. *Mol Cell Endocrinol*. **339**, 120-129.

Zhu P, Goh YY, Chin HF, Kersten S, Tan NS (2012). Angiopoietin-like 4: a decade of research. *Biosci Rep*. **32**, 211-219.

Zou MH, Shi C, Cohen RA (2002). High glucose via peroxynitrite causes tyrosine nitration and inactivation of prostacyclin synthase that is associated with thromboxane/prostaglandin H(2) receptor-mediated apoptosis and adhesion molecule expression in cultured human aortic endothelial cells. *Diabetes*. **51**, 198-203.

Zouhal H, Jacob C, Delamarche P, Gratas-Delamarche A (2008). Catecholamines and the effects of exercise, training and gender. *Sports Med*. **38**, 401-423.