Université de Montréal

Regulation of lipid metabolism in adipocytes and hepatocytes by hexarelin through scavenger receptor CD36

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Thèse présentée à la Faculté des études supérieures
en vue de l'obtention du grade de Philosophiae Doctor (Ph.D.)
en Biochimie

Avril 2011

Université de Montréal
Faculté des études supérieures

Cette thèse intitulée :

Regulation of lipid metabolism in adipocytes and hepatocytes by hexarelin through scavenger receptor CD36

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Résumé

Les sécrétines de l'hormone de croissance (GHRPs) sont de petits peptides synthétiques capables de stimuler la sécrétion de l'hormone de croissance à partir de l'hypophyse via leur liaison au récepteur de la ghréline GHS-R1a. Le GHRP hexaréline a été utilisé afin d'étudier la distribution tissulaire de GHS-R1a et son effet GH-indépendant. Ainsi, par cette approche, il a été déterminé que l'hexaréline était capable de se lier à un deuxième récepteur identifié comme étant le récepteur scavenger CD36. Ce récepteur possède une multitude de ligands dont les particules oxLDL et les acides gras à longue chaîne. CD36 est généralement reconnu pour son rôle dans l'athérogénèse et sa contribution à la formation de cellules spumeuses suite à l'internalisation des oxLDL dans les macrophages/monocytes. Auparavant, nous avions démontré que le traitement des macrophages avec l'hexaréline menait à l'activation de PPARɣ via sa liaison à GHS-R1a, mais aussi à CD36. De plus, une cascade d'activation impliquant LXRα et les transporteurs ABC provoquait également une augmentation de l'efflux du cholestérol. Une stimulation de la voie du transport inverse du cholestérol vers les particules HDL entraînait donc une diminution de l'engorgement des macrophages de lipides et la formation de cellules spumeuses. Puisque CD36 est exprimé dans de multiples tissus et qu'il est également responsable du captage des acides gras à longue chaîne, nous avons voulu étudier l'impact de l'hexaréline uniquement à travers sa liaison à CD36. Dans le but d'approfondir nos connaissances sur la régulation du métabolisme des lipides par CD36, nous avons choisi des types cellulaires jouant un rôle important dans l'homéostasie lipidique n'exprimant pas GHS-R1a, soient les adipocytes et les hépatocytes.

L'ensemble de mes travaux démontre qu'en réponse à son interaction avec l'hexaréline, CD36 a le potentiel de réduire le contenu lipidique des adipocytes et des hépatocytes. Dans les cellules adipeuses, l'hexaréline augmente l'expression de plusieurs gènes impliqués dans la mobilisation et l'oxydation des acides gras, et induit également l'expression des marqueurs thermogéniques PGC-1α et UCP-1. De même, hexaréline

augmente l'expression des gènes impliqués dans la biogenèse mitochondriale, un effet accompagné de changements morphologiques des mitochondries; des caractéristiques observées dans les types cellulaires ayant une grande capacité oxydative. Ces résultats démontrent que les adipocytes blancs traités avec hexaréline ont la capacité de se transformer en un phénotype similaire aux adipocytes bruns ayant l'habileté de brûler les acides gras plutôt que de les emmagasiner. Cet effet est également observé dans les tissus adipeux de souris et est dépendant de la présence de CD36. Dans les hépatocytes, nous avons démontré le potentiel de CD36 à moduler le métabolisme du cholestérol. En réponse au traitement des cellules avec hexaréline, une phosphorylation rapide de LKB1 et de l'AMPK est suivie d'une phosphorylation inhibitrice de l'HMG-CoA réductase (HMGR), l'enzyme clé dans la synthèse du cholestérol. De plus, la liaison d'hexaréline à CD36 provoque le recrutement d'insig-2 à HMGR, l'étape d'engagement dans sa dégradation. La dégradation de HMGR par hexaréline semble être dépendante de l'activité de PPARɣ et de l'AMPK. Dans le but d'élucider le mécanisme d'activation par hexaréline, nous avons démontré d'une part que sa liaison à CD36 provoque une déphosphorylation de Erk soulevant ainsi l'inhibition que celui-ci exerce sur PPARɣ et d'autre part, un recrutement de l'AMPK à PGC-1α expliquant ainsi une partie du mécanisme d'activation de PPARɣ par hexaréline.

Les résultats générés dans cette thèse ont permis d'élucider de nouveaux mécanismes d'action de CD36 et d'approfondir nos connaissances de son influence dans la régulation du métabolisme des lipides.

Mots-clés : Adipocytes, hépatocytes, CD36, hexaréline, PPARɣ, PGC1α, biogenèse mitochondriale, UCP-1, oxydation des acides gras, LKB1, AMPK, HMGR, insig-2, Erk, OSBPs.

Abstract

Growth hormone releasing peptides (GHRPs) are small synthetic peptides aimed at stimulating GH release from the pituitary through their binding to ghrelin receptor known as growth hormone secretagogue receptor 1a (GHS-R1a). Using the GHRP, hexarelin to study tissue distribution of GHS-R1a and its GH-independent effect, it was observed that hexarelin was capable of binding to a second receptor identified as scavenger receptor CD36. While having multiple ligands, CD36 is mainly known for binding and internalizing oxLDL and long chain fatty acids. CD36 is thought to play a detrimental role in macrophage derived foam cell formation and development of atherosclerosis. Previously, we have shown that in macrophages, expressing both GHS-R1a and CD36, hexarelin promoted an activation of PPARɣ via GHS-R1a but also through its binding to CD36. This activation led to the induction of the LXRα-ABC transporters pathway and an increase in cholesterol efflux, reducing lipid-laden macrophage content. This positive effect on macrophages was reproduced in apolipoprotein E-null mice on a high fat diet treated with hexarelin. A significant reduction in the size of atherosclerotic lesions was observed while similar increases in the expression of PPARɣ, LXRα and ABC transporters occurred in isolated peritoneal macrophages. CD36 also plays a role in fatty acid uptake, and to further investigate the impact of the interaction of hexarelin with CD36, we aimed at evaluating the role of CD36 in regulating lipid metabolism in cells devoid of GHS-R1a such as adipocytes and hepatocytes.

In the present thesis, we demonstrated through its interaction with hexarelin, the ability of CD36 to decrease intracellular lipid content in both adipocytes and hepatocytes. In adipocytes, hexarelin was able to increase the expression of several genes involved in fatty acid mobilization, fatty acid oxidation but also to induce the expression of the thermogenic markers, PGC-1α and UCP-1. In addition, hexarelin increased the expression of genes involved in mitochondrial biogenesis which was accompanied by mitochondrial morphological changes in agreement with what is usually seen in highly oxidative cells. In

support of these findings, we also observed an increase in the activity of cytochrome c oxidase (a component of the respiratory chain) which could reflect an increase in oxidative phosphorylation. The results generated with cultured white adipocytes suggest the ability of hexarelin to promote changes toward a brown fat-like phenotype which also occurred in vivo and was dependent on the presence of CD36. In hepatocytes, CD36 was capable of regulating cholesterol metabolism by rapidly phosphorylating LKB1 and AMPK which subsequently resulted in the inactivating phosphorylation of HMG-CoA reductase, the rate-limiting enzyme in cholesterol synthesis. Hexarelin via CD36 also induced the recruitment of insig-2 to HMGR, the committed step in HMGR degradation while lifting the exerted inhibitory effect of Erk on nuclear receptor PPARɣ activity, and promoting the recruitment of AMPK to PPARɣ coactivator PGC-1α, suggesting an enhanced transcriptional potential of PPARɣ.

The results generated during my graduate studies represent unique and novel mechanisms by which CD36 is capable of regulating lipid metabolism.

Keywords : Adipocytes, hepatocytes, CD36, hexarelin, PPARγ, PGC1α, mitochondrial biogenesis, UCP-1, fatty acid oxidation, LKB1, AMPK, HMGR, insig-2, Erk, OSBPs.

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Abbreviation list

9-,13-HODE	9- and 13-hydroxyoctadecadienoic acid
15-HETE	15-hydroxyeicosatetraenoic acid
$[Ca^{2+}]i$	intracellular calcium concentration
ABCA1	ATP-binding cassette transporter AI
AC	adenylate cyclase
ACC	acetyl-CoA carboxylase
acLDL	acetylated low density liprotein
AF-1, -2	activation function 1 and 2
AICAR	5-amino-4-imidazolecarboxamide ribonucleoside
AMPK	5' adenosine monophosphate-activated protein kinase
ANGPTL2	angiopoietin-like protein 2
aP2	adipocyte protein 2
ApoB100	apolipoprotein B-100
ApoE	apolipoprotein E
ATGL	adipose triglyceride lipase
ATP	adenosine triphosphate
BAT	brown adipose tissue
bHLH	basic helix-loop-helix
CaMKK	Ca2+/calmodulin-dependent protein kinase kinase
cAMP	adenosine 3′,5′-cyclic monophosphate
CAT	carnitine acylcarnitine transferase
CBP/p300	cAMP response element binding protein (CREB) binding protein
CCL2	Chemokine (C-C motif) ligand 2
CD36	Cluster of Differentiation 36
CDCA	chenodeoxycholic acid
cDNA	complementary deoxyribonucleic acid
CE	Cholesterol ester

C/EBP	CCAAT/enhancer-binding protein
CEOOH	cholesterol ester hydroperoxides
CHD	coronary heart disease
ChIP-Seq	chromatin immunoprecipitation sequencing
cLDL	carbamylated low density lipoprotein
CLESH	CD36 LIMP-II Emp sequence homology
CNS	central nervous system
CPT-I	carnitine palmitoyltransferase I
CsA	cyclosporine A
CSF	colony stimulating factor
CXCL16	Chemokine (C-X-C motif) ligand 16
DAG	diacylglycerol
DBD	DNA binding domain
DEXA	dual-energy x-ray absorptiometry
DGAT	diacylglycerol acyltransferase
EMSA	electrophoretic mobility shift assay
ER	endoplasmic reticulum
Erk	extracellular signal-regulated protein kinases 1 and 2
FA	fatty acids
FABPpm	plasma membrane fatty acid binding protein
FACS	fatty acyl-CoA synthetase
FAK	focal adhesion kinase
FAO	fatty acid oxidation
FAS	fatty acid synthase
FAT	fatty acid translocase
FATP	fatty acid transporter protein
FFA	free fatty acid
FXR	farnesoid X receptor

G-3-P	glycerol-3-phosphate
GH	growth hormone
GHRH	growth hormone releasing hormone
GHRP	growth hormone releasing peptide
GHS-R1a	GHS receptor 1a
GLUT-4	glucose transporter-4
GOAT	ghrelin O-Acyltransferase
GPAT	glycerol-3-phosphate acyltransferase
GPCR	G protein-coupled receptor
GPI-anchored	glycophosphatidylinositol
GPIV	glycoprotein IV
GS	glycogen synthase
HDL	high density lipoprotein
HFD	high fat diet
HMGR	HMG-CoA reductase
HSL	hormone-sensitive lipase
HNE	4-hydroxynonenal
IBMX	3-isobutyl-1-methylxanthine
IFN	interferon
IGF-1	insulin-like growth factor 1
IL	Interleukin
Insig-1,-2	insulin-inducible genes-1 and -2 proteins
IP3	inositol (1,4,5)-trisphosphate
JNK	c-Jun N-terminal kinase
LBD	ligand binding domain
LCFA	long chain fatty acid
LDLR	low density lipoprotein receptor
LKB1	liver kinase B1

LOX-1	lectin-like oxLDL receptor-1
LPL	lipoprotein lipase
LPS	lipopolysaccharide
LTA	Lipoteichoic acid
MAPK	mitogen-activated protein kinase
M-CPT-1	muscle carnitine palmitoyltransferase I
MEFs	mouse embryonic fibroblasts
MEKK2	mitogen-activated protein (MAP) kinase kinase kinase 2
M-FABP	muscle fatty acid binding protein
MGL	monoacylglycerol lipase
mmLDL	minimally modified low density lipoprotein
mRNA	messenger ribonucleic acid
MRP	mitochondrial ribosomal protein
mTOR	mammalian target of rapamycin
MW	molecular weight
NAFLD	non-alcoholic fatty liver disease
NADH	nicotinamide adenine dinucleotide
NAMPT	nicotinamide phosphoribosyltransferase
NASH	non-alcoholic steatohepatitis
NCoR	Nuclear receptor corepressor protein
NPY	neuropeptide Y
Nrf2	Nuclear factor E2-related factor 2
oxLDL	oxidized low density lipoprotein
$oxPC_{CD36}$	oxidized phosphatidylcholine
PEPCK	cytosolic phosphoenolpyruvate carboxykinase
PfEMP1	*Plasmodium falciparum* erythrocyte membrane protein 1
PFK2	6-phosphofructokinase-2
PGC-1α	PPARγ coactivator-1 alpha

PGC-1β	PPARγ coactivator-1 beta
PHA	Phytohemagglutinin
PI3K	phosphoinositide 3-kinase
PKA, PKC	protein kinase A and C
PL	phospholipid
PLAP	placental alkaline phosphatase
PMA	Phorbol 12-myristate 13-acetate
PPAR	peroxisome proliferator-activated receptor
PPRE	peroxisome proliferator-activated receptor response element
PRDM16	PRD1-BF1-RIZ1 homologous domain containing 16
PUFA	polyunsaturated fatty acid
RIP140	receptor interacting protein 140
RUNX3	Runt-related 3
RXR	retinoid X receptor
SCAP	SREBP cleavage-activating protein
SCD-1	stearoyl-CoA desaturase-1
SFRP5	secreted frizzled-related protein 5
siRNA	small interfering RNA
shRNA	small hairpin RNA
SMC	smooth muscle cells
SMRT	silencing mediator of retinoid and thyroid hormone receptors
SR	scavenger receptor
SR-A	scavenger receptor type A
SR-BI	scavenger receptor type B
SRC	steroid receptor co-activator
SREBP	sterol-regulatory element binding protein
SREC	scavenger receptor expressed by endothelial cells proteins
SRIF	somatotropin release-inhibiting factor

SSD	sterol-sensing domain
TAK1	TGFβ-activated kinase-1
TCA cycle	tricarboxylic acid cycle
TG	triglycerides
TGF	tumor growth factor
TIM	translocase of the inner membrane
TIP47	tail-interacting protein 47
TLR	Toll-like receptors
TM	transmembrane
TNF-α	tumor necrosis factor alpha
TOM	translocase of the outer membrane
TR4	testicular orphan nuclear receptor 4
TR4RE	testicular orphan nuclear receptor 4 response element
TRAIL	TNF-related apoptosis-inducing ligand
TRAP220	thyroid receptor associated protein 220
TSC-2	tumor suppressor tuberous sclerosis 2
TSP-1	thrombospondin 1
TZD	thiazolidinedione
UCP	uncoupling protein
UTR	untranslated region
VLDL	very low density lipoprotein
VSMC	vascular smooth muscle cells
WAT	white adipose tissue

To Sarah and Éric

Acknowledgements

The desire to return to graduate school stemmed from a few years of working in biotech and pharmaceutical companies. And while I loved that experience, the wish to return to basic research prompted me to reconsider my future. At a given point, I grabbed that opportunity and decided to finally stop asking that nagging question: Should I? So I did and I certainly don`t regret it. It seemed like such a long journey, long enough that my life no longer resembles what it was when I first started. People have entered my life while others have left forever but for most, they have remained as supportive and essential to me, especially throughout this period. I`d like to think that I`ve experienced all those wonderful interactions as best as I possibly could.

A special thanks to my research advisor, André Tremblay who has given me the opportunity to fulfill my dream. You have provided an environment and a training that permitted us to improve our skills, share our knowledge and excel in what we do. I am especially grateful to you for providing enough guidance without impeding my independence and for always being there when needed. I have learned so much from your ability to always put an interesting spin to papers and projects. Although it might seem trivial, thank you for building an incredible team of people for otherwise this experience would not have been the same.

Aux filles du labo pour lesquelles j'ai une affection sans pareil! To Mélanie Sanchez, my companion throughout my chocolate rampage and other delicious goodies: Thanks for all the laughs, for sharing the joy of simply being silly, not to mention your endless help and guidance. You are one of the few people I know who can be absolutely hilarious without overshadowing their professionalism. This journey would have not been the same without the girls. A special thanks to Nathalie Picard, Karine Sauvé, Mélanie Sanchez and Annie Demers with whom I have worked along the longest. Thanks to Véronique Caron, Stéphanie Bilodeau, Méryl Hassan, Julie Martin and Julie Lepage for all our discussions (personal and professional) and for all the help that you have provided. Because of the embedded willingness to help, this amazing quality that you all possess

almost goes unnoticed. Your team-spirit should be an example to all. I would be ever so lucky and honored to work by your side again one day. Kudos!

A special thanks to Hugues Bernard and Steve Tirrell for their much appreciated time and help.

On a more personal note, I am grateful to my wonderful love, Eric, for being my rock, my pillar of strength. Thanks for your patience, and for encouraging and supporting me throughout my graduate studies. And in particular, thanks for picking up the slack during the last few months. Your love and support are so important to me. To my greatest accomplishment of all: Sarah. My little angel with the propensity to lighten up the world around her; whom I love more than life itself. You have transformed me more than I could have ever imagined and all for the best. Thanks for reminding me to take a deep breath and enjoy the simple things especially when it seems like there`s no time left. You are too young to realize why I had so little time in the past few months but I will do my best to make it up to you. To Pop, your great knowledge, and among other things your continuous fascination and enthusiasm for science have always encouraged me to follow my own passions and dreams. To Maggie and Liam who have become incredible individuals. Thanks to my family for their continuous encouragements and support.

In memory of Cathy Way (2004), Claude René (2008), Cheryl Irving (2009) and Patrick Errada (2010) for their courage that never faltered in the face of cancer. We wish you were still here with us. To my friend Patrick who has always shown such passion and love for life, I miss you.

To my dearest friends: Isabelle Michaud, Sylvie Bernier, Johanne Laporte, Mary Donoghue, Sue Acton, Barb Sullivan and Colleen Mullen for their support, guidance and input, and above all for lending an ear.

Thank you! Merci!

CHAPTER 1: Introduction

1 Growth hormone secretagogues

Growth hormone secretagogues are a family of synthetic peptides (also called growth hormone releasing peptides) and peptidomimetic agonists designed to increase the secretion of growth hormone in GH-deficient patients.

1.1 Design of growth hormone releasing peptides

Growth hormone releasing peptides (GHRPs) research stemmed from studies on synthetic analogs of opioid peptides during the 1970s. Opioids such as enkephalins, endorphins, dynorphins, and endomorphins are produced by the body in response to pain while naturally occurring opiates such as morphine and codeine are extracted from opium poppy. Enkephalins are pentapeptides that regulate nociception or pain perception via peripheral nerves in order to control or lessen the pain signal sent to the central nervous system (CNS). There are 2 forms of enkephalin which are products of the same proenkephalin gene: leu-enkephalin (Tyr-Gly-Gly-Phe-**Leu**) and met-enkephalin (Tyr-Gly-Gly-Phe-**Met**) (Udenfriend & Kilpatrick, 1983). In 1975, enkephalins were identified as the endogenous ligands of the morphine receptor, opioid receptor μ widely distributed throughout the central and peripheral nervous system (Hughes *et al.*, 1975). With enkephalins as the prototype, opioid analogs were synthesized in order to develop more potent and less addicting analgesic compounds. Soon after, Cyril Y Bowers, Frank Momany and colleagues noticed that certain opioids stimulated growth hormone (GH) secretion (Bowers *et al.*, 1977).

Also called somatotropin, GH is 191-aa hormone secreted by the pituitary gland (hypophysis), more precisely by the anterior part of the gland (Figure 1A). The pituitary gland is controlled by the hypothalamus, connected by the pituitary stalk (or infundibular stem). The hypothalamus directly controls the endocrine system by secreting factors that stimulate or inhibit the secretion of hormones released from the pituitary. The latter

contains 2 sections: the anterior pituitary and the posterior pituitary. The anterior pituitary is responsible for the secretion of various hormones including GH. The secretion of GH is regulated by the hypothalamic-pituitary axis (Figure 1B). Somatocrinin or growth hormone-releasing hormone (GHRH) is produced by the hypothalamus (Figure 1A) and secreted towards the pituitary (Guillemin *et al.*, 1982). GHRH binds to somatotropes and induces secretion of GH into the blood circulation which will then bind to its receptor present in peripheral tissues. In response to GH, the liver secretes insulin-like growth factor 1 (IGF-1) causing a negative feedback on the production of GH. The resulting increase in somatotropin release-inhibiting factor (SRIF) production competes with GHRH for the same receptor on somatotropic cells and reduces secretion of GH. The interplay between GHRH and SRIF results in the observed pulsatile release of GH (Brazeau *et al.*, 1973).

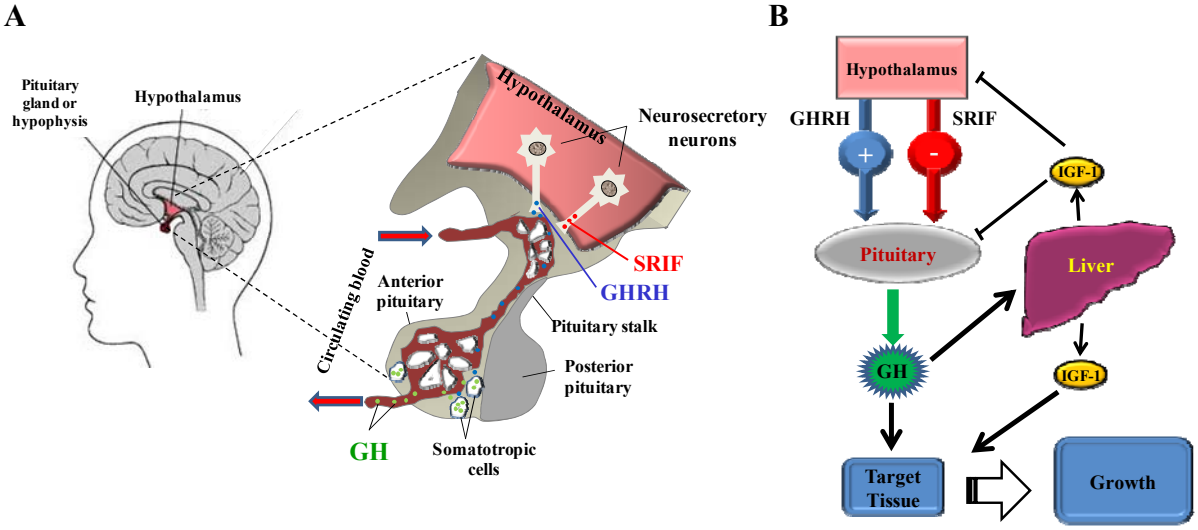


Figure 1. The hypothalamic-pituitary axis. (A) Functional anatomy of the hypothalamus and pituitary gland, (B) Regulation of the secretion of growth hormone by the hypothalamus

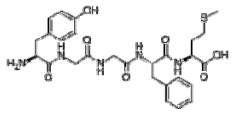
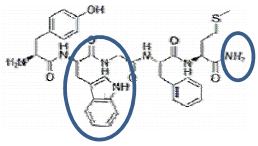
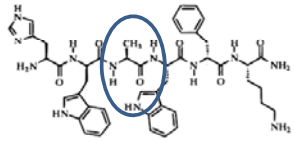
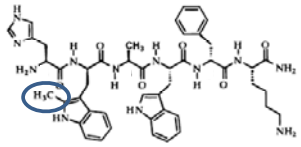
Regardless of the cause, in newborns and young children, a GH deficiency results in hypoglycemia and growth retardation. Although rare, a GH deficiency in adults (due to traumatic brain injury, cancer, radiotherapy) can result in prolonged fatigue, a loss in bone

density and a loss of muscle mass. Prior to the advent of recombinant DNA technology in 1985, the treatment of choice for GH deficiency was the rather expensive injections of purified human GH. Although due to the seriousness of contracting the degenerative neurological disorder, Creutzfeldt-Jakob disease (CJD) from contaminated human GH samples, the use of hGH was soon banned from Europe and North America (Huillard *et al.*, 1999). This hormone represents a 191-aa protein with a molecular mass of 215 kDa and a low oral absorption (Nargund *et al.*, 1998). In addition, daily injections masked the physiological regulatory feedback and therefore resulted in the absence of pulsatile release of GH. In certain cases, a deficiency in GH was not primarily due to an insufficient synthesis/secretion of GH but rather a poor signaling from the hypothalamus. Taking those two facts into account, an alternative approach was considered in which the biologically active portion of the 44-aa GHRH peptide would be used to stimulate GH secretion from the pituitary. This 29-aa synthetic bioactive truncated GHRH had the advantage of maintaining the pulsatile release of GH, and therefore several analogues of GHRH were tested in humans as an alternative to GH replacement therapy (Grossman *et al.*, 1984). Following the arrival of recombinant GH on the market, studies performed on GHRH and its analogues could not demonstrate the advantage of using GHRH in terms of efficacy and bioavailability in comparison to recombinant GH (Campbell *et al.*, 1995).

Following the previously mentioned observation in 1977 of Bowers, Momany and colleagues that enkephalins stimulated GH secretion, this team redirected their research from opioids and undertook the tedious task of synthesizing more potent analogues as a therapeutic approach to treating GH deficiency and doing so by using met-enkephalin as their prototype (Table 1). The peptide sequence was modified to produce analogues capable of eliciting a stronger GH secretion on isolated rat pituitary glands without affecting opioid receptors (Bowers *et al.*, 1980;Momany *et al.*, 1981). The analogue of met5-enkephalin, Tyr-DTrp-Gly-Phe-Met-NH_2, illustrated in Table 1, was the first to elicit a stronger *in vitro* GH secretion; however, when tested in rat, no *in vivo* activity was found. The D conformation of the substitute aromatic amino acid, tryptophan brings a stabilizing element

to this peptide making it more resistant to proteases; while the amine group (NH2) in C-terminal renders the peptide biologically active (Kreil, 1997). Indeed, it is estimated that half of peptide hormones have an amine group at their C-terminus and is required for optimal biological activity (Kim & Seong, 2001). Theoretical conformational studies by Momany helped in the design of more energetically favorable and potent GHRPs (Momany *et al.*, 1981;Momany & Bowers, 1996). While some were a thousand times more active than their predecessor, they still did not possess any *in vivo* activity. The main feature of all GHRPs synthesized that needed to be preserved in the following screenings was the presence, position and stereochemistry of the Trp residu at position 2.Using a trial and error approach, the first *in vivo* bioactive GHRP called GHRP-6 (His-D-Trp-Ala-Trp-D-Phe-Lys-NH2) was finally synthesized (Momany *et al.*, 1984). The three dimensional structure of GHRP-6 indicated that the N-terminal end of histidine and the C-terminal end of lysine were in close proximity, adopting a folded conformation. The hydrophobic indole ring of each tryptophan (D-Trp^2 and Trp^4) was directed toward one another while the phenyl unit of D-Phe^5 interacted with the amino unit of Lys^6. These particular modifications seemed to give GHRP-6 its bioactive feature. Unfortunately, GHRP-6 and others that followed had a very poor oral absorption and a short half-life. For example, oral GHRP-6 had a bioactivity of 0.3% to that of injected GHRP-6 with a half-life of 20 minutes (Bowers *et al.*, 1992). This lack of bioavailability provided the opportunity for other research teams to search for nonpeptidyl compounds imitating GHRPs but with the added feature of being orally active.

Table 1. Evolution of growth hormone releasing peptides and their unique related properties

Name: Peptide sequence	Structure	Modification(s) Applied	Function or Reported effect(s)
Met-enkephalin: Tyr-Gly-Gly-Phe-Met		N/A	Opioid μ receptor ligand
Analogue of met5-enkephalin: Tyr-D-Trp-Gly-Phe-Met-NH2		NH2 in C-terminal confers to this peptide an increase in bioavailability D-Trp, conformation renders peptide resistant to proteases	GH secretion from isolated pituitary *in vitro* but not *in vivo*
GHRP-6: His-D-Trp-Ala-Trp-D-Phe-Lys-NH2		Several modifications were made to generate hexapeptide with a second aromatic aa with a D conformation	First peptide capable of stimulating *in vitro* AND *in vivo* GH secretion
Hexarelin: His-D2MeTrp-Ala-Trp-D-Phe-Lys-NH2		Methyl added in position 2 in aromatic group of D-Trp	Methyl group provides an increased stability to this peptide. First peptide that can be taken orally.

1.2 Peptidomimetic growth hormone secretagogues

Roy Smith and colleagues from Merck assumed the task of designing such compounds. To do so, they took into consideration all of the particular features that made GHRP-6 a bioactive peptide such as the amine group, the aromatic amino acids in position 2, 4 and 5 as well as the unnatural D-Trp and combined them with benzodiazepine, known to imitate small peptides. They synthesized an array of compounds capable of secreting GH called benzolactames (Smith *et al.*, 1993). The bioavailability of benzolactames, although superior to GHRPs, turned out to be relatively low (Leung *et al.*, 1996). Focusing on the concept of "privileged structures" by Ben E. Evans and colleagues that stated that certain molecular units had the capacity of interacting with various receptors, the same team

screened different compounds within the company's various internal projects to finally focus on agonists containing a spiropiperidine group (Evans *et al.*, 1988;Jacks *et al.*, 1996). More precisely, spiroindoline sulphonamide (also called MK-0677) was found to elicit GH release in rat pituitary cell culture assay with greater potency. MK-0677 was identified as a specific GHS with an elevated bioactivity, a high bioavailability of more than 60% and a half-life of 5 to 6 hours in dogs (Jacks *et al.*, 1996). Merck selected MK-0677 for safety assessment studies which then entered clinical trials (Nargund *et al.*, 1998).

1.3 Growth hormone secretagogues today

Despite tremendous research and effort at designing efficacious GHS that will at the same time maintain the pulsatile release of GH and have an elevated oral bioavailability, injections of recombinant GH continues to be the treatment of choice for GH-deficient patients, mainly due to its low-cost production. Furthermore, in a high percentage of cases, a decrease in GH is the result of an improper activity of the pituitary gland; therefore, injection of GH is the logical approach to treat GH-deficiency. However, throughout the years, new applications were considered for GHS. For example, they have been used as a diagnostic tool to detect GH deficiency. In Japan, the second generation of GHRP-6, GHRP-2 is used as a kit to detect GH deficiency in adults (Arita *et al.*, 2008). In addition, MK-0677 is being considered for its role in counteracting the reduced basal metabolic rate resulting from aging, from calorie-restricted diets and from wasting syndrome (cachexia) seen in patients suffering from chronic diseases (Nass *et al.*, 2008;Smith *et al.*, 2007;Murphy *et al.*, 1998). However, due to its effect on GH secretion and its potential in improving athletic performance, GHS are amongst the list of banned substances published by the World Anti-Doping Agency (WADA). GHS have also found their way to the black market for their use in bodybuilding. Recently, GHRP-2 was detected in over-the-counter nutritional supplemental tablets (Thomas *et al.*, 2010).

2 Growth hormone secretagogue receptor and its natural ligand

2.1 Identification of an alternative pathway for GH release

As illustrated in Figure 1B, it was first presumed that the pulsatile GH secretion was regulated by only two hormones: GHRH and SRIF. Following the discovery of GHRPs, studies pertaining to their mechanism of action have permitted to identify a second activation pathway for the release of GH. Based on their preliminary results, Bowers and colleagues suggested early on that GHRPs acted on a different pathway than that of GHRH. They observed that combining GHRP-6 with GHRH had an additive effect on GH secretion in rats (Bowers *et al.*, 1984;Sartor *et al.*, 1985). In addition, Roy Smith and colleagues at Merck reported that in isolated rat somatotropic cells, repeated treatments with GHRP-6 resulted in the desensitization of cells to GHRP-6 without affecting its response to GHRH; and inversely, treatment of cells with a GHRH antagonist had no effect on the response of cells to GHRP-6 (Cheng *et al.*, 1989). Similarly to opioid receptors, GHRH receptor (GHRHR) belongs to the family of G protein-coupled receptors (GPCRs) (Figure 2). One main feature of the binding of GHRH to its receptor is the increase in intracellular cyclic AMP (cAMP) via stimulation of adenylate cyclase (AC) by Gsα subunit (Mayo, 1992). Cyclic AMP activates protein kinase A (PKA) which in turn activates a range of factors responsible for the expression of GH and its subsequent processing and secretion (Cohen *et al.*, 1999). In support of an alternative pathway for GH release, several studies have used GHS such as GHRP-6 and MK-0677 to reveal the following findings:

- GHS had no effect on intracellular cAMP levels (Cheng *et al.*, 1989)
- Phorbol 12-myristate 13-acetate (PMA), an activator of protein kinase C (PKC) was capable of imitating the additive effect of GHS when combined with GHRH (Cheng *et al.*, 1991)

- GHS causes a rapid increase in intracellular free calcium, $[Ca^{2+}]i$ (Herrington & Hille, 1994;Bresson-Bepoldin & Dufy-Barbe, 1994)
- The secretion of GH by GHS involved the inositol (1,4,5)-trisphosphate/ diacylglycerol (IP_3/DAG) pathway (Adams *et al.*, 1995;Mau *et al.*, 1995)

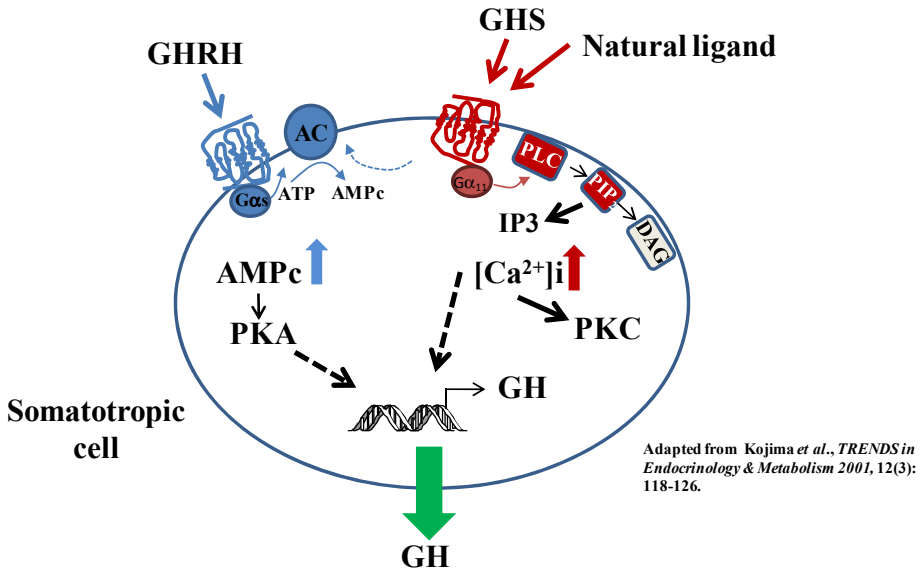


Figure 2. Growth hormone-releasing hormone and growth hormone secretagogue pathways involved in GH release from the pituitary

2.2 Identification of the receptor for GHS

In 1996, under the supervision of Lex Van der Ploeg and Roy Smith, the research team at Merck were the first to clone the receptor for GHS (Howard *et al.*, 1996). Using the expression-cloning strategy in which size-fractionated poly A^+ RNA from pig pituitaries were microinjected into *Xenopus* oocytes along with cRNA of selected G protein and aequorin. Using aequorin, a bioluminescent probe known to bind intracellular calcium, they measured levels of $[Ca^{2+}]i$ released following treatment with MK-0677. Exhaustive stepwise fractionation of positive pools finally resulted in the identification of an orphan

receptor. The gene for GHS receptor, called GHS-R1a codes for a 366-aa GPCR (41 kDa) belonging to GPCR, class A (rhodopsin-like receptors) with the typical conserved 7 transmembrane (7-TM) α-helices and classic sequence for G protein interactions. A second isoform (GHS-R1b) was also cloned that represented a truncated 289-aa protein missing TM 6 and 7, which did not respond to GHS and was a product of pre-mRNA splicing. Formation of GPCRs homo- and hetero-oligomers is thought to play an important role in ligand binding and cell signaling (Maggio *et al.*, 2005). It was recently shown that GHS-R1b acts as a dominant-negative form of GHS-R1a to lessen its constitutive activity by forming heterodimers and when its expression exceeds that of GHS-R1a, trafficking of GHS-R1a to the cell surface is attenuated (Leung *et al.*, 2007).

2.3 Distribution of GHS-R1a

The tissue distribution of GHS-R1a is more widespread than first anticipated suggesting a role beyond that of stimulator of GH secretion from the pituitary. Table 2 depicts the detection GHS-R1a in various tissues or cell types. Listed also is the expression of scavenger receptor CD36 which will be discussed in section 5.2. The expression of GHS-R1a is elevated in several sections of the brain including the hypothalamus and pituitary gland (Guan *et al.*, 1997). The hypothalamic nuclei play a major role in the regulation of food intake and energy homeostasis (Gao & Horvath, 2008;Horvath, 2005). In addition, GHS-R1a is also expressed in endocrine tissues such as pancreas, adrenal glands, thyroid, ovaries and testicles (Guan *et al.*, 1997;Gnanapavan *et al.*, 2002;Gaytan *et al.*, 2005;Tena-Sempere *et al.*, 2002). In relation to the cardiovascular system, GHS-R1a is present in the aorta, the left atrium and ventricle, and more precisely in cardiomyocytes as well as in smooth muscle cells and microvascular endothelial cells (Nagaya *et al.*, 2001;Kleinz *et al.*, 2006;Li *et al.*, 2007a). Looking at the digestive system, GHS-R1a was detected in the intestine and stomach (Dass *et al.*, 2003;Wu *et al.*, 2004;Shuto *et al.*, 2001). GHS-R1a is also found in spleen and in leukocytes such as monocytes/macrophages, lymphocytes and neutrophiles (Gnanapavan *et al.*, 2002;Demers *et al.*, 2004;Hattori *et al.*,

2001). Its presence in adipose tissue has been suggested (Choi *et al.*, 2003;Davies *et al.*, 2009) while in other studies it has not been detected (Gnanapavan *et al.*, 2002;Muccioli *et al.*, 2004). To support its absence in adipose tissue, GHS-R1a was undetectable in cultured mouse adipocytes, 3T3-L1 (Zhang *et al.*, 2004;Rodrigue-Way *et al.*, 2007). To explain this discrepancy, macrophages can be present in adipose tissue and their infiltration can contribute to inflammation in obese subjects (Weisberg *et al.*, 2003). It is therefore possible that detection of GHS-R1a in adipose tissue in certain studies is due to contaminating macrophages known to express GHS-R1a. GHS-R1a is also absent in liver, primary hepatocytes and human hepatocellular carcinoma cell line, HepG2 (Smith *et al.*, 2007;Thielemans *et al.*, 2007;Gauna *et al.*, 2005). GHS-R1a neither was detectable in skeletal muscle nor in mouse differentiated myoblastic cell line, C2C12 (Ueberberg *et al.*, 2009;Filigheddu *et al.*, 2007). It was also untraceable in colon, kidney and prostate (Ueberberg *et al.*, 2009).

It is noteworthy to mention that GHS-R1a is not expressed in tissues involved in lipid metabolism such as adipose tissue (adipocytes), liver and skeletal muscle.

Table 2. Tissue- or cell-specific distribution of GHS-R1a and CD36

Tissue or cell type	GHS-R1a	CD36	Reference(s)
Hypothalamus	√	√	Guan *et al.*, 1997; Le Foll et al., 2009
Pituitary gland	√	√	Guan *et al.*, 1997; Ong et al., 1998b
Heart or cardiomyocyte	√	√	Kleinz *et al.*, 2006; Van Nieuwenhoven et al., 1995
Vascular smooth muscle cell	√	√	Kleinz et al., 2006; de Oliveira et al., 2008
Microvascular endothelial cell	√	√	Li *et al*., 2007a; Swerlick et al., 1992
Spleen	√	√	Gnanapavan *et al.*, 2002; Memon et al., 1998
Pancreas	√	√	Guan et al., 1997; Noushmehr et al., 2005a
Stomach	√	√	Shuto *et al.*, 2001; Chen et al., 2001
Intestine	√	√	Dass *et al.*, 2003;Wu *et al.*, 2004; Chen et al., 2001
Adrenal gland	√	√	Gnanapavan *et al.*, 2002; Zhang et al., 2003
Macrophage	√	√	Demers *et al.*, 2004; Endemann et al., 1993
Lymphocyte	√	√	Hattori *et al.*, 2001; Won et al., 2008
Neutrophile	√	√	Hattori *et al.*, 2001; Suchard et al., 1992
Ovary	√	√	Gaytan *et al.*, 2005; Zhang et al., 2003
Testicule	√	√	Tena-Sempere *et al.*, 2002; Gillot et al., 2005
Adipose tissue and 3T3-L1 adipocyte	X	√	Gnanapavan *et al.*, 2002; Zhang *et al*., 2004; Harmon & Abumrad, 1993
Liver, primary hepatocyte and HepG2	X	√	Smith *et al.*, 2007; Gauna *et al.*, 2005; Thielemans *et al*., 2007; Memon et al., 1998;Malerod et al., 2002
N9 microglia cell	X	√	Bulgarelli et al., 2009
Kidney	X	√	Ueberberg *et al.*, 2009; Susztak et al., 2004
Colon	X	√	Ueberberg *et al.*, 2009; Chen et al., 2001
Skeletal muscle or C2C12 myoblast	X	√	Ueberberg *et al.*, 2009; Filigheddu *et al.*, 2007; Van Nieuwenhoven et al., 1995
Prostate	X	√	Ueberberg *et al.*, 2009; Vallbo & Damber, 2005
Platelet/megakaryocyte	?	√	Clemetson *et al*., 1977
Breast	?	√	Clezardin *et al.*, 1993
Pneumocytes	?	√	Guthmann *et al.*, 1999
Airway epithelium	?	√	Atsuta *et al.*, 1997
Dendritic cell	?	√	Juhlin, 1989
Retinal pigment epithelium	?	√	Ryeom *et al.*, 1996
Gustatory cell	?	√	Fukuwatari *et al.*, 1997
Thyroid	√	X	Patey *et al.*, 1999

√ -mRNA or protein detected
X -Undetected
? - Unknown

2.4 The natural ligand of GHS-R1a: ghrelin

2.4.1 Discovery of ghrelin: classical example of reverse pharmacology

The endogeneous ligand of GHS-R1a was isolated in 1999 by Kenji Kangawa and his team in Japan (Kojima *et al.*, 1999). The discovery of ghrelin became a classical example of reverse pharmacology (Libert *et al.*, 1991). Their approach consisted of treating

stable GHS-R1a-transfected CHO cells with tissue extracts from brain, lung, heart, kidney, stomach and gut; and to monitor for changes in $[Ca^{2+}]_i$. Following a positive response from stomach extracts, they proceeded to go through intensive chromatography purification steps, including reversed-phase HPLC. From the final purified sample, they characterized and sequenced the potential GHS-R1a ligand by mass spectrometry. Finally, they identified a 28-aa protein with a n-octanoylated ($C_7H_{15}CO$) serine residue at position 3. This unique post-transcriptional modification was essential for its GH-releasing activity. The authors names this peptide "ghrelin" after the word root "ghre" in Proto-Indo-European languages meaning "grow" and superposed with "rel" for "release" for its GH-releasing ability (Kojima, 2008). It took a little less than a decade for Joseph Goldstein and Andrew Brown and colleagues, to discover the enzyme responsible for the acylation of ghrelin with the fatty acid, octanoate (Yang *et al.*, 2008). Ghrelin O-Acyltransferase (GOAT) is the only member of its family capable of attaching a small fatty acid to a peptide. Due to its unique role, GOAT has become an interesting target in the treatment against obesity and diabetes (Chen *et al.*, 2009). The majority of ghrelin in the gastrointestinal tracts is produced by a distinct endocrine cell type called X/A-like cells in the oxyntic gland (Date *et al.*, 2000). However, ghrelin is also expressed to a smaller degree throughout the digestive system from the stomach to the colon and also in the pancreas, adipose tissue, heart, kidney, lung, adrenal gland, thyroid, pituitary and hypothalamus (Kojima *et al.*, 1999;Ueberberg *et al.*, 2009). In addition to its role in secreting GH, ghrelin is an appetite-stimulating peptide hormone and the only gastrointestinal peptide with orexigenic powers (Asakawa *et al.*, 2001;Woods, 2004).

2.4.1.1 Deacyl-ghrelin

The deacylated ghrelin does not bind to GHS-R1a and was considered as a non-functional peptide until recently (Chen *et al.*, 2009). Since deacyl-ghrelin was the major form secreted in the circulation, it seemed improbable that it had no physiological relevance. Indeed, the ratio deacyl-ghrelin:ghrelin was found to be between 2.5:1 and 9:1 (Tsubone *et al.*, 2005;Broglio *et al.*, 2004;Yoshimoto *et al.*, 2002). The ratio deacyl-

ghrelin:ghrelin turned out to have important physiological consequences on energy balance since it was discovered that deacyl-ghrelin had opposite effects to that of ghrelin on food intake, for example (Asakawa *et al.*, 2005). In obese patients, circulating deacyl-ghrelin levels decreased greatly while ghrelin levels increased; and in obese diabetic patients, this change in ratio was further accentuated (Rodriguez *et al.*, 2009). Despite its inability to bind GHS-R1a, deacyl-ghrelin is thought to bind a yet unknown receptor since some studies have shown binding sites on cardiomyocytes and C2C12 skeletal muscle cells (Filigheddu *et al.*, 2007;Lear *et al.*, 2010).

2.5 Role of ghrelin and its binding to GHS-R1a

Given the broad distribution of GHS-R1a and ghrelin, it wasn't surprising to discover that their influence on energy homeostasis went far beyond that of simply controlling GH secretion. As stated previously, a major role was given to ghrelin as an appetite-stimulating hormone (Asakawa *et al.*, 2001). In addition, central and peripheral injections of ghrelin in mice provoked a decrease in energy expenditure, an increase in respiratory quotient (RQ= CO_2 eliminated /O_2 consumed) and a decreased in oxygen consumption (Tschop *et al.*, 2000;Asakawa *et al.*, 2005). Using neuropeptide Y (NPY)-deficient mice, injections of ghrelin were shown to increase body weight and adiposity independently from its orexigenic effect due to the absence of NPY, the regulator of food intake (Tschop *et al.*, 2000). Ghrelin decreased adipocyte thermogenesis suggesting a GHS-R1a-independent effect (Tsubone *et al.*, 2005). Adipogenesis is a carefully controlled event during which the timing in the expression of specific genes is important in the induction of the differentiation program and proper functioning of adipocytes. Adipogenesis is further discussed in Section 7.2. Both chronic intracerebroventricular infusion of ghrelin in rats and treatment of human visceral adipocytes resulted in the increased expression of 2 genes involved in adipocyte differentiation, nuclear receptor peroxisome proliferator-activated receptor gamma (PPARγ) and sterol-regulatory element binding protein-1 (SREBP-1) as well as several genes involved in adipocytic function such as lipoprotein lipase (LPL),

acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS) and stearoyl-CoA desaturase-1 (SCD-1) (Rodriguez *et al.*, 2009;Theander-Carrillo *et al.*, 2006). Ghrelin is shown to activate 5' adenosine monophosphate-activated protein kinase (AMPK) in tissues expressing GHS-R1a such as the hypothalamus and the heart while inhibiting AMPK in GHS-R1a-negative tissues such as the liver and adipose tissue (Kola *et al.*, 2005). However, ghrelin had no effect on AMPK in skeletal muscle. AMPK is a key regulator of energy homeostasis further presented in Section 9.1. The GH-independent cardioprotective effect of ghrelin might be due in part to the activation of AMPK (Frascarelli *et al.*, 2003). In general, the orexigenic effect of ghrelin ties macronutrient composition with regulation of energy balance by the CNS but it also has direct GHS-R1a-dependent and -independent effects of peripheral tissues.

2.6 Therapeutic interest of ghrelin and GOAT

Based on its role in energy balance, ghrelin has become an interesting therapeutic target for certain pathophysiological conditions. In cancer patients as well as in patients with severe chronic wasting diseases such as chronic obstructive pulmonary disease and renal failure, infusion of ghrelin resulted in a marked improvement in appetite, food intake and nutrient absorption (Neary *et al.*, 2004;Nagaya *et al.*, 2005;Ashby *et al.*, 2009). Along with the identification of GOAT in 2008 and the importance in the ratio deacyl-ghrelin:ghrelin, much attention is given to GOAT and its role in controlling ghrelin's action (Romero *et al.*, 2010). However, the use of an agonist or antagonist of GOAT still remains to be determined since the physiological effects of ghrelin are multiple, sometimes opposite and difficult to interpret based on the various sites of action of ghrelin independent and dependent of GHS-R1a.

3 The growth hormone releasing peptide, hexarelin

3.1 A novel GHRP

Hexarelin made its appearance in 1994 when Vittorio Locatelli and colleagues in Italy synthesized a derivative of GHRP-6 in which they substituted D-Trp for D-2-methyl-Trp (see Table 1) making this peptide chemically more stable than GHRP-6 and above all the first orally active GHRP (Deghenghi *et al.*, 1994;Ghigo *et al.*, 1994). Initially called EP 23905, hexarelin was synthesized during the era when intensive search for a highly orally active GHS was conducted. Despite the superior bioavailability of peptidomimetic GHS, studies using GHRPs, including hexarelin, were pursued in humans and various animal models in the hopes of understanding its biological effect but also to mainly justify its use as a therapeutic or a diagnostic tool (Micic *et al.*, 1999). Hexarelin studies were quickly undertaken in humans to verify its efficacy on GH secretion. Hexarelin was well tolerated in humans without any reported side-effects and elicited a substantial elevation in plasma GH concentrations in a dose-dependent manner (Imbimbo *et al.*, 1994). Even though hexarelin was orally active, it still did not mirror the efficacy of the orally active GHS, MK-0677. Because of the highly vascularized nasal cavity, intranasal administration was proposed for hexarelin as a therapeutic tool for GH deficiency instead of IV injections (Laron *et al.*, 1994;Laron *et al.*, 1995;Pontiroli, 1998). Today, hexarelin is used mainly in research but it also has become a popular choice as a performance enhancement drug.

3.2 The dual action of hexarelin

Prior to the detection of GHS-R1a in the myocardium, GH-independent studies suggested that hexarelin possessed cardioprotective properties distinct from that of GH (Berti *et al.*, 1998;Rossoni *et al.*, 1998). It was suggested that perhaps in addition to GHS-R1a, GHRPs were capable of binding to a sub-type of GHRP receptor. In 1998, the group of Magnus Nilsson performed binding assays on membrane extracts from human, bovine

and porcine anterior pituitaries to identify hexarelin's receptor (Ong *et al.*, 1998b). They developed a photoreactive analogue of hexarelin containing a photoactivatable amino acid, p-benzoyl-L- phenylalanine and iodine-125 labeled ([^{125}I]iodoTyr-Bpa-Ala-Hexarelin) (Dorman & Prestwich, 1994). They identified a 57 kDa photolabeled protein in all samples analyzed that was distinct from the 41-kDa GHS-R1a protein. Another protein capable of interacting with hexarelin and with a molecular weight of 84 kDa was detected in heart (Ong *et al.*, 1998a). However, when the heart photolabeled protein was treated with N-glycosidase F, a decrease in its MW to ~57 kDa was observed suggesting that this receptor contained oligosaccharide chains and was therefore heavily N-glycosylated in the heart but not in the pituitary gland (Bodart *et al.*, 1999). In addition, the binding was specific to hexarelin since MK-0677 and EP51389 (another potent GHRP) were unable to compete with the photoactivatable hexarelin for this unidentified receptor. In support of these findings, another study showed that hexarelin had a different binding pattern than that of MK-0677 or ghrelin (Papotti *et al.*, 2000). Binding of hexarelin was detected in decreasing order in heart, adrenal gland, gonad, artery, lung, liver, skeletal muscle, kidney, pituitary, thyroid, adipose tissue, vein, uterus, skin and lymph node. In 2002, the second receptor for hexarelin was identified as the scavenger receptor, CD36 (Bodart *et al.*, 2002).

4 Scavenger receptors

The role of certain cell types as scavengers was first suggested by Joseph Goldstein, Andrew Brown and associates (Goldstein *et al.*, 1979). They observed that in presence of acetylated ^{125}I-labeled low density lipoprotein (^{125}I-acLDL) cultured macrophages would internalize these particles at a much higher rate than native LDL particles. This uptake resulted in the accumulation of intracellular cholesterol and a transformation of cells into foam cell-like phenotype similar to what is observed in atherosclerotic plaques. These results implied the presence of receptors capable of binding modified LDL particles and were therefore referred to as scavenger receptors.

4.1 The role of scavenger receptor in the formation of atherosclerotic lesions

It was later determined that the formation of oxidized LDL (oxLDL) particles rather than acLDL was a likely occurrence since oxLDL particles were present in atherosclerotic plaques (Palinski *et al.*, 1989;Yla-Herttuala *et al.*, 1989).The first step in the development of atherosclerotic plaques is the appearance of fatty streaks consisting primarily of foam cells loaded with lipids and T lymphocytes within the vessel's subendothelial space or intima (Daugherty & Roselaar, 1995). Oxidation of LDL particles is thought to occur not in the circulation but rather in the intima following their infiltration in specific locations of a vessel (Chow *et al.*, 1998;Rangaswamy *et al.*, 1997). The oxidation of imprisoned LDL particles is a long and complex process that is still considered a key step in the development of atherosclerosis (Stocker & Keaney, Jr., 2005;Steinberg, 2002). The core of a LDL particle contains cholesterol esters (CE) and triglycerides (TG) while the surface is covered with a single layer of phospholipids (PL), including phosphatidylcholine, molecules of non-esterified cholesterol and a single molecule of apolipoprotein B-100 (apoB100) that specifically interacts with the LDL receptor on neighboring cells (Steinberg, 2002).While cholesterol is less susceptible to oxidation, an important variety of oxidized PL was detected within lesions and especially in oxLDL (Berliner *et al.*, 2001).When phospholipids are minimally modified (mmLDL), they become negatively charged, have an anti-apoptotic effect on scavenger cells, and stimulate secretion of chemokines and cytokines from neighboring endothelial cells lining the blood vessels (Berliner *et al.*, 1995;Boullier *et al.*, 2006). Consequently, monocytes are recruited from the circulation toward the site of inflammation. Subsequently, within the intima, the infiltrated monocytes differentiate into macrophages. The constant recruitment of inflammatory cells results in increased cytokine secretion and continuous oxidation of mmLDL. Phospholipids are increasingly oxidized on LDL while apoB100 undergoes modifications and unfolds (Hamilton *et al.*, 2008). Oxidized LDL is no longer recognized by LDLR but becomes a ligand for scavenger

receptors. Macrophages, endothelial cells and even vascular smooth muscle cells (VSMC) take up oxLDL via their scavenger receptors. This process allows especially macrophages to clear the intima from the harmful presence of oxLDL. In addition, the oxidized lipids taken up by the macrophage serve as ligands to nuclear receptor, PPARγ and induce the expression of genes involved in the reverse cholesterol transport such as ATP-binding cassette transporter AI (ABCA1) which shuttles internalized cholesterol into nascent HDL particles for clearance by the liver (Chawla *et al.*, 2001). However, when macrophages become overloaded and overwhelmed by oxLDL, an imbalance occurs between the uptake and the clearance of lipids and cells become consequently lipid-laden macrophages, or foam cells (Faggiotto *et al.*, 1984). In atherosclerosis, progression in the formation of foam cells, but also in the increase in inflammation, in cellular necrosis, and thinning of the fibrotic plaque eventually lead to plaque rupture and thrombosis (Lusis, 2000). Therefore, scavenger receptors are thought to play a detrimental role in the pro-atherogenic effect of modified LDL particles.

4.2 Scavenger receptor classes

Scavenger receptors (SR) have been identified and grouped based on their capacity to bind modified lipoprotein particles and their contribution to the development of atherosclerosis. They have been categorized into different classes based mainly on their structural features and functional domains (Krieger, 1997;Horiuchi *et al.*, 2003). Figure 3 provides an illustration of the different classes of SR. Members of class A have a single TM region, a trimeric formation (α-helices) and possess an affinity for acLDL and oxLDL. This class includes SR-A type 1 and type 2 (SR-A1 and SR-A2) which are encoded by the same gene and alternatively spliced (Kodama *et al.*, 1990;Rohrer *et al.*, 1990). Members of class A, mainly expressed in macrophages, play a major role in the development of atherosclerosis (Sakaguchi *et al.*, 1998;Suzuki *et al.*, 1997). A marked decrease in lesion size was observed in SR-A knockout mice on either an apoE- or a LDLR-deficient background (atherosclerotic models). The main feature of members of Class B, such as

CD36 and SR-BI, consists of two TM domains separated by N- and C-terminal cytosolic extremities, forming a large loop-like extracellular structure. CD36 will be discussed further in the following section. SR-BI binds to oxLDL, acLDL, native LDL, apoptotic cells and also lipopolysaccharide (LPS) found on bacteria. It is however mainly known for its ability to bind HDL particles and its central role in reverse cholesterol transport (Acton *et al.*, 1996). SR-BI is expressed on macrophage, endothelial cell as well as in liver, adrenal gland, placenta and gonad (Landschulz *et al.*, 1996). SR-BI is also highly glycosylated and comparisons between SR-BI and CD36 show several conserved extracellular cysteine residues; however their TM and cytosolic domains share little resemblance (Krieger, 1999). Class C initially thought to be expressed in mammals is only found in *drosophila* (dSR-CI) and was characterized based on its ability to bind acLDL (Pearson *et al.*, 1995). Class D features CD68 which is mostly present in the macrophage endosome (Ramprasad *et al.*, 1996). When the cell is activated, CD68 is translocated to the cell surface for binding to oxLDL, acLDL and native LDL. CD68 is heavily O-glycosylated accounting for two thirds of its molecular weight (Holness & Simmons, 1993). Class E is represented by lectin-like oxLDL receptor-1 (LOX-1) expressed mainly on endothelial cells but also on macrophages, smooth muscle cells (SMC) and platelets (Sawamura *et al.*, 1997;Apostolov *et al.*, 2009). LOX-1 contains a type C lectin-like domain that recognizes specific carbohydrate structures (Drickamer, 1988). Special attention is given to LOX-1 since in addition to binding to oxLDL and acLDL, it also binds to carbamylated LDL (cLDL) particles, recently associated with oxidative stress and inflammation (Apostolov *et al.*, 2009). Class F is defined by the scavenger receptor expressed by endothelial cells proteins (SREC-I and –II) which have repeats of EGF-like cysteine-rich motifs (Ishii *et al.*, 2002;Adachi *et al.*, 1997). While their expression pattern is similar (endothelial cells, macrophages and SMC), they differ in their ligand recognition. SREC-I recognizes oxLDL, acLDL and bacterial surface proteins. SREC-II does not bind native or modified LDL particles, technically not qualifying as a scavenger receptor. However, due to its EGF-like domain, SREC-II participates in cell aggregation by interacting with SREC-I in absence of modified LDL

particles (Ishii *et al.*, 2002). Class G protein, SR-PSOX/CXCL16 binds oxLDL and phosphatidylserine, and contains a chemokine domain and mucin-like domain (Shimaoka *et al.*, 2000;Matloubian *et al.*, 2000). Phosphatidylserine, usually in the inner-leaflet of the membrane, becomes exposed on cell surface of apoptotic cells (Fadok *et al.*, 1992). Proteolysis of the chemokine portion of this receptor results in the release of the soluble CXCL16 and acts to attract CXCR6-positive lymphocytes (van der Voort *et al.*, 2010). PSOX/CXCL16 is expressed on macrophages, and also on dendritic, endothelial and smooth muscle cells (Sheikine & Sirsjo, 2008). All these classes, with the exception of class C, have been present in atherosclerotic lesions and are involved in foam cell formation. Scavenger receptors on phagocytic cells act primarily to detect abnormal specific motifs and are considered multi-ligand receptors. Each class possesses distinct properties; however, their ligand-recognition ability often overlaps and complicates our understanding of their role and downstream effects. It is clear however that in a healthy individual, the role of scavenger receptors is to clear the body of infection, of apoptotic cells and of modified lipoprotein that might be potentially harmful. For the purpose of this thesis, the focus will remain on CD36.

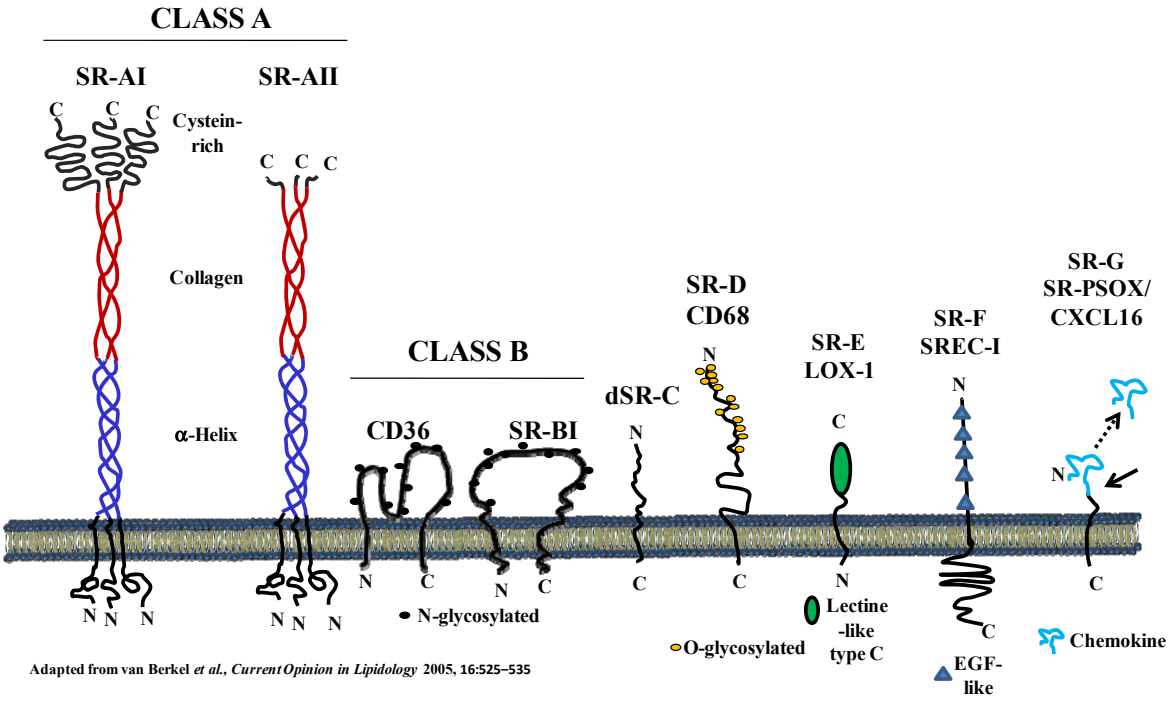


Figure 3. Classification of scavenger receptors and their proposed structural features

5 Scavenger receptor, CD36

5.1 Identification of CD36 and its various designations

Blood platelets play a crucial role in hemostasis and coagulation. When activated following an injury, platelets adhere to the solid surface of a vessel to form aggregates with other platelets and prevent hemorrhage (Cooper *et al.*, 1976). Little less than forty years ago, studies on polypeptides and glycoproteins located on the surface of platelets were undertaken to determine the physiological role and biochemical nature of this anucleated cell. Three types of glycoprotein (I, II and III) were identified as major platelet surface proteins involved in adherence and aggregation (Phillips, 1972;Nachman & Ferris, 1972). Glycoprotein IV (GPIV) also present on platelets was identified as a protein with a MW of ~88 kDa (Clemetson *et al.*, 1977). During the 1980's, more than a hundred ninety

laboratories collaborated for the 3rd International Workshop on Human Leukocyte Differentiation Antigens to characterize a little over 800 antibodies in the hopes of describing and identifying human leukocyte surface molecules. During that time, the leukocyte differentiation antigen Cluster of Differentiation 36, CD36 was identified as the antigen for anti-human monocyte antibody OKM5 (Shaw, 1987;Knowles *et al.*, 1984). It was soon determined that GPIV and CD36 were the same protein (Tandon *et al.*, 1989b). Due to its multi-ligand pattern recognition function, CD36 is also known as thrombospondin (TSP) receptor, collagen receptor, fatty acid translocase (FAT) and finally the commonly referred name, scavenger receptor CD36 (Asch *et al.*, 1987;Tandon *et al.*, 1989a;Harmon & Abumrad, 1993;Endemann *et al.*, 1993).

5.2 Tissue distribution

CD36 is expressed in various tissues and cell types. As mentioned in the previous section and listed in Table 2 (page 11), CD36 was first identified on platelets/megakaryocytes (Clemetson *et al.*, 1977;Tandon *et al.*, 1989b) but it is also found in heart and skeletal muscle (Van Nieuwenhoven *et al.*, 1995), kidney (Susztak *et al.*, 2004), pancreas (Noushmehr *et al.*, 2005a), spleen (Memon *et al.*, 1998), stomach (Chen *et al.*, 2001), liver (Memon *et al.*, 1998;Malerod *et al.*, 2002), gonad (Gillot *et al.*, 2005), adrenal gland (Zhang *et al.*, 2003), prostate (Vallbo & Damber, 2005), hypothalamic ventromedial nucleus (VMN) neurons (Le Foll *et al.*, 2009) and pituitary gland (Ong *et al.*, 1998b). CD36 is also found on the surface of different types of leukocytes such as monocytes/macrophage (Endemann *et al.*, 1993), neutrophiles (Suchard *et al.*, 1992), B lymphocytes (Won *et al.*, 2008), dendritic cells (Juhlin, 1989) and microglia cells (macrophages residing in the brain and spinal cord fluid) (Coraci *et al.*, 2002). CD36 is also expressed on vascular SMC (de Oliveira *et al.*, 2008) and microvascular endothelial cells (Swerlick *et al.*, 1992), in addition to airway epithelium (Atsuta *et al.*, 1997), pneumocytes (Guthmann *et al.*, 1999), breast (Clezardin *et al.*, 1993) and retinal pigment epithelium (Ryeom *et al.*, 1996). Moreover, it is highly expressed in cell types involved in lipid

metabolism such as adipocytes (Harmon & Abumrad, 1993), hepatocytes (Maeno *et al.*, 1994), enterocytes of the small intestine (Chen *et al.*, 2001) and even on gustatory cells (Fukuwatari *et al.*, 1997). However, thyrocytes did not seem to express CD36 (Patey *et al.*, 1999). Table 2 compares the tissue/cell type distribution between GHS-R1a and CD36. It is interesting to note that CD36 is present in tissues involved in lipid metabolism, which are negative for GHS-R1a. Therefore, in adipocytes or hepatocytes, hexarelin would only act through CD36.

5.3 The role of the multi-ligand receptor CD36 in different biological processes

Based on the different studies published, it is apparent that the role of CD36 is primarily defined by its ligand and by the cell type in which it is expressed. CD36 is known to bind TSP-1 (Silverstein *et al.*, 1989), oxLDL (Endemann *et al.*, 1993), apoptotic cells (Albert *et al.*, 1998;Fadok *et al.*, 1998), malaria parasites (Biggs *et al.*, 1990), bacteria (Hoebe *et al.*, 2005) and long chain fatty acids (Abumrad *et al.*, 1993).To better define its functions, the following section is divided by ligands and when applicable, sub-divided into reported functions.

5.3.1 Thrombospondine-1 (TSP-1) and CD36

Like CD36, TSP-1 is referred to as a multifunctional protein due to its capacity to bind to at least a dozen different receptors (Bornstein, 1995). TSP-1 is involved in cell proliferation, apoptosis, cell migration, phagocytosis, coagulation and angiogenesis. One of its receptors was identified in 1987 as an 88-kDa protein reacting with OKM5 monoclonal antibody and was later identified as CD36 (Asch *et al.*, 1987;Silverstein *et al.*, 1989). Due to this interaction, CD36 was associated with the following biological processes:

5.3.1.1 Hemostasis and platelet aggregation

Platelets, or thrombocytes, were thought to bind to collagen via CD36 on damaged arterial or vessel walls to form a platelet plug (Tandon *et al.*, 1989a). In fact, CD36 was indirectly associated with binding of platelets to collagen through its direct binding to TSP-1 (Legrand *et al.*, 1991). Therefore, one of the first roles given to CD36 was the activation and aggregation of platelets via its binding to TSP-1.

5.3.1.2 Thrombosis

The formation of a thrombus, or blood cloth, constitutes the last stage in hemostasis. However, during the development of an atherosclerotic plaque, a thrombus can pathologically lead to thrombosis, or blood vessel occlusion. In addition to its implication in platelet aggregation, CD36 was also shown to mediate the association platelet/monocyte and platelet/endothelial cell via TSP-1, supporting a role for CD36 in the development of obstructive blood clots (Silverstein *et al.*, 1989).

5.3.1.3 Angiogenesis

TSP-1 is a potent inhibitor of angiogenesis rendering endothelial cells insensible to a variety of vascularization stimulators (Good *et al.*, 1990). Since CD36 is expressed on microvascular endothelial cell surface; Noël Bouck and colleagues discovered that the antiangiogenic effect of TSP-1 was in fact due to its interaction with CD36 on endothelial cells, causing an inhibition in cell migration and capillary tube formation (Dawson *et al.*, 1997).

5.3.1.4 Endothelial cell apoptosis

TSP-1 can render microvascular cells sensitive to apoptosis in order to control angiogenesis and limit blood vessel density within normal tissue (Guo *et al.*, 1997). Induction of apoptosis by TSP-1 is dependent on activation of CD36, Fyn kinase, caspase-3 and mitogen-activated protein kinases (MAPKs) (Jimenez *et al.*, 2000).

5.3.1.5 Corneal neovascularization

Normally, the cornea is devoid of blood vessels; however, corneal neovascularisation can occur due to prolong uninterrupted contact lens wear, inflammation, infection or trauma and result in impaired vision. CD36 contributes significantly to the maintenance of corneal avascularity by inhibiting neovascularisation. Blocking CD36 activity leads to corneal neovoascularization (Mwaikambo *et al.*, 2006). While in a hypoxic state, CD36 expression is increased by the transcription factor, hypoxia-inducible factor-1 (HIF-1) as an adaptive response (Mwaikambo *et al.*, 2009).

5.3.2 Modified LDL particles, atherosclerosis and inflammation

CD36 is notoriously associated with atherosclerosis; or more precisely, the binding of CD36 on monocytes/macrophages to oxLDL and foam cell formation (Endemann *et al.*, 1993). The capture and internalization of oxidized lipids lead to the activation of PPARγ and promotion of monocyte to macrophage differentiation and eventually to foam cell differentiation (Tontonoz *et al.*, 1998). However, since CD36 is also expressed on platelets, endothelial cells and vascular smooth muscle cells (VSMC), binding of oxLDL on these cell types can also play an important role in the development of atherosclerosis. Indeed, foam cells derived from VSMC were also detected in atherosclerotic lesions and are known to play a role in its formation, in addition to its inflammatory response to oxLDL (Faggiotto *et al.*, 1984;Lim *et al.*, 2006). Binding of oxLDL to endothelial cells via CD36 is linked to endothelial dysfunction in atherosclerosis (Kopprasch *et al.*, 2004), while on monocytes and macrophages it can induce an inflammatory response and stimulate phagocytosis (Harb *et al.*, 2009).

5.3.3 Phagocytosis of apoptotic cells

The phagocytosis of apoptotic or senescent cells by specialized cells is essential for tissue remodeling or for the resolution of an inflammatory response to avoid the spilling of toxic intracellular content and amplification of tissue injury (Savill *et al.*, 1989).

Phagocytes recognize apoptotic cells via the exposed phosphatidylserines on the surface of apoptotic cells. CD36 expressed on macrophages and dendritic cells intervenes in this process (Albert *et al.*, 1998;Fadok *et al.*, 1998). The recently proposed Lipid Whisker Model states that when the cell membrane undergoes lipid peroxidation, the hydrophobic portion of fatty acids flips from the internal side of the bilayer to the extracellular surface enabling CD36 to recognize the apoptotic cell through binding with the oxidized lipid components (Greenberg *et al.*, 2008).

5.3.4 Bacterial infection

CD36 is also involved in the phagocytosis of, and the pro-inflammatory response to Gram-positive and Gram-negative bacteria. Lipoteichoic acid (LTA) and lipopolysaccharide (LPS) on the surface of Gram-positive bacteria and Gram-negative, respectively, are recognized by CD36 and contribute to the recruitment of LTA and LPS to Toll-like receptors (TLR) responsible for the activation of cells involved in the immune response (Baranova *et al.*, 2008;Triantafilou *et al.*, 2006).

5.3.5 Parasitic infection

When infected with the malaria parasite, *Plasmodium falciparum*, erythrocytes express on their surface the adhesion protein *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1). The infected erythrocytes are isolated from blood circulation due to their adhesion to microvascular endothelial cells. This sequestration contributes directly to the pathology related to malaria: hemolysis of red blood cells and severe anemia. CD36 expressed on the surface of the endothelial cells are responsible for the adherence of erythrocytes (Ockenhouse *et al.*, 1989;Biggs *et al.*, 1990).

5.3.6 Long chain fatty acids

CD36 is also known as fatty acid translocase (FAT) for its ability to bind and internalize long chain fatty acids (LCFA) (Abumrad *et al.*, 1993). A comprehensive description of the effect of CD36 in LCFA uptake is presented in Section 6.3.3.

5.3.6.1 LCFA uptake

LCFA are a main source of energy and can diffuse passively through the cell membrane lipid bilayer; however, their transfer is more efficient by means of various fatty acid transporter proteins (FATP1 to 6), fatty acid binding protein (FABPpm) and CD36. The majority of free fatty acids (FFA) are coupled to albumin while the rest is transferred from lipoprotein particles and require transporters for rapid uptake (Bierbach *et al.*, 1979). In addition, they also play a secondary role in the coupling between FA uptake and their efficient use in various required biological processes including energy production (Glatz *et al.*, 2010). Compared to other FA transporters, the uptake of LCFA by CD36 in various tissues is a key event influencing general energy metabolism (Nickerson *et al.*, 2009;Hajri & Abumrad, 2002;Ehehalt *et al.*, 2006).

5.3.6.2 Orosensory detection of fatty acids

Following the detection of CD36 on gustatory cells, a potential role in the detection of fatty acids present in nutrients was therefore proposed (Fukuwatari *et al.*, 1997). However, for a proper detection, with the help of Von Ebner`s glands secreting lingual lipase, triglycerides are hydrolyzed to release fatty acids (Field *et al.*, 1989). It was recently shown that the freed FA then binds to CD36, causing an increase in $[Ca^{2+}]_i$ and a neurosensorial stimulation along with a rapid increase in the flux and protein content of pancreatic juice (El-Yassimi *et al.*, 2008;Gaillard *et al.*, 2008). Detection of fat in the mouth cavity by CD36, allows the digestive system to prepare for a fatty meal. A decrease in the expression of CD36 on gustatory cells resulted in the absence of satiety signals following a fatty meal and favored the development of obesity in rat and mice fed *ad libitum* (Schwartz *et al.*, 2008;Zhang *et al.*, 2010).

5.4 CD36 gene

The 46-kilobase gene encoding for CD36 is located on chromosome 7q11.2 (Fernandez-Ruiz *et al.*, 1993). As illustrated in Figure 4, CD36 gene is encoded by 15 exons; however,

only part of exon 3, exon 4 to 13, and part of exon 14 encode for the protein (Armesilla & Vega, 1994). The remaining exons form the 5' and 3' untranslated regions (UTRs.)

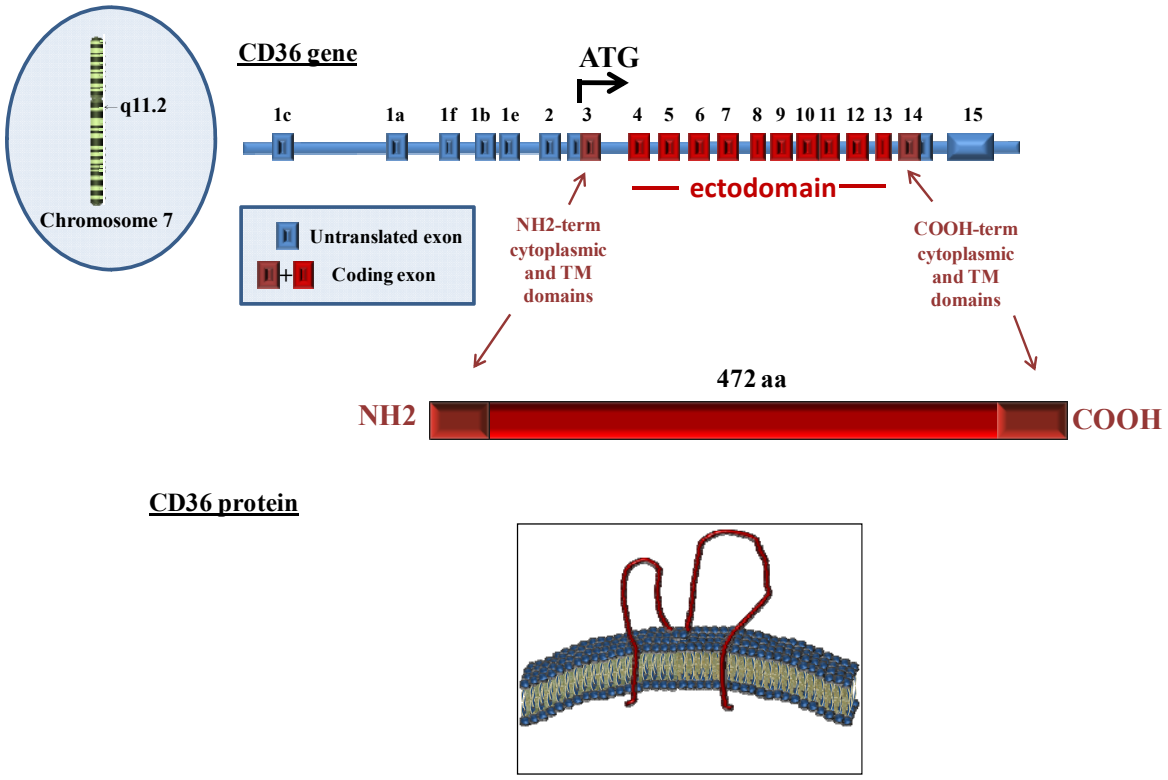


Figure 4. Schematic representation of CD36 gene and protein

5.4.1 CD36 alternative transcripts and mutations

Regulation of the expression of CD36 is complex and reflects the multifunctionality of this receptor. To date, there are 5 alternative first exons (exon 1a, 1b, 1c, 1e and 1f- Figure 4) that are controlled by 3 alternative promoters for which their usage is dependent on the tissue and the general physiological condition (Armesilla & Vega, 1994;Sato *et al.*, 2002;Zingg *et al.*, 2002;Noushmehr *et al.*, 2005b;Andersen *et al.*, 2006). For example, in THP-1 macrophages, all alternative transcripts of CD36 are upregulated in the presence of oxLDL (Andersen *et al.*, 2006). Alternative exon 1a is higher in adipose tissue followed by heart, skeletal muscle and monocytes and weakest in liver; while the transcript containing

exon 1b is higher in adipose tissue and monocytes compared to other tissues (Andersen *et al.*, 2006). In addition, treatment of mice with PPARα synthetic agonist WY-14,643, known to upregulate the expression of CD36, resulted in the differential expression of CD36 transcripts in a tissue-dependent manner (Motojima *et al.*, 1998;Sato *et al.*, 2007). In response to WY-14,643, exon 1a was increased in liver, skeletal muscle, adipose tissue and tongue. Exon 1c alternative transcript was increased in intestine, adipose tissue and tongue while exon 1b mRNA isoform remained unaffected. The induction of CD36 expression through one of its promoters in response to hormones and nutrients seems to be gender-dependent. The expression of CD36 in the liver is higher in females than in males possibly suggesting a hormonal regulation of CD36 (Cheung *et al.*, 2007). This particular study demonstrated that in female rats, a prolong fasting period had a repressive effect on CD36 expression in liver while having a stimulating effect in skeletal muscle; however in male rats no effect was observed in the liver. It is clear that these changes in the expression of CD36 and in the pattern of alternative first exon transcripts reflect the ability of the tissue to respond to precise physiological needs through the use of its different promoters. As stated earlier, while different transcripts of CD36 exist, they all contain the same coding sequence and therefore produce an identical CD36 protein.

In rare cases, functional diversity also exists for CD36 due to alternative splicing. A truncated CD36 protein was isolated from human erythroid leukemia (HEL) cells which resulted from the deletion of exons 4 and 5, producing a 369-aa protein missing the original amino acids 41 to 143 which included the phosphorylation site on CD36, three glycosylation sites, the TSP-1 binding site as well as a portion of PfEMP1 binding site (see the following section 5.5 on CD36 structure and ligand binding sites) (Tang *et al.*, 1994). It turns out that this deletion provided some protection against complications related to malaria. A polymorphism in the CD36 gene in which TG repeats in intron 3 caused a jump in the splicing of the pre-mRNA (Omi *et al.*, 2003). Table 3 (page 31) describes different reported mutations in the human CD36 gene. Nucleotide substitutions in the human gene have been reported in several studies (Kashiwagi *et al.*, 1993;Gelhaus *et al.*, 2001;Omi *et*

al., 2003;Imai *et al.*, 2002;Hanawa *et al.*, 2002;Aitman *et al.*, 2000;Lepretre *et al.*, 2004). Several other studies have reported deletions (Kashiwagi *et al.*, 1994) (Tanaka *et al.*, 2001) (Kashiwagi *et al.*, 2001) (Curtis *et al.*, 2002), short insertions (Tanaka *et al.*, 2001) (Kashiwagi *et al.*, 1996), duplications (Tanaka *et al.*, 2001), nucleotide rearrangements as well as repetitive sequences (Aitman *et al.*, 2000;Omi *et al.*, 2003) ; all capable of affecting expression or activity of CD36. Two types of deficiency have been given to CD36 solely based on their expression in monocytes and in platelets. Type I CD36 deficiency is defined by an absence of CD36 in monocytes and in platelets. Mutation C268T in exon 4 of CD36 is the most common mutation, responsible for 50% of mutated alleles in type I Asian population (Kashiwagi *et al.*, 1993;Kashiwagi *et al.*, 2001). This substitution causes a premature degradation of CD36. Although rare, other mutations such as 949insA or 329-330delAC were also reported to be the cause of a type I deficiency (Kashiwagi *et al.*, 1996;Kashiwagi *et al.*, 1994). Type II CD36 deficiency is defined by the absence of CD36 in platelets only. Type II is predominant in the African-American and Asian population (3 to 4% of each population) and is extremely rare in Caucasians (Take *et al.*, 1993;Yamamoto *et al.*, 1994). The molecular or genomic causes of type II deficiency are not well understood. Yuji Matsuzawa and his colleagues have done extensive work identifying several mutations in the Japanese population. They observed that cDNA samples from monocytes from two patients with type II deficiency were heterozygous for C268 and T268 (exon 4) while only the protein originating from the T268 isoform was found in their platelets suggesting that the expression of CD36 in platelets was controlled by other specific hereditary factors (Kashiwagi *et al.*, 1993;Kashiwagi *et al.*, 2001;Imai *et al.*, 2002).

Table 3. Exons, introns and mutations in the human CD36 gene

Exon number	Next intron length	mRNA nucleotides[a]	Amino acids encoded	Change in nucleotide sequence[b]	Change in amino acid sequence	Reference
1a (1c)	7341 (43708)[c]	−289 to −184 (−356 to −184)[c]	None			
2	470	−183 to −90	None	del *exons 1–3*	no expression of CD36 protein	(Curtis *et al.*, 2002)
3	9679	−89 to +120	1–40			
4	4362	121–281	41–94	*C268T*	Pro90Ser	(Kashiwagi et al., 1993)
5	1779	282–429	94–143	319–324delGCTGAG	inframe del AA 107–108	(Kashiwagi et al., 1994)
				329–330del *AC*	frameshift at AA 110	
				G367A	Glu123Lys	(Gelhaus et al., 2001)
				C380T	Ser127Leu	(Omi et al., 2003)
				T411C	Ala137Val	(Imai et al., 2002)
6	1236	430–609	144–203	*560insT*	frameshift at AA 187	(Tanaka et al., 2001)
7	1945	610–701	204–234	619–624del *ACTGCA* /ins *AAAAC*	frameshift at AA 207	(Kashiwagi *et al.*, 2001)
				691–696del *AAAGGT*	inframe del AA 231–232	(Aitman *et al.*, 2000)
8	3463	702–748	234–250	None reported	N/A	
9	954	749–818	250–273	*T760C*	Phe254Leu	(Hanawa *et al.*, 2002)
10	757	819–1006	273–336	845–849del *ACGTT*	frameshift at AA 282	(Aitman *et al.*, 2000)
				949ins*A*	frameshift at AA 317	(Kashiwagi *et al.*, 1996)
				T975G	Tyr325Term	(Aitman *et al.*, 2000)
11	729	1007–1125	336–375	*T1079G*	Leu360Term	(Lepretre *et al.*, 2004)
12	511	1126–1199	376–400	Del tttagAT	skipping *exon 12*	(Tanaka *et al.*, 2001)
				1140–1146delTTTACAA/ins*CCAAA*	frameshift at AA 380	(Aitman *et al.*, 2000)
				G1150C + 1155del*A*	Ala384Pro + frameshift at AA 385	
13	573	1200–1254	400–418	del *tattacagAG*	skipping *exon 13*	(Tanaka et al., 2001)
				dupl. 1204–1246	frameshift at AA 416	
				1218–1224del*GAGGAAC*	frameshift at AA 406	(Kashiwagi *et al.*, 2001)
				1228-1239del*ATTGTGCCTATT*	deletion of Ile-Val-Pro-Ile	
				A1237C	Ile413Leu	(Hanawa *et al.*, 2002)
14	2236	1255–1688	419–472			
15	—	1420–2044	None			

[a]Genbank NM 000072; the first mRNA nucleotide encoding CD36 protein is +1.
[b]Lowercase nucleotides are located in an intron.
[c]Alternatively spliced exon 1 (Genbank NM 001001547).
(Adapted from Rac *et al* ., Mol. Med. 13 (5 - 6): 288-296)

5.5 CD36 structure and post-translational modifications

CD36 nucleotide sequence predicts a 472-aa protein with a MW of 53kDa (Fernandez-Ruiz *et al.*, 1993). However, considerable post-translational modifications can generate a protein with a MW between 78 and 88 kDa, depending on the cell type and the level of modifications.

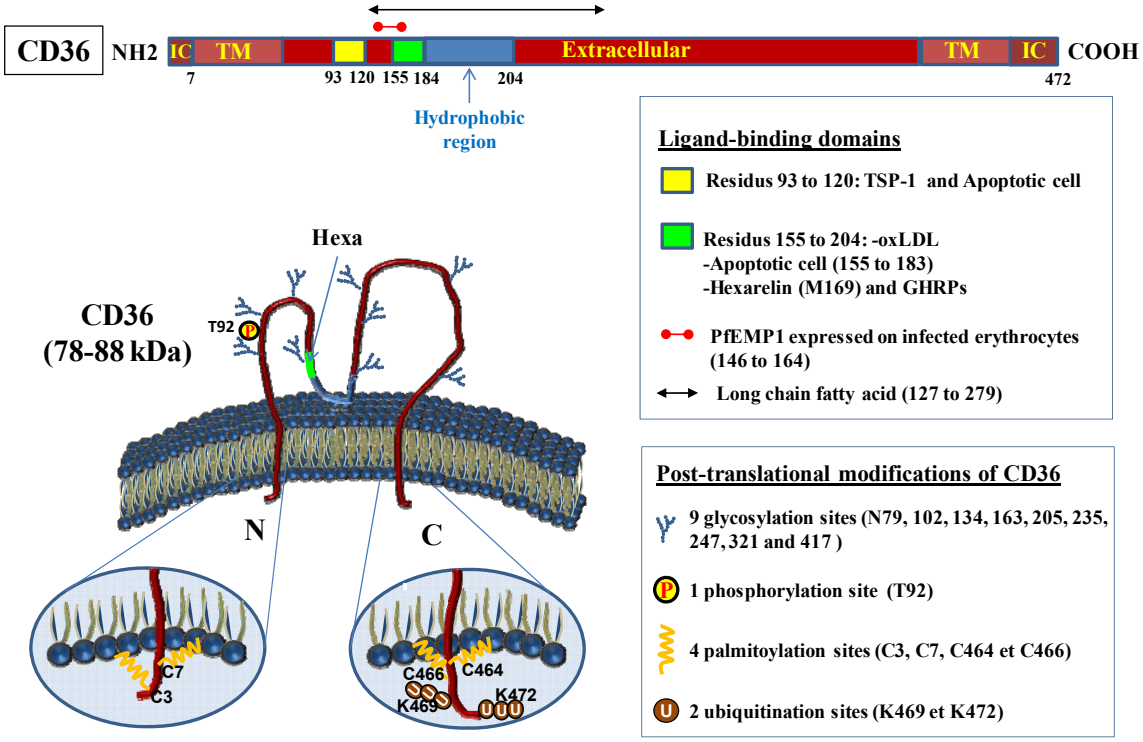


Figure 5. Schematic representation of CD3 protein, ligand-binding sites and post-translational modifications

The main structural features of scavenger receptor class B, CD36 are the large extracellular domain (ectodomain) flanked by transmembrane (TM) domains as well as short intracellular N- and C-terminal extremities (IC). As shown in Figure 5, the extracellular domain is approximatively 400-aa long and similarly to SR-BI, it is heavily N-glycosylated. Nine out of the ten possible N-glycosylation sites on asparagines residues (N-X-S/T, X≠P) were recently confirmed (Hoosdally *et al.*, 2009). Expression of recombinant CD36 in the insect cell line, *Spodoptera frugiperda* 21 (Sf21) provided an understanding on the role of glycosylation in the maturation of CD36 and its movement towards cell surface. Predicted N-glycosylation sites Asn-79, -102, -134, -163, -205, -235, -247, -321 and -417 were confirmed while no evidence of glycosylation was observed for Asn-220. In addition, glycosylation of CD36 was necessary for the efficient transport towards cell surface since

the non-glycosylated CD36 mutant was no longer located on the plasma membrane. Glycosylation of CD36 was not necessary for ligand binding, neither was it shown to influence the proper folding of CD36 protein. Contrary to SR-BI, which possesses two obligatory N-glycosylation sites for its transport to the cell surface, no similar sites were found for CD36 (Vinals *et al.*, 2003;Hoosdally *et al.*, 2009).

CD36 is constitutively phosphorylated on threonine 92 by an ectoprotein kinase and its level of dephosphorylation plays a role in the binding of certain ligands and the subsequent downstream molecular signaling (Asch *et al.*, 1993;Hatmi *et al.*, 1996). The binding of PfEMP1 expressed on the surface of *Plasmodium falciparum*-infected erythrocytes to CD36 on endothelial cells causes the dephosphorylation of CD36 by an ecto-alkaline phosphatase (Ho *et al.*, 2005). The dephosphorylated CD36 has a higher affinity for infected erythrocytes and this interaction is proposed to mimic its binding to TSP-1 (Asch *et al.*, 1993).

The N-terminal domain of CD36 contains approximately 7 aa while the C-terminal domain is ~13-aa long. Each extremity has two palmitoylated cysteine residues (Cys-3, -7, -464 and -466, Figure 5) allowing the tails to associate with the inner-layer of the cell membrane (Tao *et al.*, 1996). Palmitoylation of CD36 was recently suggested to regulate its post-transcriptional processing and maturation in the ER. Inhibition of CD36 palmitoylation by cerulenin (an antifungal antibiotic that inhibits fatty acid and steroid biosynthesis) or by mutation of its cysteine residues, caused a delay in the maturation of CD36 in the endoplasmic reticulum (ER), a decrease in its incorporation into lipid rafts, a reduction in its capacity to internalize oxLDL as well as a decrease in its half-life (Thorne *et al.*, 2010). Cytosolic lysines 469 and 472 are subjected to ubiquitination rendering CD36 susceptible to degradation (Smith *et al.*, 2008).

5.6 Binding sites on CD36

TSP-1 is an adhesive glycoprotein capable of binding among other proteins fibrinogen, collagen, fibronectin, integrin and also CD36 (Lahav *et al.*, 1982;Asch *et al.*,

1987;Taraboletti *et al.*, 1990). As stated in Section 5.3.1, the interaction CD36/TSP-1 plays a critical role in the inhibition of angiogenesis (Dawson *et al.*, 1997). Such as illustrated in Figure 5 (linear display of CD36), TSP binds to the region within residues 93 to 120 (in yellow) which is also known as the CLESH adhesion domain (CD36 LIMP-II Emp sequence homology) (Leung *et al.*, 1992;Frieda *et al.*, 1995;Simantov *et al.*, 2005). Regions 93 to 120 (yellow) and 155 to 183 (green) are involved in the recognition and endocytosis of apoptotic cells (Navazo *et al.*, 1996b;Ren *et al.*, 1995). PfEMP1 binds to the region between residues 146 and 164 (red rounded line) (Baruch *et al.*, 1999). Although the LCFA binding site on CD36 has not been characterized, comparison of the ectodomain of CD36 with that of the FA binding region on muscle fatty acid binding protein (M-FABP) showed a sequence homology as high as 73% with the region between residues 127 and 279 (black double arrowed line) (Prinsen & Veerkamp, 1996;Baillie *et al.*, 1996). Anionic phospholipids present on oxLDL particles are thought to be responsible for their binding to CD36 (Rigotti *et al.*, 1995). In 2002, Stanley Hazen and colleagues identified specific components on oxLDL capable of binding to CD36, oxidized phosphatidylcholine called $oxPC_{CD36}$ (Podrez *et al.*, 2002a;Podrez *et al.*, 2002b). Lysines 164 and 166 are indispensable to CD36's binding to $oxPC_{CD36}$ due to its electrostatic properties in which the negatively charged oxidized phospholipids interact with the positively charged lysine residues within the oxLDL binding region 155-204, shown in Figure 5, in green (Kar *et al.*, 2008;Navazo *et al.*, 1996a). Protein residue regions 28-93 and 120-155 also seemed to be important for oxLDL binding (Pearce *et al.*, 1998). Lysine 472 (K472) in the C-terminal cytosolic region of CD36 was shown to be important in the binding, internalization and degradation of oxLDL (Malaud *et al.*, 2002). Endocytosis of oxLDL following its binding to CD36 on macrophages requires the presence of lipid rafts and dynamin proteins but does not seem to involve clathrine or caveolae (Sun *et al.*, 2007a;Zeng *et al.*, 2003). And finally, hexarelin's binding site on CD36 was identified and spanned from asparagine 132 to glutamic acid 177; more particularly, the presence of methionine 169 was necessary for hexarelin interaction with CD36 (Demers *et al.*, 2004). This region overlaps with the

binding site of oxLDL and explained in part the anti-atherogenic effect of hexarelin which blocked the binding of oxLDL to CD36 (Demers *et al.*, 2004;Avallone *et al.*, 2006).

6 Regulation of CD36

The present section will discuss the regulation of CD36 through gene expression changes by various transcriptional regulators (Section 6.1) but also by factors influencing its mobilization (section 6.2). Since more is known about the regulation of CD36 and its impact in atherosclerosis, much of this section will discuss changes in CD36 expression in macrophages therefore providing an insight in the key regulators of CD36 expression. Although more limited, factors reported to influence the expression of CD36 in adipocytes and hepatocytes will be presented as well.

6.1 Regulation of CD36 gene expression

As mentioned previously, much of the regulation of CD36 expression depends on the tissue, physiological condition, gender and promoter usage.

6.1.1 In atherosclerosis

The increase in the expression of CD36 in macrophages, endothelial cell and VSMC is often associated with formation of atherosclerotic plaques. The role that CD36 plays in atherosclerosis is mainly due to its binding and internalization of oxLDL particles.

6.1.1.1 Nuclear receptors in monocytes/macrophages

The adhesion of monocytes to endothelial cells stimulates the expression of CD36 on these leukocytes. Their subsequent differentiation into macrophages stimulates furthermore its expression (Prieto *et al.*, 1994;Huh *et al.*, 1996). The presence of scavenger receptors on macrophages such as CD36 assures a proper endocytosis and clearance of such particles in the subendothelial space (Han *et al.*, 1997). The nuclear family peroxisome proliferator-activated receptor (PPAR) contains 3 members: PPARα, PPARβ/δ and PPARγ

(Laudet *et al.*, 1999). They form heterodimers with the versatile retinoid X receptor (RXR) and activate transcription by binding to their response element (PPRE) on the promoter of target genes mainly involved in lipid metabolism and energy balance (Yessoufou & Wahli, 2010). The mechanism of action of PPARγ will be further presented in Section 8. The internalization and degradation of oxLDL by monocytes/macrophages provides ligands to PPARγ, and more specifically the content of this cargo such as 9- and 13-hydroxyoctadecadienoic acid (9- and 13-HODE) are known potent activators (Nagy *et al.*, 1998) . The activation of PPARγ induces important transcriptional changes in genes, including CD36, involved in the differentiation of monocytes into macrophages and eventually into foam cells (Tontonoz *et al.*, 1998). The degree of oxidation of oxLDL lipids will influence the level of activation of nuclear receptors. The minimally and moderately modified LDL (mmLDL) particles had a greater influence on the expression of CD36 in cultured human monocyte-derived macrophages while extensively oxidized LDL had no effect on CD36 expression (Kavanagh *et al.*, 2003). Using electrophoretic mobility shift assay (EMSA), the DNA binding activity of PPARs was shown to be higher in mmLDL-exposed macrophages. PPARγ-deficient stem cells were capable of differentiating into macrophages therefore demonstrating that PPARγ is not vital to macrophage differentiation (Moore *et al.*, 2001). While the basal expression of CD36 is dependent on the presence of PPARγ, the sole activation of RXR (LG268 or retinoic acid agonists) was capable of increasing the expression of CD36 even in absence or through inhibition of PPARγ activity, demonstrating that other nuclear receptors associated with RXR are capable of controlling CD36 expression as well (Moore *et al.*, 2001;Han & Sidell, 2002). Indeed, PPARα seems also to upregulate CD36 in macrophages since exposure to cholesterol ester hydroperoxides (CEOOH) found in mmLDL increases the binding of PPARα to the PPRE site on the proximal promoter of CD36 (Jedidi *et al.*, 2006). PPARβ/δ was found to be ubiquitously expressed while its role remained mainly unknown for more than a decade (Schmidt *et al.*, 1992). It turns out that PPARβ/δ possesses properties that seemed to combine both some of

the positive effects of both PPARγ and PPARα, and is today considered also as a potential therapeutic target for treating metabolic syndrome-related diseases (Barish *et al.*, 2006). PPARβ/δ mediates the macrophages-derived inflammatory response (Lee *et al.*, 2003). Its expression was found to be increased during macrophage differentiation and following treatment of cells with its synthetic agonist, compound F, PPARβ/δ activation resulted in the increase in CD36 expression (Vosper *et al.*, 2001). Another nuclear receptor, farnesoid X receptor (FXR) was also shown to heterodimerize with RXR (Forman *et al.*, 1995a). Following binding of bile acid, FXR/RXR negatively and positively regulates many of its target genes (Makishima *et al.*, 1999;Parks *et al.*, 1999). FXR is a regulator of cholesterol homeostasis, triglyceride synthesis and lipogenesis. Its expression is elevated in liver, intestine and kidney but it is also found at lower levels in other tissues and cell types such as leukocytes. One of the side-effects of ritonavir, a protease inhibitor against HIV, is dyslipidemia which threatens patients with cardiovascular diseases such as atherosclerosis (Periard *et al.*, 1999). The accumulation of plaques and cell foam formation was shown to be associated with an elevation in the expression of CD36 (Dressman *et al.*, 2003). A recent study demonstrated that the activation of FXR by one of the major bile acids, chenodeoxycholic acid (CDCA) in the atherosclerotic mouse model ApoE-/- treated with ritonavir, resulted in a marked decrease in atherosclerotic plaque formation as well as an attenuated expression of CD36 in circulating monocytes (Mencarelli *et al.*, 2010). This effect on CD36 expression was also observed in cultured macrophages. The testicular orphan nuclear receptor 4 (TR4) also functions as a sensor for fatty acid and regulates glucose and lipid metabolism (Liu *et al.*, 2007). TR4 is activated by polyunsaturated fatty acids (PUFAs) and retinoids but does not form a heterodimer with RXR (Zhou *et al.*, 2011). TR4 induces the expression of CD36 by binding to its response element TR4RE found on the promoter of CD36 and contributes also to foam cell formation (Xie *et al.*, 2009).

6.1.1.2 Cytokines and inflammation

Inflammatory response is an essential component of the immune response to pathogens and damaged cells. Cytokines are secreted by macrophages, monocytes, T lymphocytes, platelets, endothelial cells and VSMC. Cytokines include tumor necrosis factor alpha (TNF- α), interleukins (ILs), chemokines, interferons (IFNs), tumor growth factors (TGFs), as well as colony stimulating factors (CSFs); all of which contributes to the proper control of the immune response. IL-4, secreted by T lymphocytes, increased the activity of macrophage 12/15-lipoxygenase, which synthesizes 13-hydroxyoctadecadienoic acid (13-HODE) and 15-hydroxyeicosatetraenoic acid (15-HETE) from linoleic and arachidonic acids, respectively (Huang *et al.*, 1999). Therefore, this activation via IL-4 was shown to provide ligands for PPARγ and induce expression of CD36 in a PKC-dependent manner (Feng *et al.*, 2000). Other cytokines such as TGF-β, IFN-γ and IL-10 also had an effect on CD36 expression. In monocytes, IFN-γ and TGF-β decreased the expression of CD36 (Nakagawa *et al.*, 1998;Han *et al.*, 2000b); while IL-10 increased both the expression of CD36 and ABCA1, facilitating the uptake but also the clearance of cholesterol by macrophages (Han *et al.*, 2009).

6.1.1.3 Other factors

Many different factors other than nuclear receptors were also identified as potential regulators of CD36 expression. The transcription factor Runt-related 3 (RUNX3) participates in the transcriptional reprogrammation of the dendritic cells after pathogen and apoptotic cell recognition and was shown to be a negative regulator of CD36 (Puig-Kroger *et al.*, 2006). The immunosuppressant drug, cyclosporine A (CsA), is associated with the development of atherosclerosis. A marked increase in PPARγ and CD36 was observed in THP-1 macrophages treated with CsA (Jin *et al.*, 2004). Aspirin increases expression of CD36, SR-BI and ABCA1 in cultured macrophages (Vinals *et al.*, 2005). Phytohemagglutinin (PHA) is a lectin commonly found in legumes which can be toxic at high levels. PHA induces mitosis and affects cell membrane permeability; it is known to

cause agglutination of red blood cells. PHA induces the expression of CD36 in lymphocytes which is shown to be partially dependent on phosphoinositide 3-kinase (PI3K) activation (Tassone *et al.*, 1998). The expression of CD36 in monocytes from diabetic patients is high, increasing their risk of developing atherosclerosis (Sampson *et al.*, 2003). Hyperglycemia induces oxidative stress in diabetic patients. High glucose is known to induce oxidation of LDL particles which contributes furthermore to oxidative stress and microvascular endothelial cell dysfunction. Glucose was shown to increase the expression of CD36 in endothelial cells making the diabetic patient more susceptible to cardiovascular complications related to diabetes (Griffin *et al.*, 2001;Farhangkhoee *et al.*, 2005). Nuclear factor E2-related factor 2 (Nrf2) is usually a key transcription factor in the anti-oxidative response and was found to be activated by oxLDL. More precisely, 4-hydroxynonenal (HNE), a lipid peroxidation product found in oxLDL, is a potent activator of nuclear tanslocation of Nrf2. In absence of Nrf2, it was shown that macrophage CD36 expression is only partially increased in response to oxLDL (Ishii *et al.*, 2004). Nrf2 regulates directly the expression of CD36 by binding to its anti-oxidant response element, ARE on the promoter upstream of exon 1a (Maruyama *et al.*, 2008). Despite its role in the anti-oxidative response, it was recently given a pro-atherogenic role since it was observed that in double deficient ApoE/Nrf2 mice, a decrease in CD36 expression coincided with a decrease in plaque formation (Barajas *et al.*, 2011).

6.1.1.4 Statins

Statins are inhibitors of the rate-controlling enzyme, HMG-CoA reductase (HMGR) and are prescribed for the treatment of hypercholesterolemia. However, interesting pleiotropic effects are observed for statins which were independent of changes in serum cholesterol such as reduced vascular inflammation and decreased VSMC migration and proliferation (Sadowitz *et al.*, 2010). Statins also decrease cell proliferation and oxLDL uptake by monocytes and macrophages (Senokuchi *et al.*, 2005). In addition, several studies have shown a decrease in CD36 expression in statin-treated monocytes and macrophages

adding to their anti-atherogenic properties (Hrboticky *et al.*, 1999;Han *et al.*, 2004;Mandosi *et al.*, 2010).

6.1.2 Adipose tissue and adipocytes

Studies conducted on CD36 in tissues and cell types outside the realm of atherosclerosis, such as adipose tissue are more limited. However, in recent years a greater interest in the impact of CD36 on metabolic disorders has given rise to many publications. In adipose tissue and adipocytes, reports have focused mainly on the role of CD36 in the uptake of fatty acids. PPARγ regulates the differentiation program in adipocytes and is a well known transcriptional regulator of CD36 (Teboul *et al.*, 2001). Activation of PPARγ by glitazones in mouse adipose tissue is also capable of further increasing CD36 expression (Sato *et al.*, 2002). A more complete description of adipocyte differentiation is presented in Section 7.2. The differentiation of pre-adipocytes involves the induction and activity of particular transcription factors in a time-specific manner. PPARγ is a key regulator of adipocyte differentiation but its own expression depends on the induction of CCAAT/enhancer-binding protein (C/EBP) β and δ (Wu *et al.*, 1995). Alone or together with PPARγ, C/EBP α is capable of controlling the expression of many genes involved in adipocyte function (Gregoire *et al.*, 1998). Recently, C/EBP α was shown to also regulate the expression of CD36 in 3T3-L1 (Qiao *et al.*, 2008). Fibrates, PPARα agonists, are hypolipidemic agents used for lowering cholesterol and triglyceride plasma levels. Fibrates were shown to increase expression of CD36 in adipocytes and to increase fatty acid oxidation by inducing the expression of muscle carnitine palmitoyltransferase I (M-CPT-1) and oxidative phosphorylation uncoupling proteins 2 and 3 (UCP-2 and -3) (Cabrero *et al.*, 2001;Zhao *et al.*, 2004). Nrf2 increased the expression of CD36 in 3T3-L1 adipocytes exposed to oxLDL as well (D'Archivio *et al.*, 2008). Glucocorticoids modulate glucose homeostasis and lipogenesis in adipocytes (Sakoda *et al.*, 2000;Berdanier, 1989). A recent study on dexamethasone-treated differentiated 3T3-L1 adipocytes showed an increase in the expression of CD36, and chromatin immunoprecipitation sequencing (ChIP-Seq)

experiments performed on these samples enabled the identification of glucocorticoid receptor binding regions near several genes involved in TG homeostasis and lipid transport including the CD36 gene (Yu *et al.*, 2010). In a diabetic rat model, it was shown that the expression of CD36 was increased in many organs including adipose tissue while insulin injection was able to normalize its expression (Chen *et al.*, 2006). In comparison, oral administration of vanadate, which corrects hyperglycemia without affecting insulin levels, had a similar effect, suggesting that glucose was responsible for the modulation of CD36 expression. LCFA also has an effect on CD36 expression. LCFA is capable of inducing CD36 expression in pre-adipocytes while reducing its expression in differentiated cells (Sfeir *et al.*, 1997;Yang *et al.*, 2007).

Phloretin, a dihydrochalcone that belongs to the class of flavonoids found in apple leaves, are potent inhibitor of FA uptake (Abumrad *et al.*, 1981). Treatment of 3T3-L1 adipocytes with phloretin increased the expression of CD36, although probably as a compensatory mechanism to a decrease in FA uptake (Hassan *et al.*, 2007). In rodents, diet- or cold-induced thermogenesis occurs in brown adipose tissue (BAT) to burn off excess fat and/or to produce heat (section 7.8.3). Cold exposure increases the expression of CD36 in BAT; and its activity in combination with that of LPL is crucial for the clearance of TG from the circulation and during thermogenesis (Bartelt *et al.*, 2011).

6.1.3 Liver and hepatocytes

Differential regulation of CD36 is suggested between liver and adipose tissue in response to PPARα and PPARγ agonists. WY-14,643 (PPARα) increases the expression of CD36 in rat hepatoma cell line, Fao (Motojima *et al.*, 1998). WY-14,643 and clofibrate also increase the expression of CD36 in mouse liver and intestine. This effect was dependent on PPARα since no effect was seen in PPARα-null mice. While PPARγ regulates CD36 expression in adipose tissue, troglitazone (PPARγ) had no effect on CD36 expression in Fao cells. The differential regulation of CD36 between PPAR agonists seen in liver can be explained by selective promoter occupation by PPAR α and γ in a tissue-dependent manner

(Sato *et al.*, 2002). Non-alcoholic fatty liver disease (NAFLD) regroups a wide spectrum of liver diseases with abnormal retention of lipids, and is associated with diseases related to the metabolic syndrome such as obesity, dyslipidemia and diabetes (Postic & Girard, 2008). The extreme form of NAFLD, non-alcoholic steatohepatitis (NASH) leads to inflammation and cirrhosis. In fatty liver, the expression of CD36 is elevated compared to normal liver; and in patients that develop hepatic steatosis, hepatocyte apoptosis was found to be associated with high CD36 expression (Greco *et al.*, 2008;Bechmann *et al.*, 2010). Nuclear receptors, PPARγ, LXRα, LXRβ and pregnane X receptor (PXR) are associated with the accumulation of hepatic TG (Inoue *et al.*, 2005;Lee *et al.*, 2007;Zhou *et al.*, 2006). Their respective response elements were all found in the promoter regions of CD36, establishing this gene as a common target in the promotion of liver steatosis (Zhou *et al.*, 2008). PPARγ coactivator-1-beta (PGC-1β) co-activates SREBPs and stimulate lipogenic gene expression contributing to hepatic lipid synthesis. However, the increased expression of PGC-1β due to a high fat diet (HFD) results ironically in the reduction of hepatic fat accumulation (Lin *et al.*, 2005). This is described by an increase in circulating TG and cholesterol (VLDL) influenced by PGC-1β co-activation of LXRα and stimulation of lipoprotein transport. Its overall action leads to hyperlipidemia and atherosclerosis. Hepatic overexpression of PGC-1β alone was sufficient to induce hyperlipidemia while decreasing the expression and activity of PPARα and increasing the expression of CD36 (Lelliott *et al.*, 2007). In rodents on a HFD, treatment with the PPARβ/δ agonist, NNC61-5920, attenuated hepatic insulin resistance and decreased the expression of SCD-1, LPL and CD36 (Ye *et al.*, 2011). Hepatic stellate cells are specialized pericytes that occupy the perisinusoidal space (between the sinusoidal blood vessel and hepatocytes) and are responsible to storing retinoids derived from vitamin A in their intracellular lipid vesicles. Esterification of vitamin A is dependent on the newly formed esters in lipid vesicles and therefore dependent on LCFA uptake (Moriwaki *et al.*, 1988). Treatment of activated stellate cells with another PPARβ/δ agonist, L165041 further induced CD36 expression (Hellemans *et al.*, 2003). The Aryl hydrocarbon receptor (AhR) belongs to the family of basic-helix-loop-helix

transcription factors and plays a role in hepatic growth and development (Schmidt *et al.*, 1996). Activation of AhR induces spontaneous hepatic steatosis which is explained in part by the binding of AhR to its element on CD36 promoter and the resulting increased in its expression (Lee *et al.*, 2010).

6.2 CD36 localization

6.2.1 In lipid rafts and caveolae

Lipid rafts are organized in dynamic microdomains that travel freely within the cell membrane. Rafts are composed of sphingolipids and cholesterol in the outer-layer connected with phospholipids and cholesterol of the membrane's inner-layer. Permanent lipid raft residents are also found such as glycophosphatidylinositol (GPI-anchored) proteins, Gα subunits of heterotrimeric G proteins and tyrosine kinases of the Src family (Src, Fyn, Lyn, Yes). Certain members are temporary residents, dependent on the presence of their ligand. Cholesterol serves as a spacer between saturated hydrocarbon chains of sphingolipids and helps in maintaining components of the lipid raft in close proximity to each other (Simons & Toomre, 2000). Cholesterol depletion or inhibition of sphingolipids causes a disassembly of lipid rafts and proteins (Ehehalt *et al.*, 2003;Ehehalt *et al.*, 2008).

Caveolae are a sub-type of lipid rafts represented as small invaginations formed by the polymerization of their palmitoylated integral membrane proteins, caveolins (1, 2 or 3), which also bind cholesterol (Smart *et al.*, 1999). Caveolae are present on various cell types and are particularly abundant on adipocytes (Scherer *et al.*, 1994). Lipid rafts play a central role in several cellular processes such as membrane trafficking, cellular polarization and signal transduction, regulating cell growth and survival, involved in the defence against pathogens but also in the uptake of glucose and fatty acid (Janes *et al.*, 2000;Kolesnick, 2002;van der Goot & Harder, 2001;Ortegren *et al.*, 2007). CD36 is detected in lipid-raft- and/or caveola-rich fractions (Lisanti *et al.*, 1994). CD36 distribution on cellular membrane of CHO cells and human melanoma C32 cells is more homogeneous compared

to caveolin-1, which has a punctuated cell distribution due to its presence in caveolae (Zeng *et al.*, 2003). However, isolation of lipid rafts yielded samples containing CD36; suggesting that in these cell types, CD36 was associated with lipid rafts but not with caveolae. This same study showed that endocytosis of oxLDL via CD36 requires neither caveolin-1 nor clathrin (protein involved in endocytosis of coated vesicles). Endosomal structures containing CD36 and oxLDL were devoid of caveolin-1 and transferrin but contained the lipid raft protein, GPI-anchored protein decay accelerating factor, DAF. Endocytosis of oxLDL by CD36 was however dependent on dynamin, a GTPase playing a role in endocytosis in lipid rafts, clathrin-coated vesicles and caveolae (Sun *et al.*, 2007a). In other cell types, such as in pneumocytes, and transfected COS-7, HEK-293 and CHO cells, studies have shown that CD36 can be co-localized and even associated with caveolin-1 (Scherer *et al.*, 1995b;Frank *et al.*, 2002;Eyre *et al.*, 2008). Moreover, the muscle specific caveolin-3 was found to be co-localized with CD36 in human skeletal muscle (Vistisen *et al.*, 2004). Caveolin-1 is particularly expressed in adipocytes and is thought to play a role in FA uptake (Scherer *et al.*, 1994;Trigatti *et al.*, 1999). The interrelationship between CD36 and caveolins seems to be crucial in the internalization of LCFA. In caveolin-1-deficient mouse embryonic fibroblasts (MEFs), CD36 was no longer present on the cell surface and FA uptake was greatly affected (Ring *et al.*, 2006). Adenoviral expression of caveolin-1 in these cells redirected CD36 to the cell membrane and rescued FA uptake. Caveolin-1-deficient mice have a dramatic reduction in aortic CD36 expression and share similar phenotypes with CD36-null mice such as elevated circulating TG and FA, and a reduced clearance of TG while in contrast being protective against the development of aortic atheromas by reducing oxLDL uptake (Razani *et al.*, 2002;Frank *et al.*, 2004).

Src kinases have been found to associate with the C-terminal extremity of CD36 in platelets and in microvascular endothelial cells (Huang *et al.*, 1991;Bull *et al.*, 1994). Linoleic acid binds to CD36 on gustatory cells inducing phosphorylation and activation of Src family of kinases (Fyn and Yes) and the downstream signaling cascade involved in the orosensory detection of fatty acid (FA) (El-Yassimi *et al.*, 2008). While CD36 is not a

target of Src kinases, several reports have shown that caveolin-1 is targeted by Src kinases in response to variety of growth factors such as VEGF, EGF and PDGF (Li *et al.*, 1996;Fielding *et al.*, 2004;Kim *et al.*, 2000;Labrecque *et al.*, 2003). Palmitoylation of caveolin-1 on Cys-156 is essential for its interaction with Src and its subsequent phosphorylation on Tyr-14 (Lee *et al.*, 2001). In addition, the mere presence of CD36 on caveolae was sufficient to abrogate caveolin-1 phosphorylation by Src. Overall it seems that the presence of caveolin-1 is necessary for CD36 localization to the cell surface; while inversely, CD36 has an inhibitory effect on Src-mediated phosphorylation of caveolin-1, potentially having an effect on growth. Palmitoylation of CD36 has also been shown to influence the localization of CD36 on lipid rafts/caveolae and affecting FA uptake (Thorne *et al.*, 2010).

Different subclasses of caveolae have been isolated in adipocytes based on their protein content. A subclass of caveolae on adipocytes was suggested to be specialized in FA uptake and conversion to TG; and harbored many proteins involved in these processes such as fatty acid transport protein-1 and -4 (FATP-1 et -4), fatty acyl-CoA synthetase (FACS), hormone-sensitive lipase (HSL), perilipin and glucose transporter-4 (GLUT4); however, CD36 was not mentioned as being a member of this subclass (Örtegren *et al.*, 2006). Two populations of CD36 have been found on cells, a raft-associated one which leads to FA uptake in a cholesterol/sphingolipid-dependent manner and another population dissociated from lipid rafts. Cross-linking experiments of CD36 with GPI-anchored protein placental alkaline phosphatase (PLAP) resulted in the marked internalization of FAs, reaching the conclusion that FA uptake by CD36 is increased when associated to lipid rafts (Ehehalt *et al.*, 2008). Interestingly, FATP-4 and CD36 were not found to be co-localized which suggests that the previously mentioned subclass involved in TG storage containing FATP-4 might not include CD36 or an absolute need for CD36 in TG synthesis. Therefore, the presence of CD36 on lipid rafts/caveolae seems to be essential to its role in FA uptake but not necessarily in TG synthesis.

In skeletal muscle and heart, CD36 is present in intracellular compartments and studies have reported that its translocation to the cell surface is stimulated by muscle contraction and insulin (Bonen *et al.*, 2000;Schwenk *et al.*, 2008). No direct association of CD36 with caveolins have been reported in hepatocytes; however, a recent study indicated that overexpression of caveolin-1 increased oxLDL uptake by CD36 (Truong *et al.*, 2009).

6.2.2 Mitochondrial CD36

LCFA must enter the mitochondrion to be oxidized and for ATP to be produced. In 2004, Arend Bonen and colleagues stated that the inhibition of carnitine-palmitoyl transferase-1 (CPT-1) by malonyl CoA was not sufficient to explain the changes in FA transport especially when energy demand dramatically increased such as during an intensive exercise. Therefore, they proposed the existence of an alternative LCFA transportation system. They identified fractions of purified mitochondria containing CD36 which also co-immunoprecipitated with CPT-1 (Campbell *et al.*, 2004). In addition, they demonstrated that thirty minutes of electrical stimulation of hindlimb muscles did not increase CD36 expression but increased the presence of CD36 in mitochondria. Since then, several studies have confirmed the presence of CD36 in mitochondria; however, its influence on FA oxidation remains controversial. Maria Febbraio and colleagues also isolated mitochondria from skeletal muscle and heart in CD36-null mice and compared the mitochondrial respiration with isolated mitochondria from wild-type mice and found no differences between groups (King *et al.*, 2007). Arend Bonen's team used the same experimental approach but challenged the muscles with electrical stimulation. While they observed no differences between the groups at rest, they did detect a decrease in mitochondrial respiration in the CD36-deficient challenged group (Holloway *et al.*, 2009).

6.3 Ligand-dependent signaling pathways and CD36 movement

The intracellular extremities of CD36 are relatively short, with no reported kinase or phosphatase activity, no interaction with GTPases and no known scaffolding domains.

Regardless of those facts, several studies have shown that CD36 is capable of activating various signaling pathways depending on its ligand and on the cell type in which CD36 is expressed. The response of CD36 to its ligand possibly involves its C-terminal cytoplasmic tail since mutations or Tyr-463 and Cys-464 results in the loss of its phagocytic potential in response to S.*aureus* (Stuart *et al.*, 2005). LTA present on the Gram-positive bacterium binds CD36 and causes the association of CD36 with TLR2/TLR6 resulting in the secretion of pro-inflammatory cytokines; while in the Tyr-463/Cys-464 mutants this response is lost (Triantafilou *et al.*, 2006). A common factor among CD36 signaling in platelets, macrophages, microsvascular endothelial cells, microglial cells and gustatory cells is the interaction of its C-terminal tail with Src family kinases, Fyn, Lyn and Yes (Huang *et al.*, 1991;Moore *et al.*, 2002;El-Yassimi *et al.*, 2008). This association is important in apoptosis, cell migration, inflammation, and foam cell formation. Although much is still unknown about the detailed mechanism(s) of action of CD36, the following sub-sections will focus on reported signaling cascades involving specific ligands. Figure 6 illustrates some of the ligand-specific binding to CD36 and the resulting downstream signaling events.

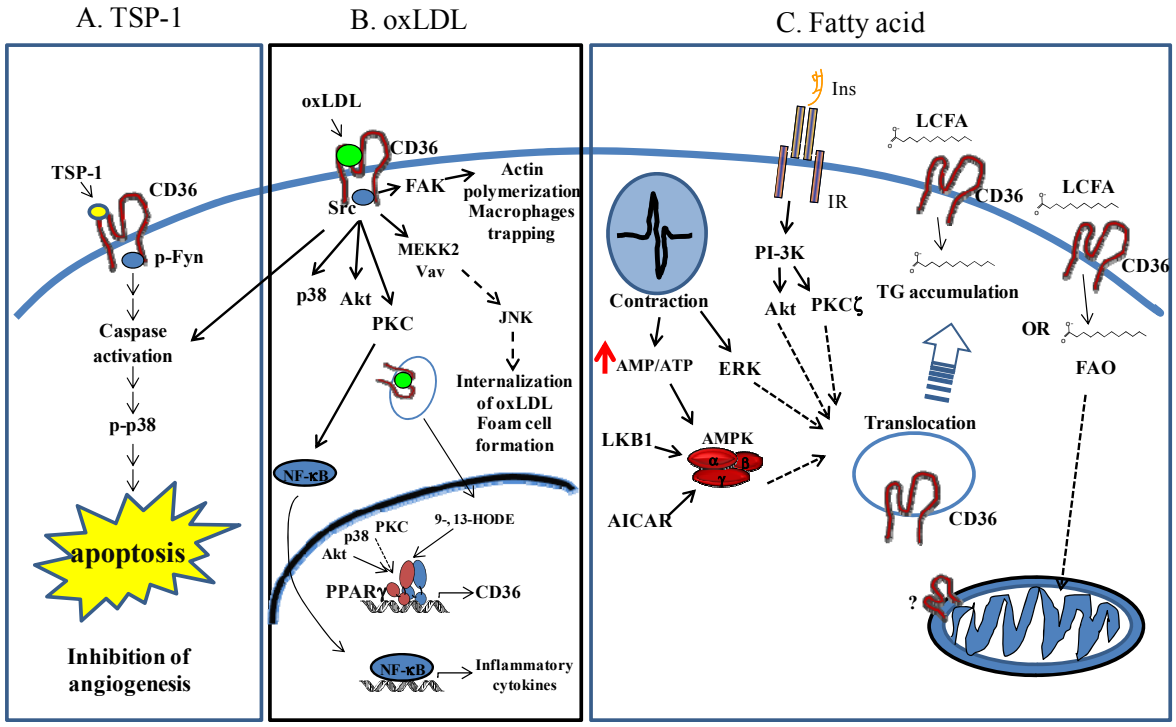


Figure 6. CD36 ligand-specific downstream events

6.3.1 TSP-1/platelets

When TSP-1 binds to CD36 on endothelial cells, an association with, and phosphorylation of Fyn occurs, which in turn results in the phosphorylation of caspases and MAP kinase p38 inducing cellular apoptosis and inhibition of angiogenesis, Figure 6A (Jimenez *et al.*, 2000).

6.3.2 LDLox/macrophages

Activation of CD36 on macrophages by oxLDL involves Src kinase, Lyn but also MAPKs, JNK and p38 (Figure 6B). Roy Silverstein and colleagues have shown by co-immunoprecipitation experiments and pull-down assays that CD36 formed a complex with Lyn and MEKK2, and that activation of JNK via CD36 was necessary for oxLDL uptake and foam cell formation (Rahaman *et al.*, 2006). Members of the Vav family of guanine

nucleotide exchange factors (GEFs) were recently shown to interact with Lyn and are considered key player(s) in foam cell formation (Rahaman *et al.*, 2011). Activation of Src amplifies CD36 response to oxLDL in turn by activating numerous signaling pathways. For example, Src activates focal adhesion kinase (FAK) inducing actin polymerization, macrophage spreading while inhibiting cell migration from the intima (Park *et al.*, 2009). The interaction CD36/oxLDL on foam cells can also induce apoptosis via activation of caspase-3 (Wintergerst *et al.*, 2000). As previously mentioned, internalization of oxLDL components, such as 9- and 13-HODE, serve as ligands to PPARγ and its activation in turn increases the expression of CD36 causing a positive autoregulatory loop with increased foam cell formation (Nagy *et al.*, 1998) . In addition, the binding of oxLDL to CD36 activates various other kinases shown to have a direct effect on PPARγ in a ligand-independent manner. Indeed, several kinases such as Akt, PKC and p38 were activated in response to oxLDL and were shown to affect PPARγ activity (Munteanu *et al.*, 2006;Feng *et al.*, 2000;Zhao *et al.*, 2002). PKC also targets NF-κB which responds to oxLDL by stimulating the transcription of inflammatory cytokines (Han *et al.*, 2000a). Extracellular signal-regulated protein kinases 1 and 2 (Erk1/2) are also activated in response to oxLDL but in macrophages were not dependent on the presence of CD36 (Rahaman *et al.*, 2006). However, activated Erk1/2 is capable of phosphorylating and inhibiting PPARγ (Hu *et al.*, 1996). Phosphorylation of PPARγ by Erk is further discussed in Section 8.3. Activation of PPARγ is normally considered to have an anti-inflammatory effect; however, its activation becomes pro-inflammatory and pro-atherogenic in the context where oxLDL is abundant and macrophages overwhelmingly try to clear these particles from the intima.

6.3.3 Fatty acids

Studies on the signaling events via CD36 are often centered around its interaction with oxLDL due to its impact on atherosclerosis. However, emerging reports on signaling events involving LCFA show certain similarities with oxLDL while others depict a very different mechanism of action.

6.3.3.1 Fatty acids and gustatory cells

Binding of linoleic acid to CD36 on gustatory cells induces the phosphorylation of Src kinases, Fyn and Yes (El-Yassimi *et al.*, 2008). This activation led to an increase in $[Ca^{2+}]_i$ and secretion of neurotransmitters,5-hydroxytryptamine and noradrenaline. It is possible that in other cell types involved in FA uptake, Src kinases play a role in the response of CD36 to LCFA. However, studies have mainly focused on the mechanisms involved in LCFA internalization and CD36 movement.

6.3.3.2 Fatty acids and muscles

Studies on the role of CD36 in cardiac and skeletal muscles are focused on the events surrounding LCFA influx, FA homeostasis and energy production. The heart takes 70% of its energy from oxidation of fatty acids while the remaining originates from glucose, lactose and pyruvate oxidation (Stanley *et al.*, 2005). However, in pathophysiological conditions such as ischemia, hypoxia and diabetes, the heart turns towards the hypoxic foetal program in which glucose becomes the main source of energy (Taegtmeyer *et al.*, 2010). In the heart and skeletal muscle, the majority of LCFA are taken up by CD36 (Luiken *et al.*, 2002a;Bonen *et al.*, 1998). An important mechanism regulating fatty acid uptake by CD36 is the translocation of CD36 from intracellular compartments to the cell surface of muscle cells called the sarcolemma (Bonen *et al.*, 2000;Luiken *et al.*, 2002a). In the non-stimulated cardiomyocytes, CD36 distribution between the sarcolemma and the endosomes is relatively equal (Luiken *et al.*, 2004). Translocation of CD36 and transport of LCFA in the cell are increased within minutes following the first contractions (Bonen *et al.*, 2000;Luiken *et al.*, 1999). Certain chronic conditions can influence LCFA transport by modifying the expression of CD36 but also by modifying CD36 cell surface translocation. In chronic electrically stimulated rat hindlimb muscles, the expression of CD36, its presence on the sarcolemma and LCFA transport are all increased (Koonen *et al.*, 2004;Bonen *et al.*, 1999). While in cardiomyocytes and in perfused hearts, treatment with insulin for 2 hours also resulted in increased presence of sarcolemmal CD36 and LCFA

uptake (Luiken *et al.*, 2002a;Chabowski *et al.*, 2004). Interestingly, insulin causes a decrease in the ubiquitination of CD36 and therefore a decrease in the degradation rate of CD36 while FA had the opposite effect (Smith *et al.*, 2008). Contractions are also known to stimulate FA oxidation (FAO) and stimulate glucose uptake via translocation of glucose transporter 4 (GLUT4) to the cell surface from intracellular compartments (Merrill *et al.*, 1997). In absence of contraction, activation of AMPK by 5-amino-4-imidazolecarboxamide ribonucleoside (AICAR), an analogue of adenosine, results in the translocation of CD36 to the sarcolemma (Bonen *et al.*, 2007;Luiken *et al.*, 2003;Chabowski *et al.*, 2005).

In muscle cells, insulin and AMPK regulate therefore CD36 translocation (Figure 6C). In addition, CD36 seems to regulate FA metabolism since in absence of CD36, FAO stimulation by AMPK or esterification of FA by insulin (storage) is impeded (Bonen *et al.*, 2007). Overexpression of CD36 in mouse muscles resulted in a decrease in total body weight caused by a decrease in adipose tissue volume, an increase in muscle FAO, a decrease in circulating TG and FFA, and even a slight decrease in total plasma cholesterol level (Ibrahimi *et al.*, 1999). Compared to other fatty acid transporters, only the overexpression of CD36 in muscle was capable of increasing FA uptake and oxidation (Nickerson *et al.*, 2009). Knowledge of CD36 signaling events in muscle, adipose tissue and liver are relatively limited. Instead, Figure 6C presents a summary of different factors that were shown to play a role in CD36 translocation and related FA metabolism but mostly in muscle. As previously mentioned, AMPK and insulin are capable of influencing CD36 translocation through two different pathways. Contractile activity temporarily increases AMP/ATP ratio causing the phosphorylation and activation of AMPK by liver kinase B1 (LKB1), a key regulator in energy homeostasis (Woods *et al.*, 2003;Habets *et al.*, 2009). Insulin's binding to its receptor activates PI3K and Akt and also stimulates CD36 translocation; while inhibition of PI3K was shown to impede the translocation of CD36 by insulin (Luiken *et al.*, 2002a). Translocation induced by insulin seemed to be dependent on PKCζ but not on PKA (Luiken *et al.*, 2009;Luiken *et al.*, 2002b). Erk is also activated by muscular contraction and its inhibition prevented cell surface translocation of CD36 in

skeletal muscle while having no effect in the heart (Turcotte *et al.*, 2005;Chabowski *et al.*, 2006b). In the rat intact perfused heart, it was shown that activation of PPARα or PPARδ induced CD36 translocation without affecting the activity of AMPK or Erk while PPARγ agonists had no effect on translocation (Kalinowska *et al.*, 2009). Finally the transcription factor, FoxO1 regulates metabolic adaptation by inhibiting glucose oxidation in muscles, was shown to recruit CD36 to the sarcolemma and to stimulate FAO (Bastie *et al.*, 2005). In extreme metabolic conditions such as obesity and type 2 diabetes, dissociation occurs between regulation of CD36 expression, cell surface translocation, and FA uptake and metabolism. In obese subjects, the expression of CD36 in muscles is not changed compared to normal individuals. However, its translocation to the sarcolemma is increased resulting in the increase in LCFA uptake and the accumulation of intramuscular TG due to increased esterification of FA but unchanged FAO (Bonen *et al.*, 2004;Luiken *et al.*, 2001). In obese Zucker rats, a permanent relocation of CD36 to cell surface of cardiomyocytes was responsible for the observed accumulation of intracellular TG (Coort *et al.*, 2004). This increase in CD36 translocation was explained by elevated insulin levels and by insulin resistance in cardiomyocytes, a phenomenon usually observed in obese models. In type 2 diabetic models, the expression of CD36 is increased along with its sarcolemmal translocation in muscles, while dysfunctional cardiac contractions are accompanied by increased internalization and esterification of FA (Chabowski *et al.*, 2006a;Ouwens *et al.*, 2007).

6.3.3.3 Fatty acid uptake by CD36 in adipose tissue and liver

In adipocytes, translocation of CD36 to the cell surface was also demonstrated following treatment of 3T3-L1 either with insulin or with chromium picolinate, an activator of AMPK (Wang *et al.*, 2010;Wang *et al.*, 2009). Similarly to adipocytes, studies on hepatic CD36 signaling events are limited. Initially, the level of CD36 was considered low and even absent in liver (Maeno *et al.*, 1994;Abumrad *et al.*, 1999) ; however present in hepatocytes, CD36 levels were found to be influenced by metabolic demands, gender and

genetic background (Abumrad *et al.*, 1993;Pelsers *et al.*, 1999;Zhang *et al.*, 2003;Stahlberg *et al.*, 2004;Truong *et al.*, 2010). The LCFA transporters in liver are FATP2 and FATP5 (Hirsch *et al.*, 1998). They account for the majority of LCFA being internalized in the liver (Doege *et al.*, 2006;Falcon *et al.*, 2010). CD36-null mice show even an increase in hepatic LCFA uptake (Coburn *et al.*, 2001;Coburn *et al.*, 2000;Febbraio *et al.*, 1999). However, the overexpression of hepatic CD36 caused an increase in LCFA uptake and intracellular TG accumulation similarly to what is observed in obesity and type 2 diabetes (Koonen *et al.*, 2007). Indeed, in humans high expression of CD36 in the liver is associated with insulin resistance, hyperinsulinemia and hepatic steatosis (Miquilena-Colina *et al.*, 2011). The expression of CD36 was increased in the liver of mice on a HFD, in ob/ob mice (leptin deficient) and in db/db mice (deficient in leptin receptor) (Ge *et al.*, 2010). The expression of CD36 was positively correlated with LCFA in the HFD group. In the leptin signaling deficient groups (ob/ob and db/db), CD36 expression was not accompanied by an increase in LCFA uptake suggesting that perhaps leptin played a role in hepatic CD36 translocation. In HepG2, CD36 is found in intracellular compartments and plays a minor role in cholesterol efflux (Truong *et al.*, 2010). On the other hand, overexpression of CD36 resulted in the increase in its participation in cholesterol efflux into HDL3 particles. CD36 is also known to bind HDL particles and a recent study demonstrated that uptake of HDL by CD36 in the liver and cultured hepatocytes accounted for approximately 30% of total HDL uptake (Brundert *et al.*, 2011). It was recently shown that CD36 is capable of internalizing oxLDL in liver while possibly delaying native LDL clearance (Luangrath *et al.*, 2008;Truong *et al.*, 2009). In adipocytes and hepatocytes combined, much more has been done to study the impact of CD36 expression and CD36 translocation on TG accumulation and LCFA uptake than studies regarding the actual signaling events following ligand binding such as LCFA. Such findings might provide further insight on its role and impact in adipocytes and hepatocytes.

6.4 Mouse and human CD36 deficiency

The generation of CD36-null mice by the team of Roy Silverstein in 1999 provided an insight into the functional role of CD36 (Febbraio *et al.*, 1999). CD36-null mice were considered asymptomatic and generally healthy. However, accompanied by a reduced binding and uptake of oxLDL in macrophages, plasma lipid levels were also elevated. When they crossed CD36-null strain with the atherogenic ApoE-null strain, they observed a net decrease in aortic sinus lesion area (Febbraio *et al.*, 2000). However, in CD36-null mice, internalization of LCFA was greatly affected in cardiac and skeletal muscles, and also in adipose tissue; resulting in an overall deregulation of circulating lipids (Coburn *et al.*, 2000;Coburn *et al.*, 2001). Mutations occurring in the human CD36 gene, as discussed previously, offered an understanding of the impact of CD36 on the development of atherosclerosis, inflammation, malaria and lipid metabolism (Yamashita *et al.*, 2007;Omi *et al.*, 2003). Althought increased CD36 expression is considered proatherogenic, CD36 deficiency in humans is associated with increased plasmatic TG, decreased HDL-cholesterol, increased LDL-cholesterol, high blood pressure, elevated fasting plasma glucose and insulin resistance leading to a higher incidence of coronary heart disease (CHD) (Miyaoka *et al.*, 2001;Yanai *et al.*, 2000;Yamashita *et al.*, 2007). A deficiency in CD36 implies a poor internalization and use of FA by various organs such as heart, muscle and adipose tissue while favoring an abnormal use of glucose as a source of energy and promoting development of metabolic syndrome-related diseases (Fukuchi *et al.*, 1999;Kintaka *et al.*, 2002;Kushiro *et al.*, 2005;Kuwasako *et al.*, 2003;Corpeleijn *et al.*, 2006;Love-Gregory *et al.*, 2008). While it is clear that CD36 plays an important role in lipid metabolism; somewhat, it remains unclear whether the inhibition or the activation of CD36 would have an overall physiological beneficial effect. It seems that in a healthy individual, the absence or decrease in CD36 could eventually lead to CHD; while an increased expression of CD36 could render the individual more susceptible of developing

atherosclerosis. In any case, when the body is challenged metabolically, the presence of CD36 plays a detrimental role on the physiological outcome.

Regardless, the study of the effect of hexarelin on CD36 function provides a unique opportunity to understand the downstream events in adipocytes and hepatocytes which have been left mainly unexplored.

7 Fatty acid metabolism in adipocytes

Adipose tissue was traditionally viewed as a reservoir for triglycerides, a mere storage device for the body. However, with the discovery of numerous proteins being secreted by adipocytes called adipokines, the adipose tissue was quickly in the forefront of influencing systemic metabolism by regulating appetite, controlling energy expenditure and influencing immunological responses. Two types of adipocytes exist; white adipocytes that serve as storage depot for excess energy whereas brown adipocytes generate heat through thermogenesis. White adipose tissue was thought to be the sole type of fat in human adults; however, after much debate on the issue, it was recently confirmed that brown adipocytes were indeed present in humans (Cypess *et al.*, 2009). Brown adipocytes are found also in rodents for hibernation and in infants. The main characteristic of brown adipocytes is its adaptive thermogenesis potential through mitochondrial uncoupling fatty acid oxidation. As seen in Figure 7, differences between adipocyte types show that brown fat cells have a higher number of mitochondria than white adipocytes and numerous smaller lipid vesicles. Depot of white adipose tissue is found throughout the body but the most abundant forms are visceral and subcutaneous adipose tissues that produce adipokines shown to contribute to the development of metabolic disorders (Samaras *et al.*, 2010).

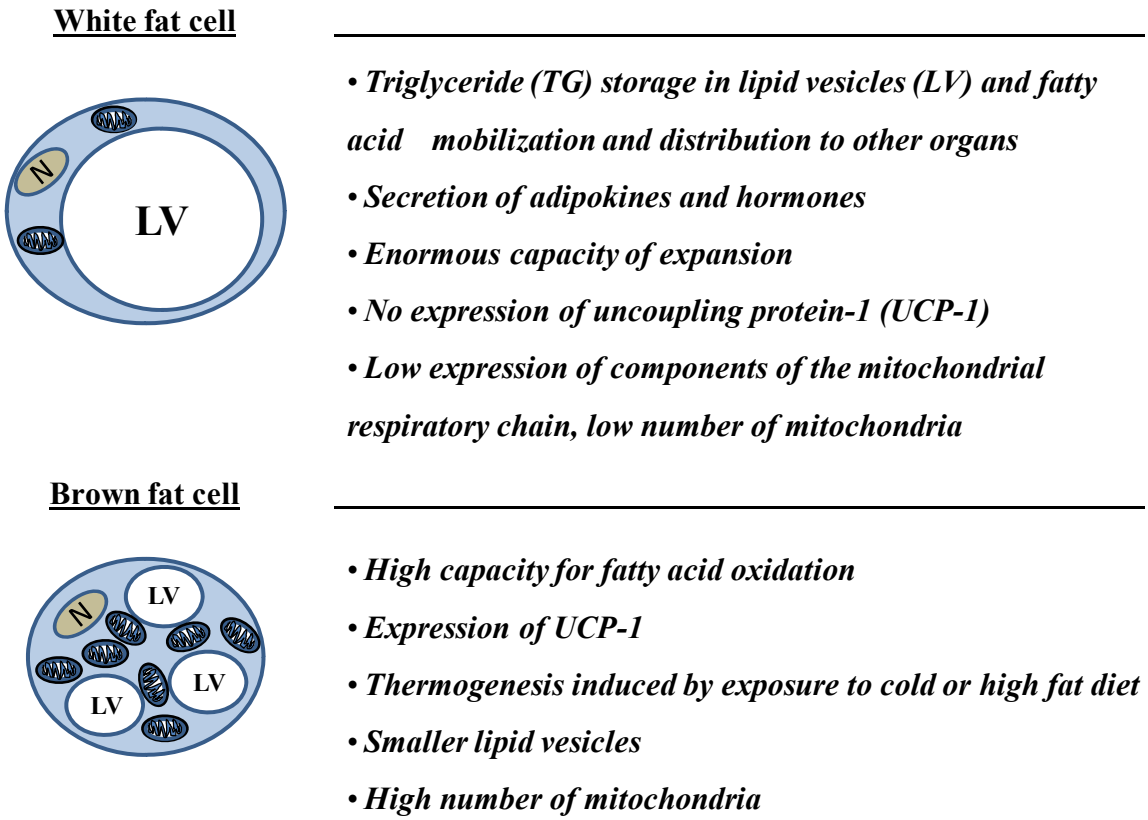


Figure 7. Adipocytes: Differences between white and brown adipocytes

7.1 The origin of adipocytes

The origin of adipocytes and the molecular events leading to the commitment of mesenchymal precursor cells to the lineage of adipocytes have been analyzed in depth. Because the master regulator of adipocyte differentiation, PPARγ controls both white and brown adipocytes differentiation, it was thought that these cells arose solely from a common mesenchymal progenitor. However, as shown in Figure 8A, it was recently discovered by Bruce Spiegelman and colleagues that brown adipocytes can originate from Myf-5-expressing myogenic precursors as well (Seale *et al.*, 2008). These precursors represent the crossroad of differentiation toward either brown adipocytes or skeletal myocytes. The transcriptional regulator PRDM16 (PRD1-BF1-RIZ1 homologous domain

containing 16) controls the fate between these two cell types mainly by interacting with PPARγ and stimulating brown adipogenesis from myogenic precursors. Brown fat cells can also emerge within white adipose tissue in response to chronic cold exposure or β-adrenergic stimulation (Cousin *et al.*, 1992;Himms-Hagen *et al.*, 2000). Therefore, factors influencing the switch between the white towards the brown fat phenotype has been of great interest as a therapeutic option for treating obesity and metabolic diseases.

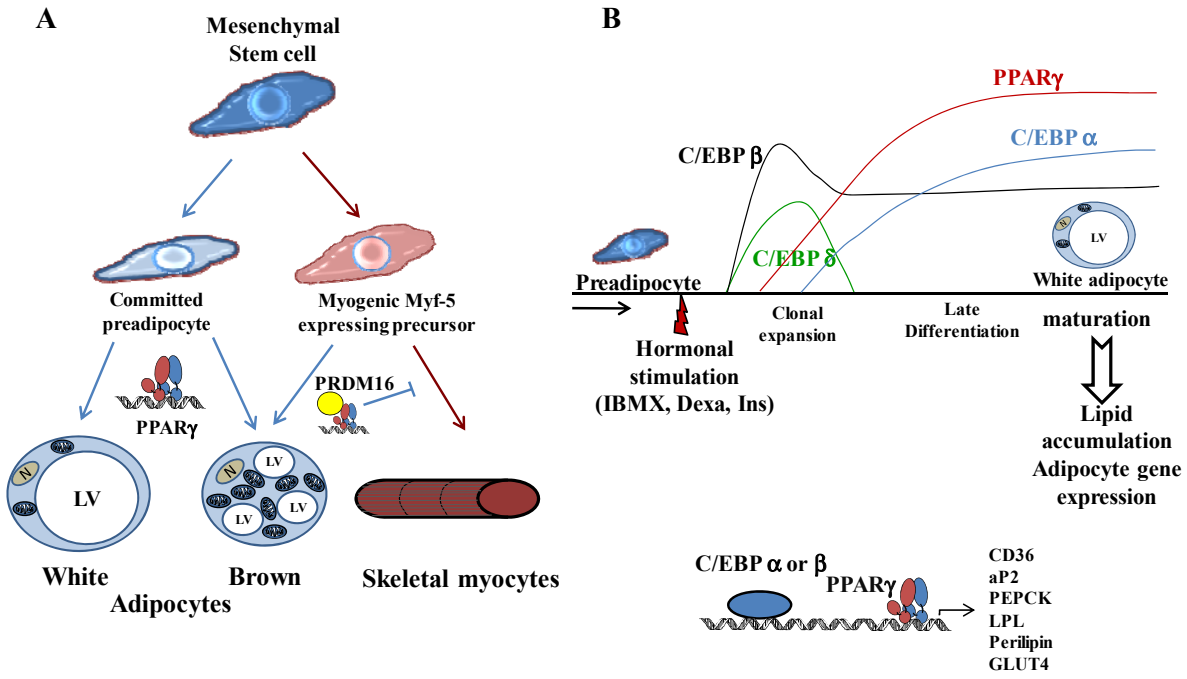


Figure 8. Adipocyte differentiation (A) Determination of adipocyte fate from mesenchymal precursors, (B) factors regulating white adipocyte differentation

7.2 Differentiation of adipocytes

The elucidation of the precise molecular events involved in the differentiation of white adipocytes was achieved mostly by using in vitro models such as 3T3-L1 preadipocytes (Green & Kehinde, 1974;Green & Kehinde, 1979). Optimized differentiation of 3T3-L1 fibroblast-like preadipocytes is achieved upon treatment with a combination of insulin, dexamethasone, 3-isobutyl-1-methylxanthine (IBMX), fetal bovine serum and high

percentage of CO_2 (Student *et al.*, 1980). Insulin acts on the IGF-1 receptor which is essential for adipocyte differentiation. Dexamethasone is a synthetic agonist that stimulates the glucocorticoid receptor pathway. IBMX, a cAMP-phosphodiesterase inhibitor, stimulates the cAMP-dependent protein kinase pathway. The very early event in differentiation of the committed preadipocyte arises from cellular confluence and growth arrest (Figure 8B). Following hormonal induction, expression of transcription factors at specific differentiation times come into play, in turn inducing the expression of genes that will define the function of adipocytes. CCAAT/enhancer binding proteins β and δ (C/EBP β and δ) are among the first to be implicated in the differentiation process (Cao *et al.*, 1991). C/EBP β and δ are responsible for the induction of the expression of PPARγ and of C/EBP α occurring during intermediate differentiation. C/EBPα is abundant in mature adipocytes and is responsible for insulin-dependent glucose uptake (Wu *et al.*, 1999b). Genome-wide analysis reveals that C/EBPα,β and PPARγ co-localizes on many genes to orchestrate their expression and regulate the function of adipocytes (Lefterova *et al.*, 2008). However, in C/EBPα-deficient mouse embryonic fibroblasts, C/EBPα was shown to be dispensable since introduction of PPARγ in these cells still promoted adipogenesis (Rosen *et al.*, 2002). PPARγ is an absolute requirement for adipogenesis and no other factor to date has been identified capable of rescuing adipogenesis in absence of PPARγ (Tontonoz *et al.*, 1994b). PPARγ will be further discussed in the context of adipocytes and hepatocytes in sections 8.4 and 8.5.

7.3 Adipokines and adipose tissue

Table 4 lists examples of secreted adipokines and their role in energy homeostasis and inflammation. Factors secreted by adipose tissue are collectively called adipokines whether they are adipocyte-specific or not (Haque & Garg, 2004). In 1993, Bruce Spiegelman and colleagues showed that TNF-α, a pro-inflammatory factor could also be produced by the adipose tissue and directly contribute to insulin resistance in obese rats

(Hotamisligil *et al.*, 1993). The potential influence of adipokines on overall energy balance came with the identification of leptin capable of regulating food intake and its association with obesity (Zhang *et al.*, 1994). The identification of adiponectin, exclusively expressed in adipocytes, demonstrated a new potential for adipocytes at reducing pro-inflammatory response while increasing insulin-sensitivity and providing protection against several metabolic disorders related to obesity (Scherer *et al.*, 1995a). The only other anti-inflammatory adipokine identified to date is the secreted frizzled-related protein 5 (SFRP5) recently associated with obesity and diabetes (Ouchi *et al.*, 2010).The pro-inflammatory resistin has been shown to induce insulin resistance in mice and linking obesity to diabetes (Steppan *et al.*, 2001). Other pro-inflammatory adipokines are IL-6, IL-18, lipocalin 2, angiopoietin-like protein 2 (ANGPTL2), CC-chemokine ligand 2 (CCL2) and nicotinamide phosphoribosyltransferase (NAMPT) (Esposito *et al.*, 2003;Yan *et al.*, 2007;Tabata *et al.*, 2009;Kanda *et al.*, 2006;Revollo *et al.*, 2007). Adipose tissue is comprised not only of adipocytes but also to a smaller extent of preadipocytes, macrophages, lymphocytes, fibroblasts, capillaries and blood vessels (vascular smooth muscle and endothelial cells). In mild and advanced metabolic dysfunctions, there is an increase in macrophage and lymphocyte infiltration caused by the release of adipokines in the circulation due to the proximity of blood vessels within the adipose tissue (Weisberg *et al.*, 2003).

Table 4. Adipokines and their role in inflammation, energy metabolism or insulin resistance

Adipokine	Receptor or Partner	Role	Reference
TNF-a	TNF receptor	Pro-inflammatory reaction	Hotamisligil *et al.*, 1993
Leptin	Leptin receptor	Appetite inhibition, energy expenditure	Zhang *et al.*, 1994
Adiponectin	ADPN receptor-1,-2 T-cadherin, calreticulin-CD91	Anti-inflammatory reaction, insulin sensitizer	Scherer *et al.*, 1995a
SFRP5	WNT5a	Anti-inflammatory reaction, WNT signaling	Ouchi *et al.*, 2010
Resistin	Unknown	Insulin resistance, pro-inflammatory reaction	Steppan *et al.*, 2001
IL-6	IL-6 receptor	Both pro- and anti-inflammatory reaction	Esposito *et al.*, 2003
IL-18	IL-18 receptor, IL-18 BP	Pro-inflammatory reaction	Esposito *et al.*, 2003
Lipocalin 2	Unknown	Insulin resistance, pro-inflammatory reaction	Yan *et al.*, 2007
ANGPTL2	Unknown	Pro-inflammatory reaction	Tabata *et al.*, 2009
CCL2	CCR2	Monocyte recruitment	Kanda *et al.*, 2006
NAMPT	Unknown	Monocyte chemotactic activity	Revollo *et al.*, 2007

7.4 Fatty acid transport into adipocytes

In addition to CD36 as a fatty acid transporter, adipocytes express FATP1 and FATP4 which unlike CD36 possess also a FACS activity that allows esterification of FA for proper storage as TG (Hall *et al.*, 2003;Hall *et al.*, 2005). It was recently shown in 3T3-L1 adipocytes that silencing by CD36 siRNA resulted in a 30-40% decrease in basal palmitate uptake and a 40-45% drop in insulin-stimulated FA uptake (Lobo *et al.*, 2009). FATP1 silencing resulted in a 25% reduction in FA uptake and a complete loss of FA uptake stimulation by insulin; however, the silencing of FATP4 had no impact on FA influx (Lobo *et al.*, 2007). The absence of FATP4 resulted in an increase in basal lipolysis and its activity is proposed to be linked with the reesterification of FA following lipolysis in adipocytes. The difference between insulin stimulation effect in absence of either CD36 or FATP1 can be explained by the localization of CD36 in the bulb of caveolea for fatty acid

uptake whereas FATP1 is not (Pohl *et al.*, 2005). Interestingly, the insulin receptor is found at the neck of caveolae but not in the bulb and could interact more favorably with FATP1 (Foti *et al.*, 2007). Overall, CD36 and FATP1 play a major role in FA uptake in adipocytes (Figure 9).

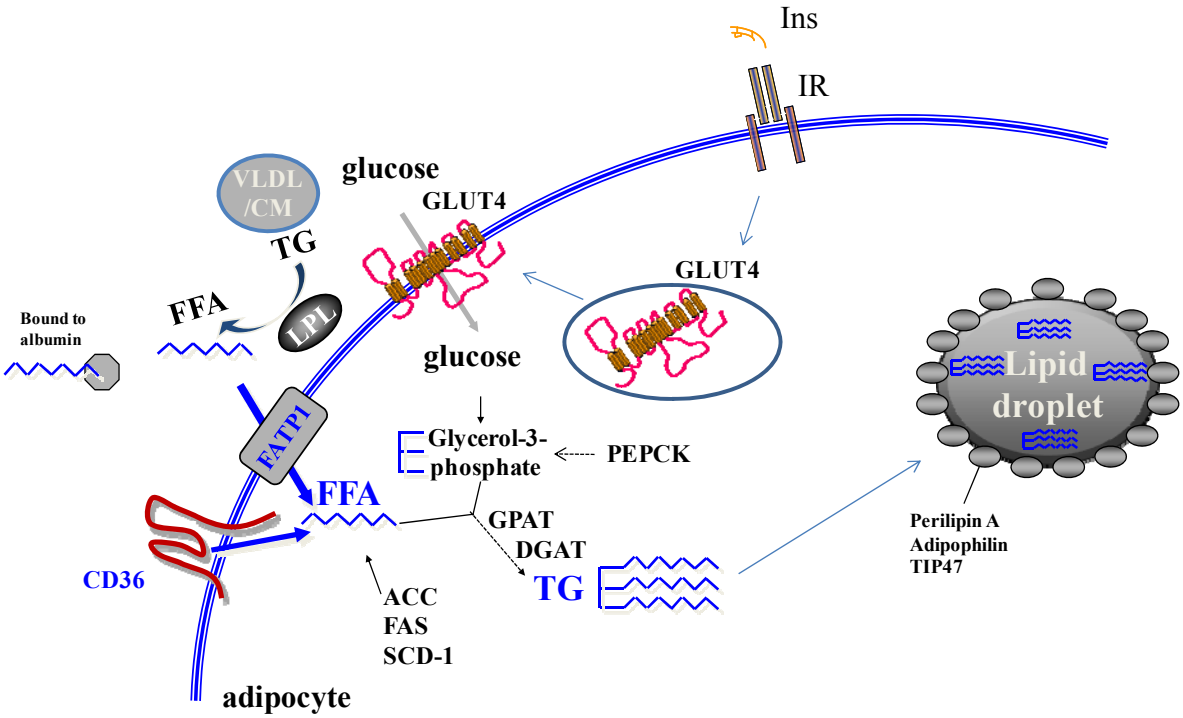


Figure 9. Fatty acid uptake, triglyceride synthesis and lipid droplets

7.5 Storage of fatty acids in lipid droplets

Figure 9 illustrates the conventional steps in FA uptake and esterification, and the subsequent storage of FA as triglycerides in lipid droplets in white adipocytes. LCFAs generated from hydrolysis of triglycerides from chylomicrons (CM) or VLDL by LPL are taken up by transporters (CD36 and FATP1). Circulating FFA is coupled to albumin. Fatty acids are stored in lipid droplets of adipocytes in the form of triglycerides which constitutes a glycerol backbone to which three molecules of fatty acids have been esterified. Since white adipocytes have little to none glycerol kinase, dihydroxyacetone phosphate (DHAP),

produced during glycolysis, becomes the precursor for production of glycerol-3-phosphate (G-3-P) and TG synthesis. Therefore, a supply of glucose is crucial for synthesis of TG and is controlled by insulin via GLUT4 translocation to the adipocyte membrane from intracellular compartments. Once the fatty acid is transported inside the cell, it undergoes esterification for the synthesis of triglycerides. Diacylglycerol acyltransferase (DGAT) catalyzes the terminal step converting DAG into TG. Interestingly, adipocytes of CD36-null mice accumulate DAG and have low levels of TG compared to wild type control (Coburn *et al.*, 2000). DGAT activity was similar in both groups and therefore could not explain the accumulation of DAG in cells. However, lipolysis enzyme activity was not measured in this study. During fasting, the glyceroneogenesis pathway (pyruvate carboxylase and cytosolic phosphoenolpyruvate carboxykinase (PEPCK)) is the major source of triglyceride glycerol (Nye *et al.*, 2008). Mitochondrial- or endoplasmic reticulum-associated glycerol-3-phosphate acyltransferase (GPAT) catalyzes the committed step in de novo TG synthesis (Cao *et al.*, 2006). Interestingly, hepatic deficiency in mitochondrial GPAT resulted in redirection of FA towards oxidation (Hammond *et al.*, 2005). FA synthesis is also possible through activity of (1) fatty acid synthase (FAS) which catalyzes the conversion of acetyl-CoA and malonyl-CoA into long-chain saturated fatty acids, (2) acetyl-CoA carboxylase (ACC) which synthesizes malonyl-CoA the first committed step in FA synthesis and (3) stearoyl-CoA desaturase-1 (SCD-1) which is the rate-limiting enzyme involved in the synthesis of the major monounsaturated fatty acids oleic acid and palmitoleic acid (Girard *et al.*, 1994). Newly synthesized TGs are stored in actively forming lipid droplets near the ER which are surrounded by the PAT family of scaffold proteins: perilipin A, adipophilin and tail-interacting protein 47 (TIP47) (Nagayama *et al.*, 2007;Wolins *et al.*, 2005). Perilipin A is the major protein found in lipid droplets and through its phosphorylation state by PKA, it plays a major role in lipolysis by providing a protective barrier from lipases (Greenberg *et al.*, 1991). Perilipin A-null mice have elevated basal lipolysis, reduced WAT mass, display significant resistance to diet-induced obesity and have increased fatty acid oxidation (Saha *et al.*, 2004).

7.6 Lipolysis

Adipocyte lipolysis represent the hydrolysis of triglycerides into fatty acids and their subsequent release into circulation for use as energy source by other tissues such as the heart and skeletal muscles. In order to respond quickly to energy demands, the lipid pool is in a constant cycle of lipolysis and re-esterification (Kalderon *et al.*, 2000). When changes in metabolic conditions arise, adipocytes respond by a shift in lipolysis to satisfy the energy demand. Lipolysis is carefully regulated and represents a skillfully orchestrated event, as observed in Figure 10. Fasting stimulates lipolysis through secretion and binding of the primary activators, catecholamines such as norepinephrine to their receptor on adipocytes. β-adrenergic receptor coupled to Gs-proteins transcends this signal to adenylyl cyclase. The produced cAMP gives rise to the activation of protein kinase A. PKA catalyzes the phosphorylation of hormone-sensitive lipase (HSL) on Ser-659 and -660 causing activation and translocation of HSL to the lipid droplet (Su *et al.*, 2003). PKA also targets Ser-563 on HSL, which is a site aimed at directly preventing phosphorylation of Ser-565 by AMPK, known to inactivate HSL (Djouder *et al.*, 2010). PKA also targets perilipin A at up to six identified sites: Ser-81, Ser-223, Ser-277, Ser-434, Ser-492, and Ser-517 (Greenberg *et al.*, 1993). The phosphorylation of perilipin A alone is sufficient to increase lipolysis (Tansey *et al.*, 2003). Phosphorylation of perilipin facilitates its interaction with translocated HSL on lipid droplets and favors lipolysis (Miyoshi *et al.*, 2006). The action of PKA results therefore in the movement of perilipin away from the lipid droplet, a remodelling of lipid droplet surface available for lipolytic attack and an activation of HSL mediated by its interaction with perilipin (Clifford *et al.*, 2000;Marcinkiewicz *et al.*, 2006;Sztalryd *et al.*, 2003).

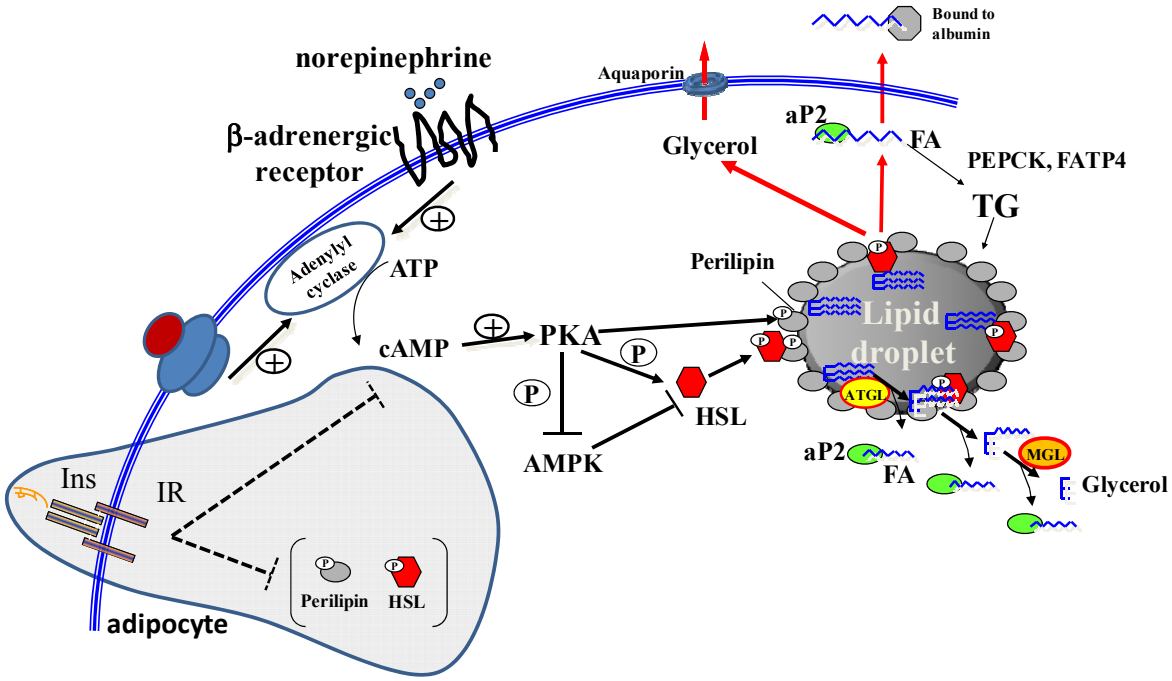


Figure 10. Regulation of lipolysis in adipocytes

In contrast, AMPK demonstrates an anti-lipolytic effect in adipocytes through phosphorylation of HSL at Ser-565 (Daval *et al.*, 2005). However, it was recently demonstrated by Whilhelm Krek and colleagues, that PKA also targets inactive AMPK (Ser-173) and prevents further activation of AMPK at Thr-172 as a mean to control lipolysis (Djouder *et al.*, 2010). When the ratio AMP/ATP rises from the energy consuming reesterification process, AMPK is activated and targets HSL for inactivation (Daval *et al.*, 2005;Gauthier *et al.*, 2008). Until the advent of HSL-null mice, it was thought that HSL was the rate-limiting enzyme in adipocyte lipolysis, hydrolyzing triacylglycerol (TAG or TG) and DAG while monoacylglycerol lipase (MGL) was responsible for the hydrolysis of MAG. However, HSL-null mice showed massive accumulation of DAG demonstrating that HSL was not mainly involved in hydrolysis of TAG into DAG but rather DAG into MAG (Osuga *et al.*, 2000). This finding prompted the search and discovery of the adipose triglyceride lipase (ATGL) (Zimmermann *et al.*, 2004). The released FA is then transported out of the cell by fatty acid carrier, adipocyte protein 2 (aP2). Glycerol produced cannot be

recycled since white adipocytes do not express glycerol kinase and therefore is secreted out of the cell through aquaporin for utilization by other tissues. Reesterification of fatty acids is part of the mechanism that controls FA release from the cell. Reesterification requires glycerol-3-phosphate which cannot be synthesized from glycolysis since it is reduced during fasting; therefore, glyceroneogenesis and PEPCK also play a role in the recycling of fatty acids (Forest *et al.*, 2003). Insulin is the main negative regulator of lipolysis. Upon feeding, insulin binds to its receptor on adipocytes, causing the recruitment and phosphorylation of its substrate, IRS-1. Subsequent activation of PI3K and Akt results in the targeted activation of phosphodiesterase 3 and phosphatases 2A, 2C and 1 which will hydrolyze cAMP and dephosphorylate HSL and perilipin, respectively (Figure 10, shaded area) (Langin, 2006). The basal lipolysis in obese patients is increased while cathecholamine-stimulated lipolysis is reduced in part due to a decreased in the expression and function of HSL (Large *et al.*, 1999). The increased basal lipolysis can be explained by an impaired sensitivity to insulin and an increase in TNF-α secretion in adipocytes. Through TNF receptor, Erk1/2 and JNK are activated and causes the downstream decrease in the expression of perilipin (Ryden *et al.*, 2004;Ryden *et al.*, 2002).

7.7 White to brown transdifferentiation of adipocytes

With the appearance of brown adipocytes in visceral fat pads of mice exposed to cold, the question remained whether these cells originated from a pool of precursor cells already present in WAT or converted into brown adipocytes from white fat cells (Young *et al.*, 1984). Today, there is mounting evidence that white adipocytes can be converted or transdifferentiated into brown adipocytes even in humans (Cinti, 2002;Puigserver *et al.*, 1998;Himms-Hagen *et al.*, 2000;Tiraby *et al.*, 2003). While PPARγ plays a central role in the differentiation of white and brown adipocytes, it is not the primary determinant whether a cell assumes either phenotype since PPARγ is present in both cell types. Nonetheless, brown fat cells can appear within WAT using PPARγ agonists (Nedergaard *et al.*, 2005;Sell *et al.*, 2004). PPARγ coactivator-1α (PGC-1α) is expressed at extremely low levels in

white adipocytes but is highly expressed in brown fat cells. PGC-1α plays a critical role in initiating the thermogenic program by inducing oxidative metabolism and mitochondrial biogenesis (Spiegelman & Heinrich, 2004). Ectopic expression of PGC-1α in white adipocytes was shown to induce the expression of UCP-1, of several mitochondrial enzymes of the respiratory chain and to increase mitochondrial DNA cellular content (Puigserver *et al.*, 1998). Perilipin overexpression in white adipose tissue of transgenic mice resulted in the induction of a brown fat-like phenotype (Sawada *et al.*, 2010). In addition to its role in brown adipogenesis in Myf-5-positive cells, PRDM16 is capable of inducing, in white pre-adipocytes, brown adipocyte differentiation (Seale *et al.*, 2007). PRDM16 coactivates PGC-1α, PPARγ and other transcription factors and suppresses the expression of white adipocyte markers (Kajimura *et al.*, 2008). Moreover, shRNA-mediated knockdown of PRDM16 in brown preadipocytes allows normal differentiation into fat cells while losing altogether their brown fat phenotype (Seale *et al.*, 2007). PRDM16 induction has become therefore an interesting approach in treating obesity-related disorders.

7.8 Mitochondria and fatty acid oxidation

Fatty acid β-oxidation takes place in the peroxisome and the mitochondrion and both organelles work closely together in order to efficiently produce ATP (Schrader & Yoon, 2007). Peroxisomes and mitochondria contain their own set of β-oxidation enzymes and show different substrate specificity; therefore, very long chain fatty acids (VLCFA) are first oxidized in the peroxisome and after a few rounds, the shortened fatty acid chain is shuttle towards the mitochondrion for further oxidation while LCFA are readily transferred into mitochondria. Among other roles, mitochondria are considered power plants of the cell to produce ATP for various in-house reactions. Despite a lower number of mitochondria in white fat cells compared to brown, mitochondrial biogenesis occurs during white adipocyte differentiation (Ducluzeau *et al.*, 2011). In support of an important role in white adipocytes, mitochondrial dysfunction in white adipocytes is associated with obesity and insulin resistance (Choo *et al.*, 2006;Sutherland *et al.*, 2008;Gao *et al.*, 2010).

7.8.1 Mitochondrial Biogenesis

Mitochondrial biogenesis represents the adaptation of any cell to a rise in energy demand by increasing the overall mitochondrial network. This definition was initially applied by John Holloszy who observed a 60% increase in protein content in mitochondrial fractions, accompanied by an increase in oxidative phosphorylation, extracted from muscles of rats subjected to a strenuous exercise routine (Holloszy, 1967). The mitochondrion houses over a thousand different proteins distributed among four different compartments: the inner and outer membranes, the intermembrane space and the matrix. Mitochondrial DNA code for 13 out of the 80 different proteins of the respiratory chain, 2 ribosomal RNAs and 22 transfer RNAs; therefore 99% of its proteins are encoded by the nuclear genome. Consequently, these newly synthesized mitochondrial preproteins are escorted into the mitochondrion through specialized protein import machinery (figure 11). All preproteins must pass the translocase of the outer membrane (TOM complex) and from the intermembrane space, four specific import pathways branch off to shuttle the preproteins into one of the four compartments. The sorting and assembly machinery (SAM) transfers proteins into the outer membrane, while translocase of the inner membrane 22 (TIM22 complex) transfers selected proteins into the inner membrane. The TIM23 complex distributes soluble proteins into the matrix and the mitochondrial intermembrane space import and assemble (MIA pathway) takes care of the remaining protein location (Gabriel *et al.*, 2007;Pfanner *et al.*, 2004;van *et al.*, 1999).

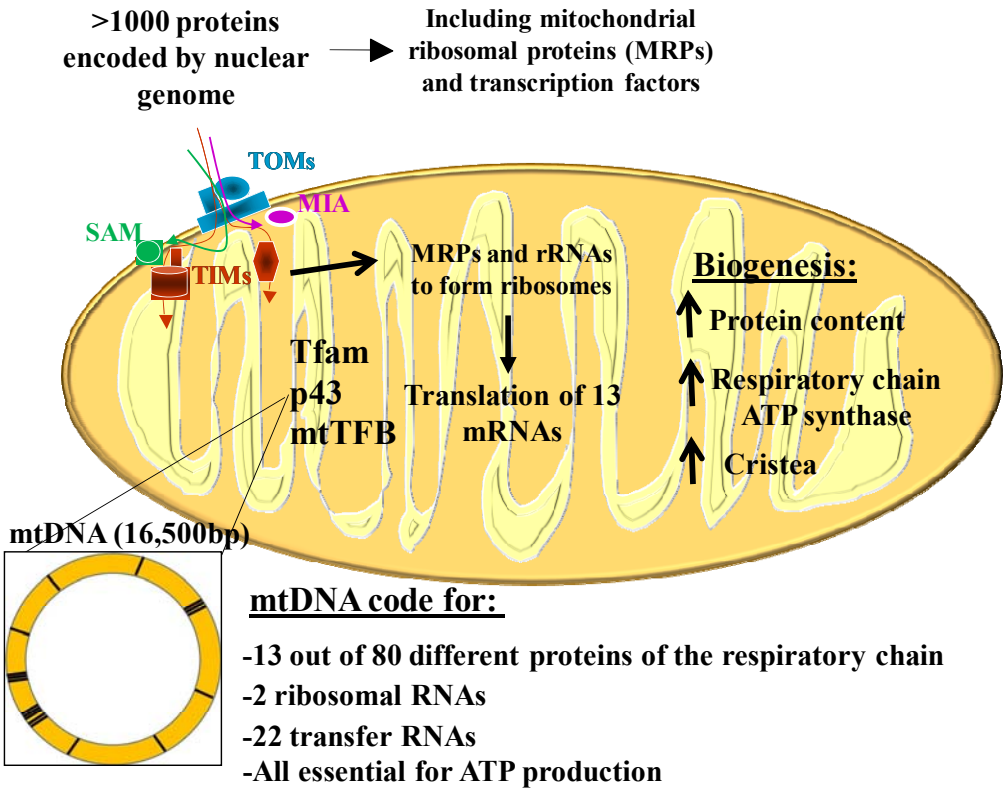


Figure 11. Mitochondrial Biogenesis, protein import and translation

Mitochondrial biogenesis begins with external stimuli to induce changes in the expression of various nuclear genes encoding mitochondrial proteins predominantly through the activation of transcription factors and co-activators. PGC-1α is a master regulator of mitochondrial biogenesis and respiration (Wu *et al.*, 1999a). Transcriptional activation of mitochondrial genes of the respiratory chain is increased following the escort of 3 known transcription factors into the mitochondrial matrix, Tfam, p43 and mtTFB which are required for biogenesis (Gordon *et al.*, 2001). Mitochondrial ribosomal proteins for the translation of mitochondrial mRNAs are also amongst the proteins being imported. High density crystal formation within the mitochondrion is an indication of highly oxidative tissue and changes during mitochondrial biogenesis (Gilkerson *et al.*, 2003). Increase in the expression of PGC-1α, mitochondrial transcription factors and proteins such

as Tfam, Tim(s) and Tom(s) and MRPs, and changes in morphology are all indicative of an increase in oxidative phosphorylation and mitochondrial biogenesis.

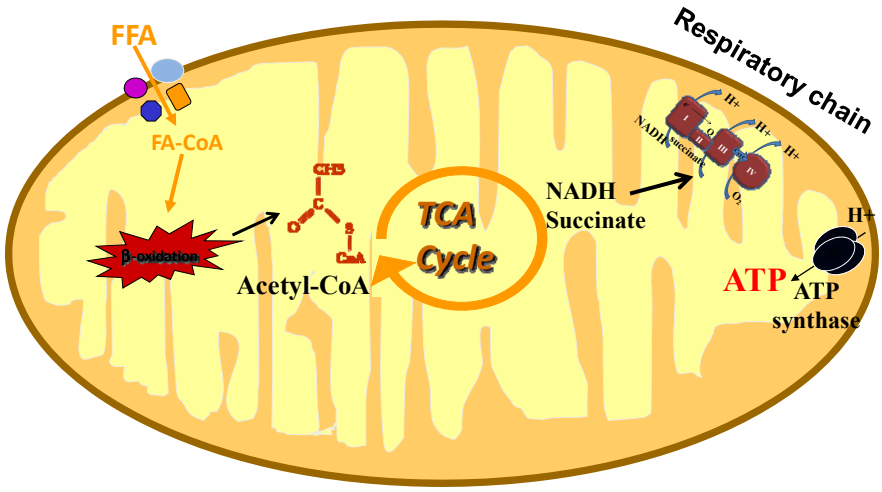


Figure 12. Steps in β-oxidation of fatty acids, oxidative phosphorylation coupled with ATP production

7.8.2 FAO and oxidative phosphorylation in white adipocytes

Figure 12 depicts all necessary steps from transport of FA into mitochondria, β-oxidation, tricarboxylic acid cycle (TCA cycle), electron transport chain and production of ATP. The initial step in fatty acid oxidation is the conversion of FA into fatty acyl-coA by acyl-CoA synthase (ACS) for its transport across the outer and inner mitochondrial membranes (Figure 13). The inner membrane is impermeable to CoA therefore carnitine becomes the carrier to transport acyl group across the inner membrane. CPT-I transfers the acyl group to carnitine then the acyl-carnitine exchanges with free carnitine across the inner membrane by carnitine acylcarnitine transferase (CAT). Finally, the fatty acyl is transferred back to CoA by CPT-II and enters the β-oxidation pathway.

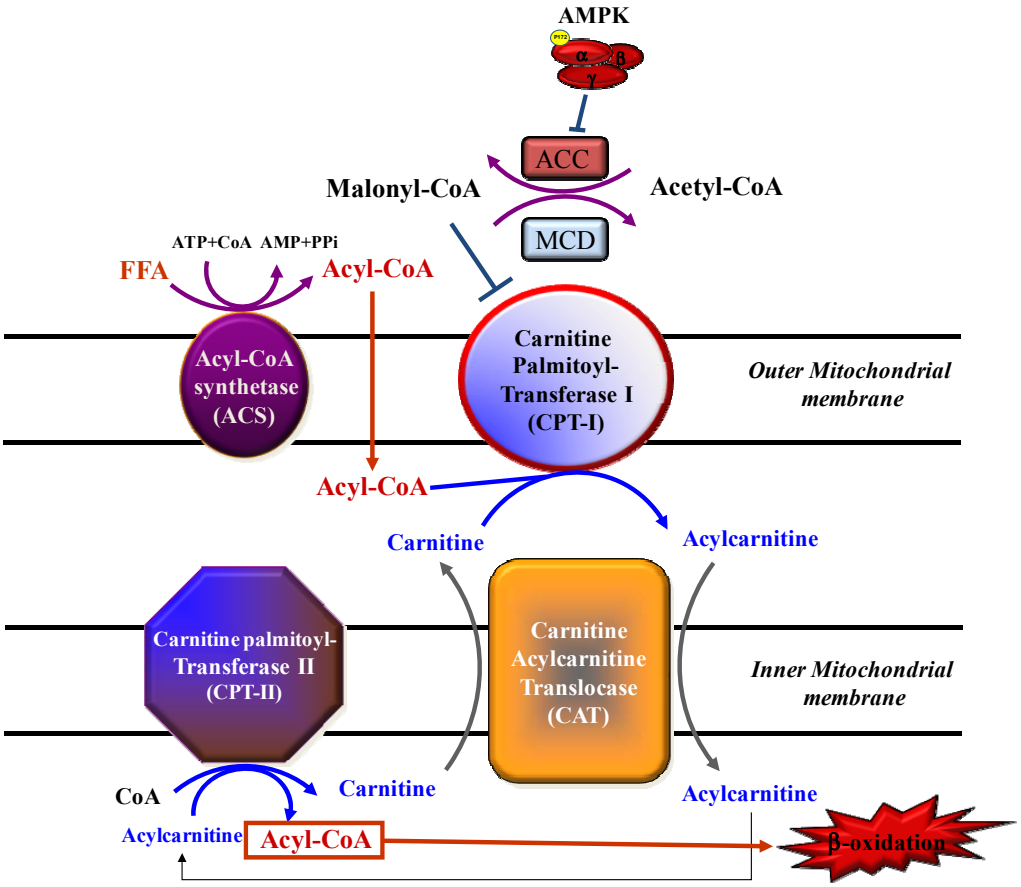


Figure 13. Initial Steps Fatty Acid Oxidation

Fatty acid entry into the mitochondrion is mainly controlled through the activity of the rate-limiting enzyme in FA uptake and oxidation, CPT-I. ACC catalyzes the rate-limiting reaction in the synthesis of LCFA by synthesizing from acetyl-CoA, malonyl-CoA, the substrate for FA synthesis. Binding of malonyl-CoA to CPT-I inhibits its activity (Murthy & Pande, 1987). Hence, ACC controls the activity of CPT-1 by producing malonyl CoA (Ha *et al.*, 1996). In turn, acetyl-CoA carboxylase (ACC) can be phosphorylated and inactivated at Ser79 by AMPK when energy supply is low, demonstrating an intricate system to control energy expenditure (Ha *et al.*, 1994). Malonyl-CoA decarboxylase (MCD) also controls the level of malonyl-CoA, while depletion of MCD was shown to increase malonyl-CoA and decrease FAO (Dyck *et al.*, 1998;Bouzakri *et al.*, 2008). Two

isoforms exist for CPT-I. CPT-1a is expressed primarily in the liver but is also found in other tissues including adipose tissue while CPT-1b in human is expressed in adipose tissue, heart, skeletal muscle and testis. However, gender- and species-differences in the expression of both isoforms exist. Interestingly, CPT-1b is the predominant form expressed in rat and mouse white and brown adipose tissue; however in mouse 3T3-L1, CPT-1a is the major isoform (Brown *et al.*, 1997;Esser *et al.*, 1996). CPT-1b is far more sensitive to malonyl-CoA than CPT-1a; whereas unlike CPT-1b, CPT-1a sensitivity to malonyl-CoA is influenced by thyroid hormone and insulin (Park *et al.*, 1995;Saggerson & Carpenter, 1981).

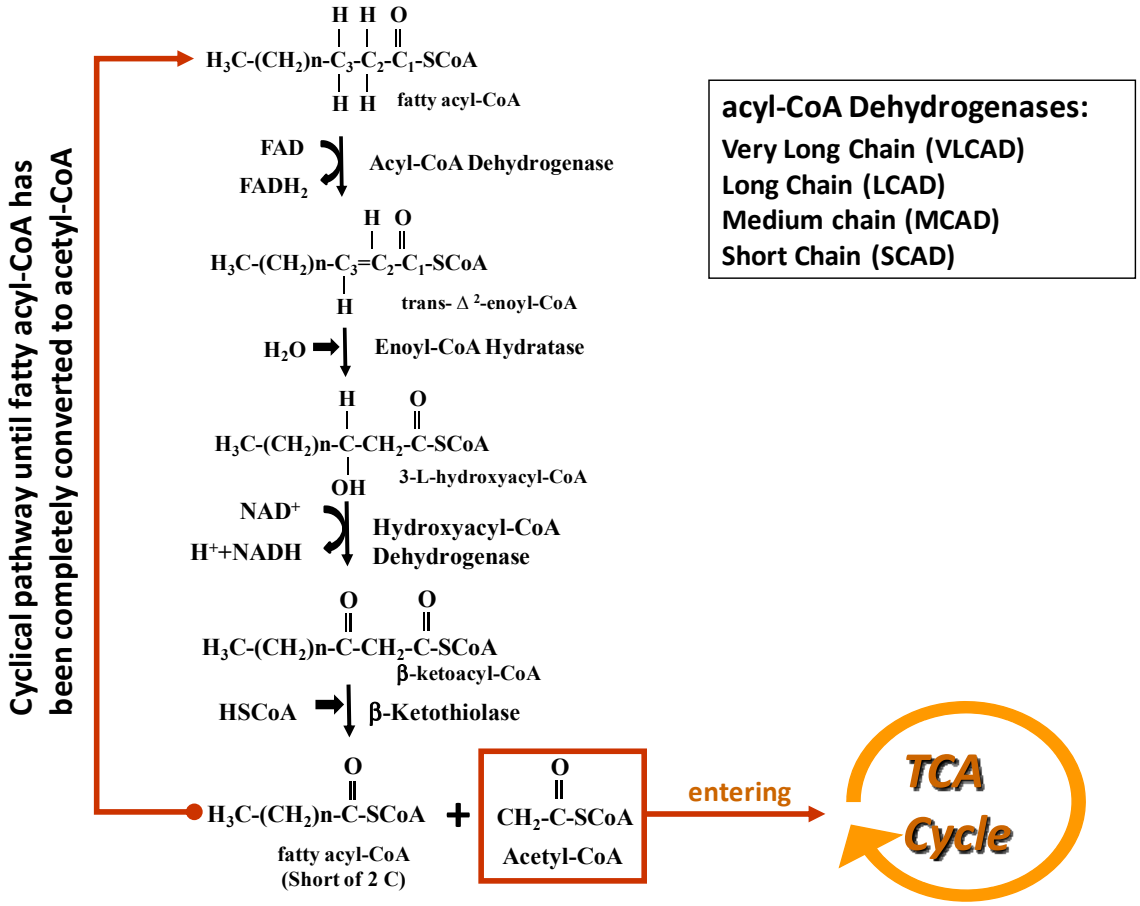


Figure 14. β-oxidation reaction of fatty acids

Fatty acid oxidation occurs in all tissues with the exception of brain, erythrocytes and adrenal medulla which utilize mainly glucose as its source of energy (Sabyasachi Sircar, 2007). As seen in Figure 14, fatty acyl-CoA once inside the mitochondrion is broken down to generate acetyl-CoA which then enters the TCA cycle. Typically, each cycle of β-oxidation produces one molecule of acetyl-CoA and leaves the fatty acid short of 2-carbon unit. As the chain shortens, various acyl-CoA dehydrogenases come into play depending on its affinity for the fatty acyl-CoA length.

Succinate and NADH (electron donor) produced by the TCA cycle are then oxidized by the electron transport chain. Electrons are transferred from donors to acceptors carried across protein complexes and the energy released by this flow is used to transport protons across the inner membrane and into the intermembrane space, forming a pH gradient and an electrical potential. This allows the protons to flow back into the matrix down this gradient through a large protein complex called ATP synthase that converts ADP into ATP from the energy created with the proton movement (Figure 15). Oxidative phosphorylation is a coupled event where energy-releasing reactions are used for energy-required reactions for the purpose of producing ATP. Each complex of the respiratory chain consists of several subunits that are defined by their substrate such as NADH-coenzyme Q oxidoreductase (complex I), succinate-Q oxidoreductase (complex II), electron transfer flavoprotein-Q oxidoreductase, Q-cytochrome c oxidoreductase (complex III) and cytochorme c oxidase (complex IV). Cytochrome c oxidase activity is often used to evaluate the level of oxidative phosphorylation leading to ATP production.

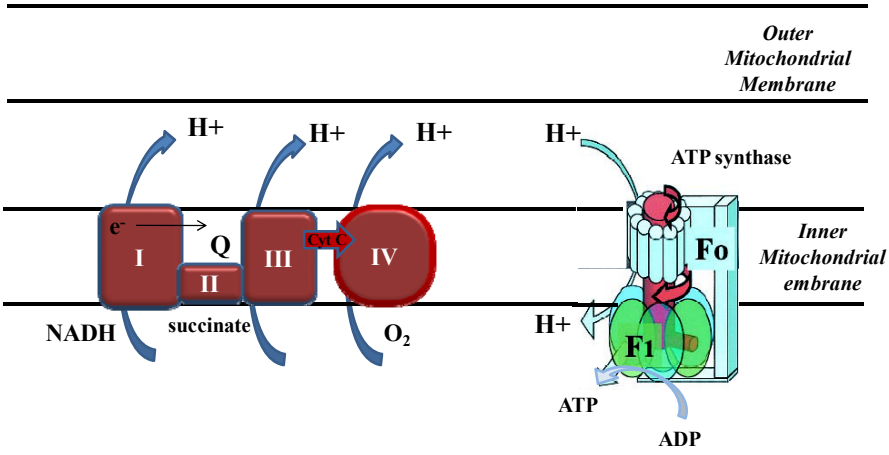


Figure 15. Oxidative phosphorylation

7.8.3 Respiratory uncoupling and thermogenesis in brown adipocytes

The brown adipocyte phenotype is essentially conferred by the presence of uncoupling protein-1 and the large amount of mitochondria. UCP-1 also called thermogenin decreases proton gradient in the intermembrane space built up by the respiratory chain by allowing protons back into the mitochondrion matrix and therefore releasing the energy from fatty acid oxidation as heat (Figure 16, box). The sympathetic nervous system controls the mobilization of stored energy and adaptive thermogenesis in adipose tissue through the activation of β-adrenergic receptor (Figure 16). UCP-1 is a 32-KDa BAT-specific protein present in mitochondria as a homodimer, not typically found in white adipocytes (Nicholls *et al.*, 1978). However, as stated in section 7.7, under the control of PGC-1α, the induction of UCP-1 is possible in white adipocytes (Puigserver *et al.*, 1998). Overexpression of UCP-1 in transgenic mice using the promoter of aP2 resulted in leaner mice with thermogenically active white adipocytes (Kopecky *et al.*, 1995). Thyroid hormones play a role in energy expenditure and adaptive thermogenesis in brown adipocytes (Bianco & Silva, 1987). Two thyroid responsive elements are found in the promoter region of UCP-1 and it was recently found that the expression of UCP-1 is reduced in mice suffering from thyroid resistance due to a frameshift mutation in the thyroid hormone receptor β (TRβ) (Ribeiro *et al.*, 2010).

Several mouse models have identified negative regulators of UCP-1 expression such as the co-repressor receptor interacting protein 140 (RIP140), LXRα and raptor, an essential component of mTORC1 (key regulator of cell growth and metabolism) (Wang *et al.*, 2008;Polak *et al.*, 2008). The small 220-bp enhancer region in the promoter of UCP-1 harbors a number of important sequences such as the cAMP-responsive element 2 (CRE2) which is recognized by ATF2, the response element of PPARs (PPRE) and the newly identified response element for LXRα (LXRE) (Cao *et al.*, 2004;Wang *et al.*, 2008). In presence of LXRα ligands, RIP140 is recruited to the transcriptional complex therefore facilitating transcriptional repression. Since PPRE and LXRE are at close proximity, it is suggested that LXR displaces PPAR preventing transcriptional activation of UCP-1. Chronic treatment of mice with ghrelin downregulated the expression of UCP-1 in brown adipocytes (Tsubone *et al.*, 2005). Overexpression of ATGL increased lipolysis and also the expression of UCP-1 in white adipocytes rendering mice resistant to diet-induced obesity (Ahmadian *et al.*, 2009). Therefore, the presence of UCP-1 is positively linked to increased lipolysis and resistance to diet-induced obesity. The discovery of functional human BAT certainly offers a new possibility of regulating energy expenditure valuable to treat obesity and other metabolic disorders.

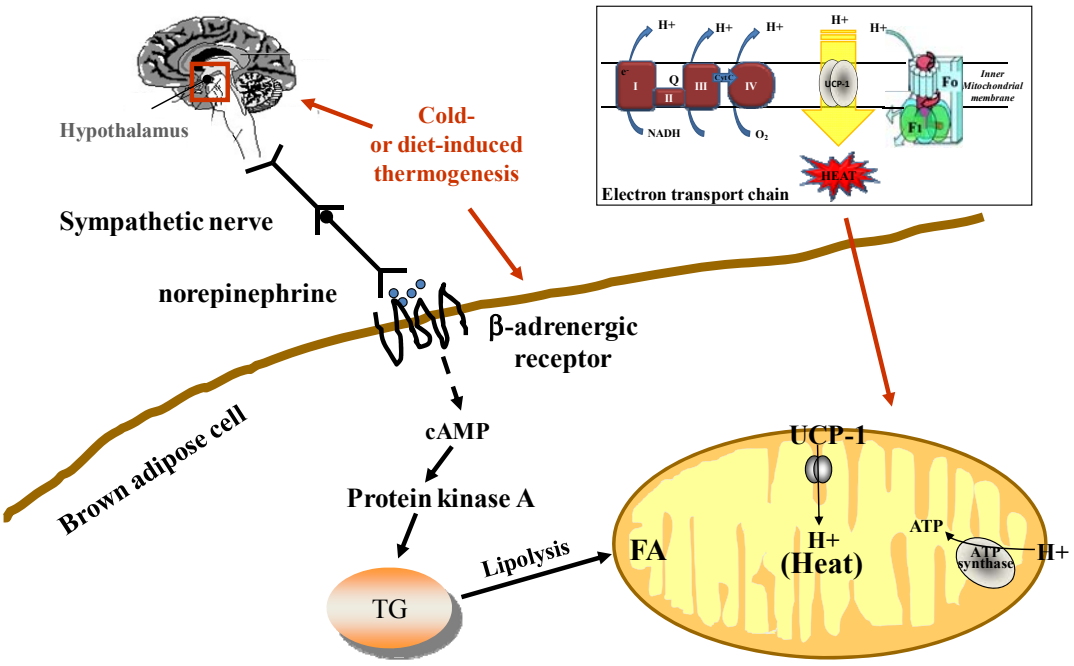


Figure 16. Adaptive thermogenesis and uncoupling protein-1 in brown adipocytes

8 PPAR gamma

The peroxisome proliferator-activated receptor family comprises of PPARα, PPARβ/δ and PPARγ (Laudet *et al.*, 1999). They are lipid-sensing receptors involved in lipid and glucose metabolism, and energy homeostasis. PPARα was discovered for its ability to bind a class of hepatocarcinogens capable of inducing peroxisome proliferation (Issemann & Green, 1990). PPARα is primarily expressed in brown adipocytes, liver, kidney, heart and skeletal muscle and plays a role in the regulation of fatty acid oxidation (Kliewer *et al.*, 1994;Lemberger *et al.*, 1996;Kersten *et al.*, 1999). PPARβ/δ is ubiquitously expressed but is present in liver at a lower level and plays a broad role in fatty acid catabolism and energy homeostasis (Kliewer *et al.*, 1994;Braissant *et al.*, 1996). PPARγ is expressed at high level in adipose tissue, macrophages, endothelial cells and large intestine but is also found in liver (Vidal-Puig *et al.*, 1996;Auboeuf *et al.*, 1997). PPARγ, in addition

to its role in lipid and glucose metabolism, in macrophage foam cell formation and inflammatory response (section 6.3.2), it also plays a major role in adipogenesis (section 7.2) (Kubota *et al.*, 1999;Tontonoz *et al.*, 1994b).

8.1 PPARγ isoforms, structure and heterodimerizaton

Two isoforms exist for PPARγ resulting from different promoters and alternate splicing (Sundvold & Lien, 2001;Chen *et al.*, 1993). PPARγ1 is expressed in all PPARγ-positive tissues; however, PPARγ2 is mainly present in adipocytes (Mukherjee *et al.*, 1997;Tontonoz *et al.*, 1994a). PPARγ shares a common structure with other nuclear receptors (Laudet *et al.*, 1992;Beato *et al.*, 1995). Figure 17 represents functional domains of PPARγ and differences between isoforms. PPARγ1 and γ2 only differ in their N-termini, PPARγ2 having an extra 28 amino acids encoded by a single exon (Zhu *et al.*, 1995;Yanase *et al.*, 1997). Nuclear receptors were originally divided into 6 regions containing functional domains (A to F) based on the sequence homology between human and chicken oestrogen receptor alpha (Krust *et al.*, 1986). Region A/B contains the ligand–independent activation function 1 (AF-1) which is 5 times more active in PPARγ2 than in γ1 (Werman *et al.*, 1997). Insulin can activate the transcriptional activity of PPARγ through AF-1 domain. Region C contains the DNA binding domain (DBD) that recognizes the PPAR response element (PPRE) while D represents the hinge region. Finally, region E/F contains the ligand binding domain (LBD) followed by a second activation function (AF-2). This region is important for the assembly of co-activators (Nolte *et al.*, 1998). Regions C and E also allows the dimerization of PPARγ with its partner RXR (Gearing *et al.*, 1993;Miyata *et al.*, 1994).

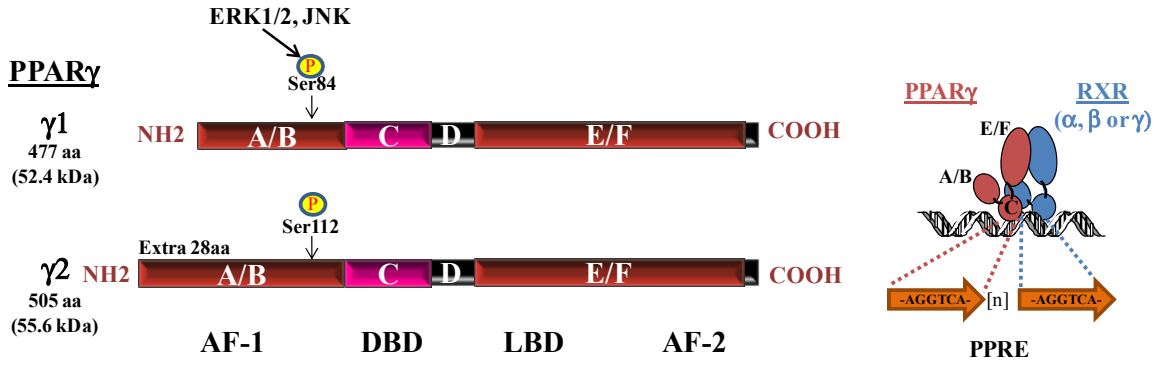


Figure 17. PPARγ isoforms, structure, and heterodimerization with RXR

All three isoforms of RXR (α, β and γ) can form heterodimers with PPARγ (Figure 17) and are activated by 9-cis retinoic acid (Issemann *et al.*, 1993). The heterodimer binds to the PPRE site upstream of its target gene which consists of a direct tandem repeat of the consensus AGGTCA element with one intervening nucleotide (Palmer *et al.*, 1995). PPRE are often found in an upstream enhancer region rather than in the proximal promoter (Tontonoz *et al.*, 1994a;Tontonoz *et al.*, 1995). Either ligands for PPARγ or for RXR can independently activate the heterodimer; however, a co-activation of PPARγ and RXR has an additive response (Cha *et al.*, 2001). Interestingly, it was previously assumed that PPAR and RXR only heterodimerize in the presence of ligand; however, it was demonstrated by fluorescence resonance energy transfer (FRET) that PPARs form heterodimers with RXRs even in absence of ligand which supports the possibility of ligand-independent activation of PPAR (Feige *et al.*, 2005).

8.2 Regulation of PPARγ activity

8.2.1 Natural and synthetic ligands

The large binding pocket of PPARγ rearranges itself to accommodate a large variety of agonists (Kallenberger *et al.*, 2003;Togashi *et al.*, 2005). The identity of the true biological ligand(s) of PPARγ remains unknown. However, a putative endogenous ligand is

produced early in adipogenesis of 3T3-L1 cells, and an organic extract containing this unidentified ligand was capable of inducing lipid accumulation as efficiently as the typical differentiation cocktail (Tzameli *et al.*, 2004). Component(s) of this extract competed for the binding of rosiglitazone. Compared to the other PPARs, PPARγ does not respond to native fatty acids while modified fatty acids elicit a stronger activation. PUFAs such as arachidonic and linoleic acids as well as eicosanoids 15-deoxy-Δ 12,14 prostaglandin J2 (15-dPGJ2) are considered natural ligands of PPARγ (Krey *et al.*, 1997;Keller *et al.*, 1993;Forman *et al.*, 1995b;Kliewer *et al.*, 1995). *In vivo* level of ligands such as 15-dPGJ2 is so low that they are unlikely to be considered biologically significant ligands.

Major oxidized lipids on oxLDL particles 9- and 13-hydroxyoctadecadienoic acid (9- and 13-HODE) are potent ligands of PPARγ (Nagy *et al.*, 1998). Thiazolidinediones (TZD) antidiabetic effect was observed in humans treated with troglitazone, glucose tolerance was increased while insulin resistance was reduced (Nolan *et al.*, 1994). With the exception of troglitazone which was later shown to have high liver toxicity, TZDs are used regularly to treat type 2 diabetes (Gale, 2006;Cohen, 2006). Soon after the determination of its potential as an antidiabetic drug, TZD was shown in adipocytes to increase the expression of aP2 through increased transcriptional activity of ARF6 DNA-binding complex on its promoter. It was later determined that ARF6 was in fact the PPARγ/RXR heterodimer and that TZD was a ligand of PPARγ (Lehmann *et al.*, 1995;Tontonoz *et al.*, 1994a;Harris & Kletzien, 1994).

8.2.2 Corepressors

No specific co-factor for PPARγ has been identified; however, several common coactivators and corepressors for the nuclear receptor family of proteins are known to interact with PPARγ. In absence of ligand, corepressors are bound to the receptor complex and recruit histone deacetylases (HDACs). Nuclear receptor corepressor protein (NCoR) and silencing mediator of retinoid and thyroid hormone receptors (SMRT) have been shown to interact with PPARγ and inhibit adipogenesis in 3T3-L1 (Horlein *et al.*,

1995;Chen & Evans, 1995;Yu *et al.*, 2005). RIP140 is capable of repressing PPARγ even in presence of ligand (Treuter *et al.*, 1998).

8.2.3 Coactivators

Ligand binding increases PPARγ affinity for coactivators and results in chromatin remodelling leading to the recruitment of the basal transcriptional machinery at the PPARγ site. The short alpha-helical motif LXXLL is an essential motif for co-factors to bind to nuclear receptors (Heery *et al.*, 2001). These include co-activators such as cAMP response element binding protein (CREB) binding protein (CBP/p300), steroid receptor co-activator (SRC) family, and the critical thyroid receptor associated protein 220 (TRAP220) (Mizukami & Taniguchi, 1997;Leo & Chen, 2000;Ge *et al.*, 2002).

8.2.3.1 PPARg coactivator-1α (PGC-1α)

Since PPARγ was expressed in brown adipocytes and played a role in the expression of UCP-1, Bruce Spiegelman and colleagues searched for components that were present in brown adipocytes capable of regulating the expression of UCP-1 while absent in white adipocytes (Puigserver *et al.*, 1998). Using a yeast two-hybrid system, they identified a 92-kDa protein called PPARγ coactivator 1 (PGC-1α) that interacted not only with PPARγ but with other nuclear receptors. PGC-1α was dramatically induced in BAT and skeletal muscle following exposure to cold. PGC-1α initiated a broad program of mitochondrial gene expression, thermogenesis and cellular respiration. Interestingly, the expression of PGC-1α does not lead to an increased expression of all PPARγ targets such as aP2, expressed in both white and brown adipocytes (Puigserver *et al.*, 1998). Figure 18 illustrates the regulation of PGC-1α by different factors. Cytokines in muscle activates p38 MAP kinase, which in turn activates PGC-1α by phosphorylation on 3 possible sites (Thr262, Ser265 and Thr298) causing an increase in the expression of mitochondrial genes and energy expenditure (Puigserver *et al.*, 2001). Also, activation of PGC-1α by p38 was shown to be essential for its recruitment to UCP-1 promoter (Robidoux *et al.*, 2005). In

liver, PGC-1α is mainly known for promoting gluconeogenesis (such as the expression of PEPCK and glucose-6-phosphatase) and repressing glycolysis, resulting in increased glucose output during fasting (Yoon *et al.*, 2001). In addition, in skeletal muscle activated AMPK targets PGC-1α on Thr177 and Ser538 (Jager *et al.*, 2007). PGC-1α activation is also triggered by arginine methylation by protein arginine methyltransferase 1 (PRMT1), another nuclear receptor coactivator (Teyssier *et al.*, 2005). While acetylation of PGC-1α by the histone acetyltransferase GCN5 (general control nonderepressible 5) was shown to inhibit its activity; its inhibition can be reversed by the protein deacetylase SIRT1 (Rodgers *et al.*, 2005;Lerin *et al.*, 2006). Through the action of insulin, the activated Akt2/PKB-β phosphorylates and inhibits PGC-1α (Ser570) and prevents its recruitment to target promoters, impairing its ability to promote gluconeogenesis and fatty acid oxidation (Li *et al.*, 2007b). It was recently demonstrated that GCN5-mediated lysine acetylation required phosphorylation of PGC-1α at Ser570 by Akt (Xiong *et al.*, 2010).

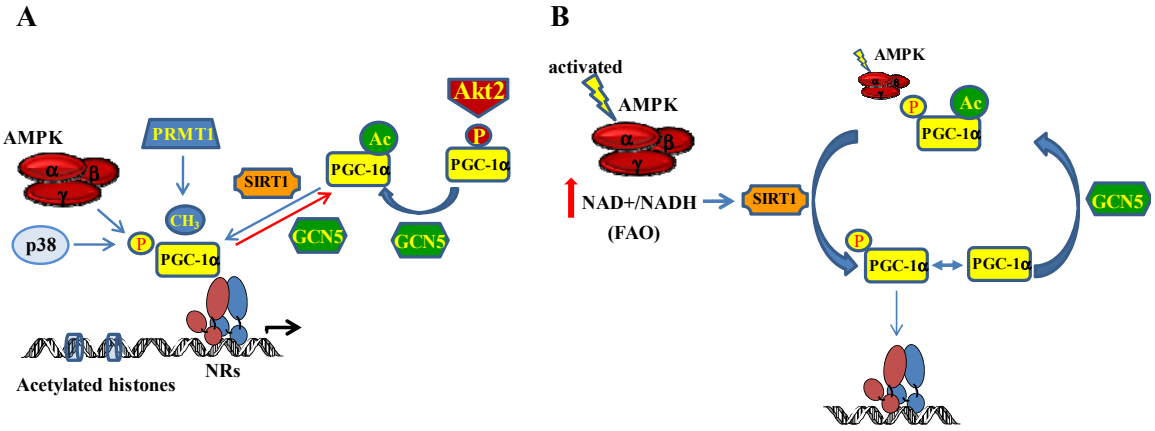


Figure 18. Regulation of PGC-1α activity

Figure 18B depicts different possible regulation of PGC-1α dependent on energy demand. SIRT1 requires NAD+ as a cofactor and is inhibited by NADH (proton donor for the respiratory chain) (Rodgers *et al.*, 2005). AMPK stimulates fatty acid oxidation which results in the production of NADH and oxidation by the respiratory chain therefore

increasing NAD+/NADH ratio. Activation of AMPK was found to positively modulate SIRT1 activity by increasing NAD+/NADH ratio (Canto *et al.*, 2009). These mechanisms influencing PGC-1α activity demonstrate the carefully orchestrated events that ultimately control the metabolic response to a variety of nutrients and physiological signals.

8.3 Post-translational modifications of PPARγ

Similarly to other nuclear receptors and their cofactors, the transcriptional activity of PPARγ can be regulated by post-translational modifications independent of ligand binding. Such modifications include ubiquitination, sumoylation, nitration and phosphorylation (Hauser *et al.*, 2000;Pascual *et al.*, 2005;Shibuya *et al.*, 2002); however, due to space constraint, only phosphorylation of PPARγ will be discussed in this section. Phosphorylation of nuclear receptors can favor the recruitment of co-factors and components of the transcriptional machinery but can also inhibit response to ligands (Rochette-Egly, 2003). Peter Tontonoz, Erding Hu and Bruce Spiegelman observed that when the N-terminal region of PPARγ2 containing the AF-1 domain was removed, this truncated protein had a greater transcriptional activity (Tontonoz *et al.*, 1994b). It was later determined that adipogenesis could be inhibited by Erk activation via phosphorylation of PPARγ2 at Ser112 (Hu *et al.*, 1996). Phosphorylation of PPARγ1 on Ser84 by Erk and by JNK was also confirmed while PPARγ was weakly phosphorylated by p38 (Figure 17) (Adams *et al.*, 1997;Camp *et al.*, 1999). Whereas Erk and JNK were known to inhibit PPARγ, Akt was shown by us and others to phosphorylate and activate PPARγ; however, the phosphorylation site of Akt has not been identified (Feige *et al.*, 2006;Demers *et al.*, 2009). Since insulin was previously shown to potentiate the activity of PPARγ AF-1 domain, it is possible that Akt targets the AF-1 domain as well (Werman *et al.*, 1997).

8.4 Role of PPARγ in adipocytes

As mentioned in section 7.2, the master regulator role in adipogenesis is attributed to PPARγ. C/EBP-β and –δ in cooperation with sterol response element binding protein 1c (SREBP-1c) induce of the expression of PPARγ and upon ligand activation, PPARγ itself induces the expression of many target genes involved in lipogenesis and adipogenesis such as LPL, aP2, PEPCK, GLUT4, adiponectin and C/EBPα (Figure 8B, page 57) (Kim & Spiegelman, 1996). C/EBPα in turn is able to bind to its site on the promoter of PPARγ and assure a positive feedback regulation loop for PPARγ expression (Tontonoz *et al.*, 1998;Nagy *et al.*, 1998). C/EBPα also induces the expression of lipin-1, a newly discovered co-factor for PPARγ playing a role in maturation of adipocytes (Koh *et al.*, 2008). PPARγ is also expressed in the brown adipocyte and plays a role in its differentiation and expression of target genes such as UCP-1 (Tai *et al.*, 1996;Sears *et al.*, 1996). PPARγ is essential for the viability of BAT and WAT and the maintenance of mature adipocytes (Tamori *et al.*, 2002;Imai *et al.*, 2004). PPARγ-targeted deletion in adipocytes resulted also in the loss of fat mass, in elevated circulating FFA and TG, and in the induction of insulin resistance in adipose tissue and liver but not in muscle (He *et al.*, 2003). The discovery of TZDs as ligands of PPARγ brought further understanding of its role in insulin sensitivity and glucose metabolism as well as its role in mature adipocytes. The effect of antidiabetic effect of TZDs is thought to stem from adipose tissue. Type 2 diabetes is associated with increased circulating FFA and storage of lipids in tissues other than adipose such as in the liver and skeletal muscle. Accumulation of FFA and TG is linked with insulin resistance and impaired glucose metabolism (Sinha *et al.*, 2002). Activation of PPARγ in adipocytes is thought to increase FA storage, TG synthesis and glucose uptake through increased expression of its target genes in addition to increasing the secretion of adipokines (such as adiponectin) that will signal to decrease lipid accumulation and to increase glucose uptake and FAO in other tissues. In adipocytes, TZDs also increases FA reesterification by

inducing the expression of glycerol kinase while also inducing FAO and mitochondrial biogenesis in adipocytes (Guan *et al.*, 2002;Wilson-Fritch *et al.*, 2003;Wilson-Fritch *et al.*, 2004). Those effects are thought to be PPARγ–dependent since mice lacking adipose-specific PPARγ are markedly deficient in their response to TZDs (He *et al.*, 2003). In vivo, TZDs promote adipogenesis and increased fat mass (Tai *et al.*, 1996). However, rather than increasing adipocyte volume, the increased in fat mass was explained by an increase in the number of adipocytes which incidentally were smaller and more sensitive to insulin (Okuno *et al.*, 1998). Due to the high expression of PPARγ in adipose tissue, it is considered the primary target of TZDs.

8.5 Role of PPARγ in hepatocytes

However present, PPARγ in hepatocytes is expressed at lower levels compared to other tissues and therefore its role or influence in liver is not fully known. Much negative attention was given to PPARγ and the hepatotoxicity effect of troglitazone. Other generations of glitazones are now used to treat diabetes such as rosiglitazone (GSK-Avandia®) and pioglitazone (Takeda-Actos®). It is thought that the toxic effect of troglitazone is independent of PPARγ activity. It was recently shown that primary human hepatocytes treated with troglitazone for 24 hours at concentration equal or exceeding 20 uM resulted in mitochondrial DNA damage, mitochondrial dysfunction and cell death while rosiglitazone had no effect (Rachek *et al.*, 2009). Troglitazone effect was not abrogated by PPARγ antagonist GW9662. A recent study confirmed that the TZD ring of troglitazone may be partially responsible for its liver toxicity in humans by inducing the production of toxic reactive metabolites (Saha *et al.*, 2010). Nonetheless, PPARγ expression level in hepatocytes is linked with liver steatosis in rodent but not necessarily in human (Yu *et al.*, 2003;Gavrilova *et al.*, 2003). Indeed, a pilot study was performed on 18 non-diabetic patients with non-alcoholic steatohepatitis (NASH) treated with pioglitazone for 48 weeks (Promrat *et al.*, 2004). Patients gained weight during treatment due to increased adiposity seen by dual-energy x-ray absorptiometry (DEXA) while magnetic resonance imaging of

the liver showed a marked decrease in volume and in fat content. In addition, glucose and FFA sensitivity to insulin were improved. Patients with fatty livers are significantly more likely to develop type 2 diabetes within the next 5 years than those with healthy livers (Sung & Kim, 2011). Interestingly, in PPARα-null mice on a HFD, PPARγ hepatic expression was increased 20-fold; however, which isoform of PPARγ was affected was not determined (Patsouris *et al.*, 2006).To further investigate the role of PPARγ in the liver, adenoviral overexpression of PPARγ1 in liver of PPARα-null mice resulted in the expression of several lipogenic and adipogenic genes but also of PPARα target genes involved in FAO. While PPARγ1 is the predominant form in hepatocytes, it was found that insulin or oleic acid, found in olive oil, increased the expression of PPARγ2 in mouse cultured hepatocytes (Edvardsson *et al.*, 2006). Normal mice fed a HFD had an increased expression of PPARγ2 in the liver (Vidal-Puig *et al.*, 1996). Induction of PPARγ2 in the liver might be an adaptive response to increase lipid utilisation. Due to the more active ligand–independent AF-1 domain in PPARγ2, perhaps it is more responsive to signaling events induced by insulin, glucose and lipids (Werman *et al.*, 1997). Understanding the precise role of PPARγ in the liver remains an interesting research avenue.

9 Regulation of cholesterol synthesis

9.1 AMP-activated protein kinase (AMPK)

AMPK is the major cellular energy sensor and master regulator of cellular energy homeostasis. AMPK is a heterotrimeric enzyme comprised of a catalytic subunit (α1 or α2) and two regulatory subunits (β1 or β2 and γ1, γ2 or γ3), all encoded by separate genes (Kahn *et al.*, 2005). AMPK is activated by three distinct pathways either by a calcium-dependent pathway involving Ca2+/calmodulin-dependent protein kinase kinase (CaMKKβ), by AMP-dependent pathway mediated by liver kinase B1 (LKB1) and recently by TGFβ-activated kinase-1 (TAK1) (Shaw *et al.*, 2004;Momcilovic *et al.*, 2006;Sanders *et*

al., 2007). CaMKK expression unlike LKB1 and TAK1, is more abundant in neuronal and haematopoietic cells. As seen in Figure 19, CaMKKβ is activated by an increase in intracellular Ca^{2+} released by the ER which in turn activates AMPK. Although still unknown, in activated T lymphocytes, it is speculated that the rise in Ca^{2+} stimulates a feed-forward signal that anticipates a large demand for ATP (Tamas *et al.*, 2006). Autophagy is the degradation of the cell's own internal organelles in development, differentiation, and tissue remodelling (Shintani & Klionsky, 2004). It is a major mechanism by which a cell recycles nutrients from unnecessary processes towards indispensable ones. Tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a multifunctional cytokine originally identified as an apoptosis-inducing member of the TNF superfamily. TRAIL-induced autophagy is dependent on AMPK which is phosphorylated by TAK1 (Herrero-Martin *et al.*, 2009). LKB1 is an important tumor suppressor and the LKB1-AMPK pathway is activated by the elevation in the AMP/ATP ratio (Hardie *et al.*, 1999). Inactive LKB1 is predominantly found in the nucleus (Nezu *et al.*, 1999).

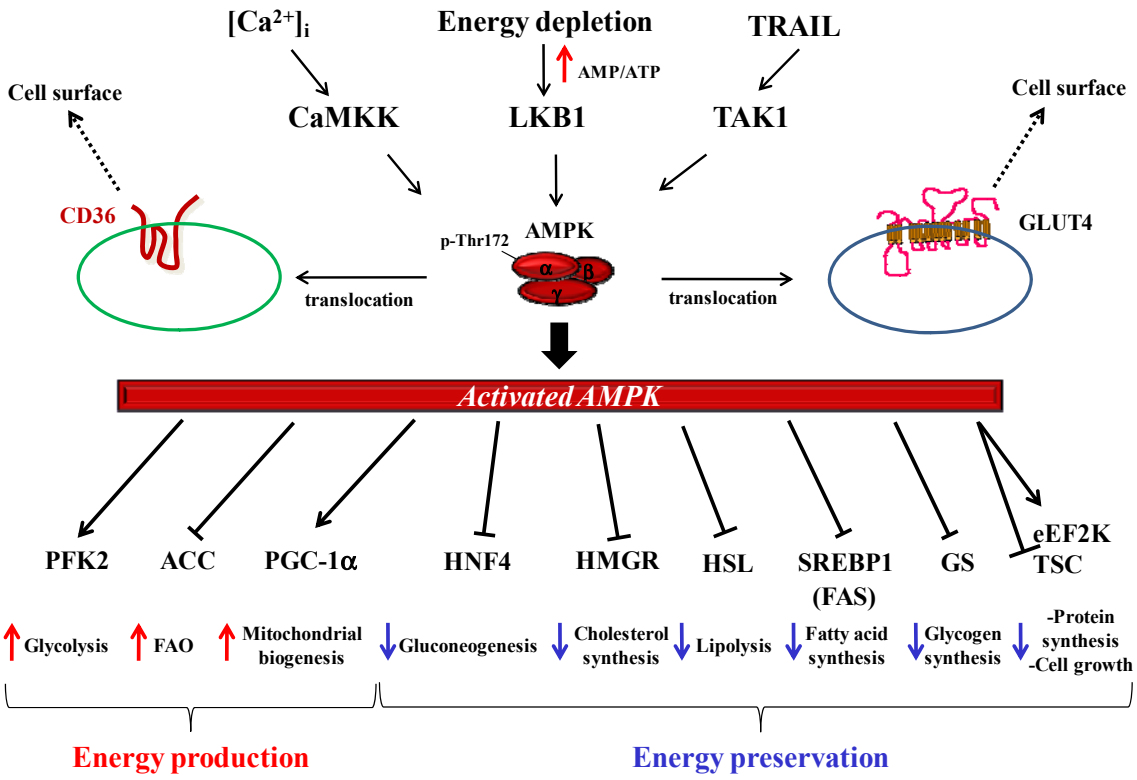


Figure 19. Activation of AMPK and its effect on downstream targets and events

LKB1 is activated through obligatory phosphorylation of Ser428 and translocated to the cytoplasm following changes in the AMP/ATP ratio (Shaw *et al.*, 2004;Xie *et al.*, 2008). AMP binds to the regulatory γ subunit of AMPK and induces a conformational change rendering the kinase less susceptible to dephosphorylation of Thr-172 (Riek *et al.*, 2008). All 3 kinases phosphorylate AMPKα subunit at Thr-172 which is an absolute requirement for AMPK activity (Hawley *et al.*, 1996). Figure 19 displays some important targets of AMPK and their downstream effect. Understandably, depending on the tissue, some of these pathways more than others might be greatly affected by AMPK activation. As discussed previously, activation of AMPK can result in the translocation of GLUT4 or CD36 to the cell surface promoting glucose and fatty acid uptake, respectively (Merrill *et al.*, 1997;Bonen *et al.*, 2007;Luiken *et al.*, 2003;Chabowski *et al.*, 2005). Glycogen is an important source of energy and an important substrate in muscle (exercise) and in liver

(fasting). In a state of increased energy demand, glycogen synthesis is inhibited through the inhibition of its key enzyme, glycogen synthase (GS) by AMPK (Carling & Hardie, 1989). In addition, AMPK promotes the breakdown of glucose by activating phosphorylating 6-phosphofructokinase-2 (PFK2) (Marsin *et al.*, 2000). As previously presented in section 7.8.2, AMPK decreases the content of malonyl-CoA by inhibiting acetyl-CoA carboxylase (ACC) and consequently stimulates fatty acid oxidation and inhibits fatty acid synthesis (Ha *et al.*, 1994). In addition, AMPK also decreases the expression of SREBP-1c and subsequently fatty acid synthase (FAS), although the mechanisms by which AMPK acts on both are still unknown (Foretz *et al.*, 2005). Following AMPK activation, FAS was found to be phosphorylated and inactivated in 3T3-L1 adipocytes (An *et al.*, 2007). As presented in Figure 10 and section 7.6, AMPK inactivates HSL and reduces lipolysis rate in cells (Djouder *et al.*, 2010). AMPK also has an impact on protein synthesis and cell growth. AMPK phosphorylates and activates eukaryote elongation factor 2 kinase (eEF2K), which in turn phosphorylates eEF2 and inhibits protein synthesis (Browne *et al.*, 2004). AMPK activates the tumor suppressor tuberous sclerosis 2 (TSC2) and results indirectly in the inactivation of mammalian target of rapamycin (mTOR), which controls among other things protein synthesis and cell growth (Reiter *et al.*, 2005;Inoki *et al.*, 2006). Activation of AMPK also leads to targeted phosphorylation of PGC-1α and its subsequent activation for induction of mitochondrial gene expression, biogenesis and fatty acid oxidation (Jager *et al.*, 2007).

Overall, once activated, AMPK's main goal is to switch on catabolic pathways that generate ATP, while switching off ATP-consuming processes such as biosynthesis, cell growth and proliferation. The remainder section on AMPK will focus on its role in hepatocytes as it pertains to the present thesis and the second part of my project.

9.1.1 AMPK in the liver

The liver is central in maintaining glucose homeostasis and energy storage. Its energy metabolism changes drastically throughout the day between physiological

conditions such as when nutritionally depleted or replenished. The liver is therefore the mediator between endogenous/dietary energy sources and extrahepatic organs. Due to its high energy demand, hepatic AMPK plays an important role in fatty acid oxidation and controls lipogenesis and glucose production. Gluconeogenesis represents the generation of glucose from sources such as lactate, glycerol, pyruvate and glucogenic amino acids (alanine and glutamine). Glucose production is controlled by transcription factor hepatic nuclear factor 4α (HNF-4α) which regulates the expression of key enzymes such as L-type pyruvate kinase, and PEPCK (Hall *et al.*, 1995;Leclerc *et al.*, 2001). AMPK influences glucogenic gene expression by phosphorylating and inhibiting HNF-4α and therefore gluconeogenesis (Figure 19) (Hong *et al.*, 2003). De novo cholesterol synthesis occurs in all cells; however, it is prominent in the liver due to its role in lipid distribution (Babin & Gibbons, 2009). HMG-CoA reductase (HMGR) is the rate limiting enzyme for isoprenoid and cholesterol synthesis and its activity is regulated by states of phosphorylation and dephosphorylation (Beg *et al.*, 1978). AMPK is the only kinase to date known to phosphorylate and inactivate HMGR at Ser-872 (Clarke & Hardie, 1990).

9.2 HMG-CoA reductase

HMGR, the rate-controlling enzyme in cholesterol synthesis, is a 97-kDa integral protein of the endoplasmic reticulum (ER) which catalyzes the synthesis of mevalonate from HMG-CoA (Liscum *et al.*, 1983). Statins are inhibitors of HMGR and are the most widely prescribed cholesterol-lowering drugs in humans. Statins are competitive inhibitors of HMGR, possessing a HMG-like moiety which occupies a portion of the HMG-CoA binding site (Endo *et al.*, 1976). HMGR contains two distinct structural domains: a C-terminal catalytic domain of 549 aa and a N-terminal of 339 aa (Liscum *et al.*, 1985). As shown in Figure 20, HMGR is integrated into the ER membrane with the help of its eight membrane-spanning N-terminal region while the C-terminal domain projects into the cytosol where it exerts its catalytic activity (Roitelman *et al.*, 1992). The N-terminal region is extremely well conserved among mammalian species indicating a role beyond that of a

membrane anchorage domain (Luskey & Stevens, 1985). Helices 2 to 6 of HMGR show sequence resemblance to helices 2 to 6 of SREBP cleavage-activating protein (SCAP) which will be presented later. The effects of sterols on the activity of HMGR and SCAP are mediated through this section called the sterol-sensing domain (SSD) within the N-terminal region (Hua *et al.*, 1996).The complexity of HMGR regulatory system was first exposed with the use of compactin, a member of the statin family by the Nobel Prize laureates, Joseph Goldstein and Michael Brown (Brown *et al.*, 1978). Treatment of human fibroblasts with compactin blocked the synthesis of mevalonate. Cells strongly responded by drastically increasing the level of HMGR owing to the increased transcription of its gene, the efficient translation of its mRNA and the prolonged half-life of HMGR.

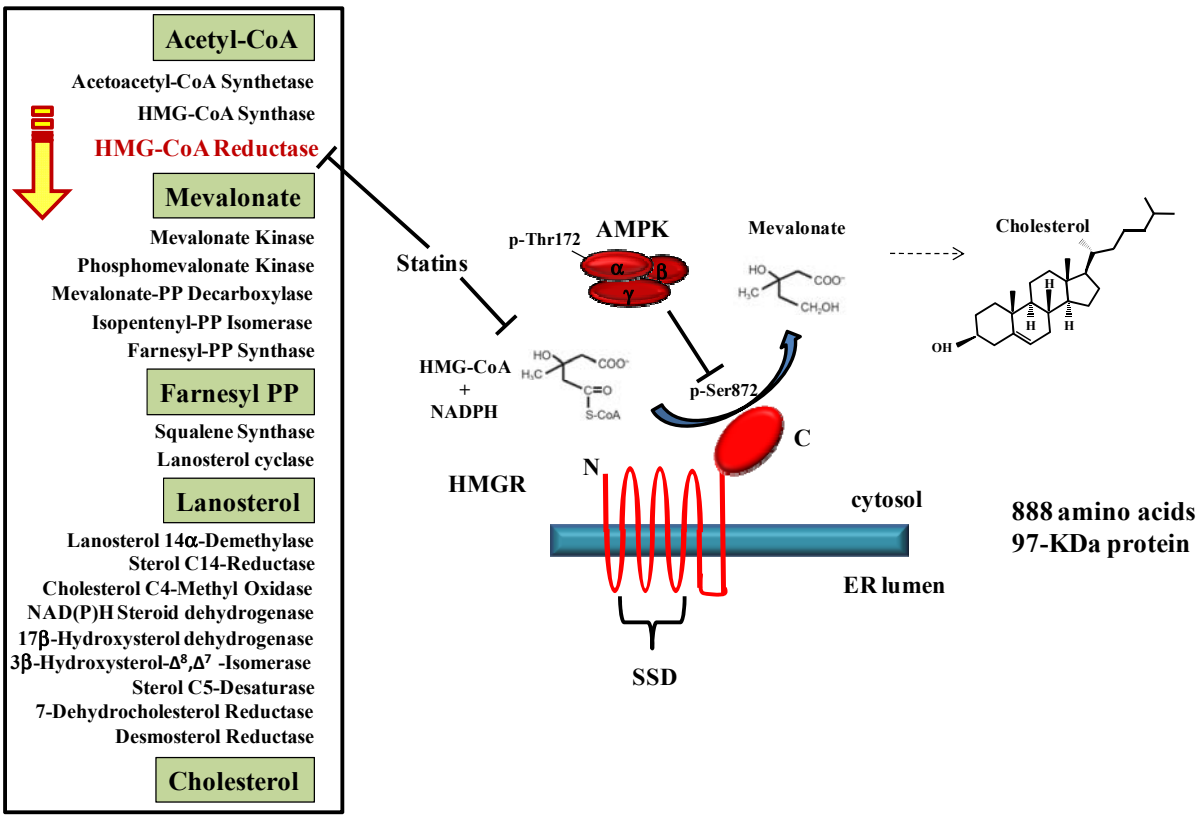


Figure 20. Cholesterol synthesis pathway and structure of its rate-controlling enzyme, HMG-CoA reductase

9.2.1 Phosphorylation of HMGR

Over the years, HMGR has proven to be an exceedingly regulated enzyme. Early on, it was determined that HMGR activity in the liver was decreased following feeding or in fibroblasts treated with LDL; however the rapid decline in activity could not be explained by degradation or reduced protein synthesis (Brown *et al.*, 1973;Higgins & Rudney, 1973). In addition, HMGR activity was found to be rapidly modulated by ATP/Mg^{2+} (Beg *et al.*, 1973). As mentioned in the section on AMPK, it was later proven that phosphorylation of HMGR by the so called HMGR kinase was in fact AMPK which targeted HMGR at Ser-872 and resulted in rapid inhibition of cholesterol synthesis (Clarke & Hardie, 1990).

9.2.2 Gene expression regulation of HMGR

The main transcriptional regulators of HMGR are the basic helix-loop-helix (bHLH) leucine zipper sterol regulatory element-binding proteins (SREBPs). There are 3 major isoforms of SREBP encoded by 2 genes: SREBP1a and 1c produced from the same gene by two different promoters and SREBP-2 (Hua *et al.*, 1995;Miserez *et al.*, 1997). SREBP-1a and -1c are similar with the exception of their N-terminal activation domain responsible for their differing co-activator interaction (Toth *et al.*, 2004). SREBP-1a and SREBP-2 have similar potent activation domains. SREBPs regulate the expression of target genes involved in cholesterol, fatty acid and triglyceride syntheses. SREBP-1c and -2 predominates in the liver (Shimomura *et al.*, 1997). SREBP-2 has a preference for cholesterol synthesis genes while SREBP-1 is associated with fatty acid synthesis genes; however, all 3 isoforms can drive the expression of HMGR by binding to its SRE in the proximal promoter region of this gene (Osborne *et al.*, 1988;Horton *et al.*, 2002;Horton *et al.*, 2003;Bennett *et al.*, 2008).

In one study, PPARγ was suggested to regulate the expression of HMGR through the use of a PPRE decoy in THP-1 macrophages (Iida *et al.*, 2002). Tro- and pioglitazone were found to upregulate the expression of HMGR while the presence of PPRE decoy

competing for PPARγ binding suppressed the expression of HMGR. Thyroid hormone (T3) is known to increase the expression of HMGR despite a lack of thyroid response element in HMGR promoter (Simonet & Ness, 1988). Recently, thyroid-stimulating hormone was found to increase the expression of HMGR through the cyclic adenosine monophosphate/protein kinase A/cyclic adenosine monophosphate–responsive element binding protein (cAMP/PKA/CREB) signaling system (Tian *et al.*, 2010).

The synthesis of one molecule of cholesterol from acetyl-CoA requires 11 molecules of O_2; therefore in a hypoxic state, the shutdown of the cholesterol synthesis pathway is mandatory to prevent accumulation of methylated sterols such as lanosterol and 24,25-dihydrolanosterol without affecting the activity of SREBP (Nguyen *et al.*, 2007). In such a condition, hypoxia-inducible factor α escapes degradation and modulates the expression of more than 70 genes involved in oxygen deprivation. Paradoxically, while HIF-1α stimulates degradation of HMGR, it also stimulates its expression by interacting with HRE found in the promoter region of HMGR (Pallottini *et al.*, 2008). Since SREBPs are not affected by hypoxia, HMGR expression can also increase through SREBP when sterol levels decrease.

9.3 Regulation of cholesterol synthesis by sterols

It was observed early on that incubation of cultured cells with LDL (cholesterol), 25-hydroxycholesterol and mevalonate resulted in the decrease of HMGR activity and its cellular protein content (Faust *et al.*, 1982;Edwards *et al.*, 1983). This drastic decrease in cholesterol synthesis could be explained by two major and distinct mechanisms: (1) decrease in the expression of HMGR (Luskey *et al.*, 1983) and (2) accelerated degradation of HMGR (Chin *et al.*, 1985). A major breakthrough in the understanding of the mechanism of regulation of cholesterol synthesis by sterols came with the discovery of the insulin-inducible genes-1 and -2 (insig-1 and -2) proteins and the subsequent elucidation of their role in HMGR degradation and, SREBP processing and maturation.

9.3.1 Insig-1 and -2

Insig-1 was first cloned in rat as the most highly insulin-induced gene in liver (Diamond *et al.*, 1993). The human gene was then cloned and insig-1 was found to be highly expressed in adipose tissue and liver especially during differentiation and regeneration, respectively (Peng *et al.*, 1997). Later, insig-1 was cloned from HepG2 cells and identified as an ER protein interacting with SCAP thus identifying insig-1 as a key player in cholesterol homeostasis (Yang *et al.*, 2002). Insig-2 was cloned shortly after and shown to play a similar role to insig-1 (Yabe *et al.*, 2002). Insig-1 and -2 have comparable expression patterns, expressed in multiple tissues but at higher level in liver (Yabe *et al.*, 2002). Despite their functional similarities, insig-1 and -2 conceal important differences. Insig-1 and insig-2 are encoded by two different genes and their proteins are 59% identical. Insig-1 is a target of SREBP and therefore insig-1 mRNA levels are high in sterol-depleted cells (Janowski, 2002). Insig-2 is not a target of SREBPs and is more stable than insig-1. Recently, it was found that insig-2 expression was mediated by the phosphorylated Ets family member of transcription factors involved in cell proliferation and differentiation, SRF accessory protein-1a (SAP1a) (Fernandez-Alvarez *et al.*, 2010). Insig-1 is rapidly ubiquitinated and degraded in comparison to insig-2 (Lee & Ye, 2004). In a sterol-depleted environment, insig-1 is degraded 15 times faster than insig-2 and this difference is due to optimal ubiquitination sites present in insig-1 but absent in insig-2 (Lee *et al.*, 2006). Since insig-2 has a longer life span and its degradation is not mediated by sterols, insig-2 is an interesting target in modifying cholesterol homeostasis outside the tightly regulated mechanism observed with insig-1 (Lee & Ye, 2004;Gong *et al.*, 2006).

9.3.2 Sterol-regulated SCAP-SREBP pathway

As mentioned previously, similarly to HMGR, SCAP possesses a SSD region within its N-terminal transmembrane region while the C-terminal region mediates constitutive association with SREBP (Nohturfft *et al.*, 1998). In addition to SCAP's role as a sterol

sensor, it also serves as an escort protein bringing SREBP to the Golgi for processing (Hua *et al.*, 1996). When depleted in cellular sterols (Figure 21, upper box), SCAP enables SREBP to cluster with coat protein complex II (COPII consisting of Sar-1, Sec23/Sec24 complex, and Sec13/Sec31 complex) and form a vesicle that will be carried to the Golgi where SREBP will then be cleaved by 2 proteases (Site-1 protease (S1P) and Site-2 protease (S2P)) releasing the basic helix-loop-helix leucine zipper (bHLH) active fragment (Horton *et al.*, 2002). The NH2-terminal domain nuclear SREBP (nSREBP) translocates to the nucleus and activates transcription of target genes. Sec24 binds to the hexapeptide sorting signal, MELADL on SCAP, this interaction is required for the translocation of SREBP to the Golgi (Sun *et al.*, 2005).

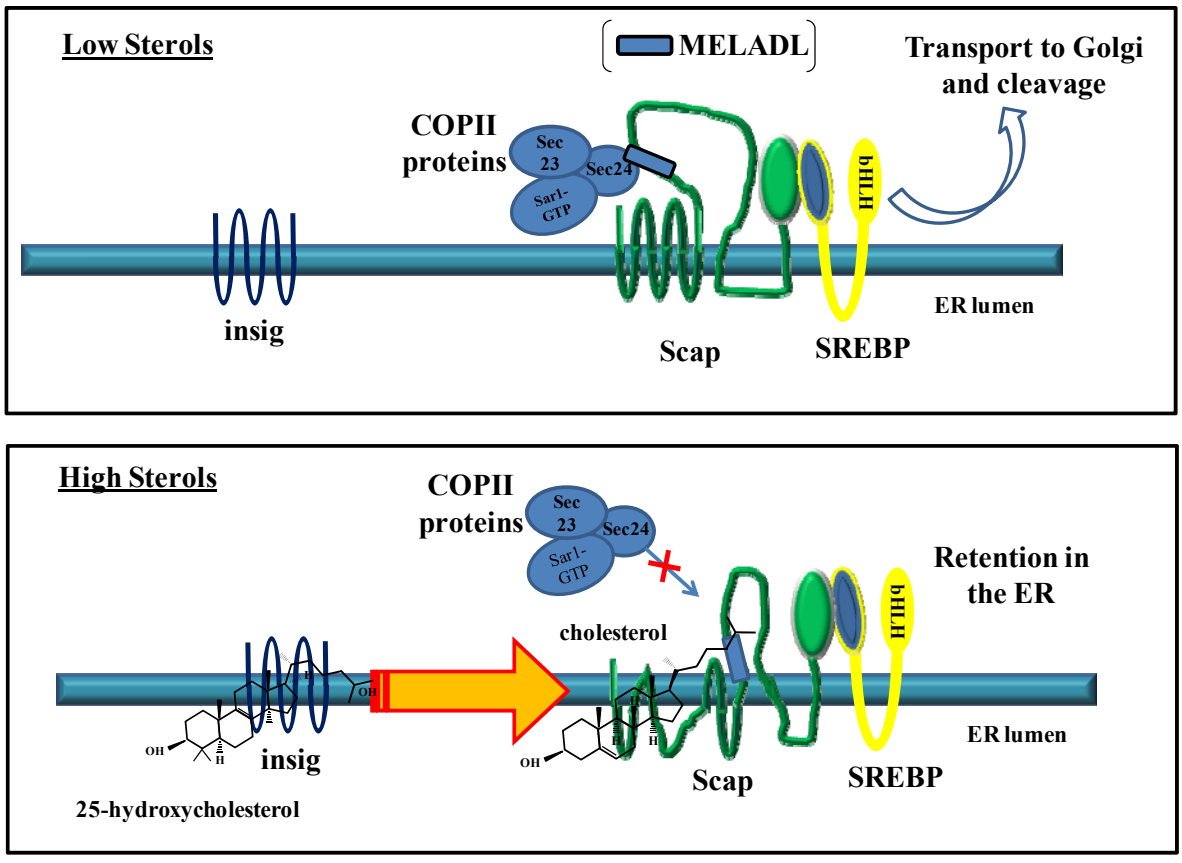


Figure 21. Mechanistic schema showing how sterols and insigs regulate post-translational proteolysis and activation of SREBPs

As shown in Figure 21, lower box, cholesterol and oxysterol block this binding by inducing an interaction between insig and SCAP but through different mechanisms (Yabe *et al.*, 2002;Yang *et al.*, 2002;Adams *et al.*, 2003). With a rise in ER sterols, cholesterol binds to SCAP and induces a conformational change in SCAP ,making MELADL no longer available to Sec24 while insig interacts with SCAP resulting in the overall retention of the SCAP/SREBP complex in the ER (Sun *et al.*, 2007b). Alternatively, insig can interact with oxysterol triggering insig to bind to SCAP. The end-result of this interaction is a decrease in the level of nuclear transcriptionally active SREBP and a decrease in the expression of SREBP target genes.

9.3.3 Degradation of HMGR

Much of our understanding of the mechanism for sterol-accelerated degradation of HMGR stemmed from the comparison between SCAP and HMGR. Both insig-1 and insig-2 are capable of inducing the degradation of HMGR (Sever *et al.*, 2003). As shown in figure 22, when sterols accumulate in the ER, a conformational change occurs in the sterol-sensing domain of HMGR and promotes the binding of either insig-1 or insig-2 to HMGR resulting in a rapid ubiquitination and degradation of HMGR through the ubiquitin-proteosome system (Song *et al.*, 2005;Sever *et al.*, 2003). Accumulation of lanosterol mediates HMGR binding to insig while oxysterol mediates interaction of insig with HMGR (Song *et al.*, 2005). Overall, the swift degradation of HMGR protein and the retention of SREBP in the ER prevent the accumulation of sterols to toxic levels within the cell. Not much has been shown to directly affect the degradation of HMGR independently of the effect of intracellular sterol levels. Only a few studies have demonstrated that it is possible to bypass the firm control exerted by sterol levels. Such an example show forms of vitamin E that can induce degradation of HMGR by mimicking sterols (antioxidants) demonstrating the potential to increase the degradation of HMGR independent of the influence of intracellular cholesterol levels (Song & DeBose-Boyd, 2006).

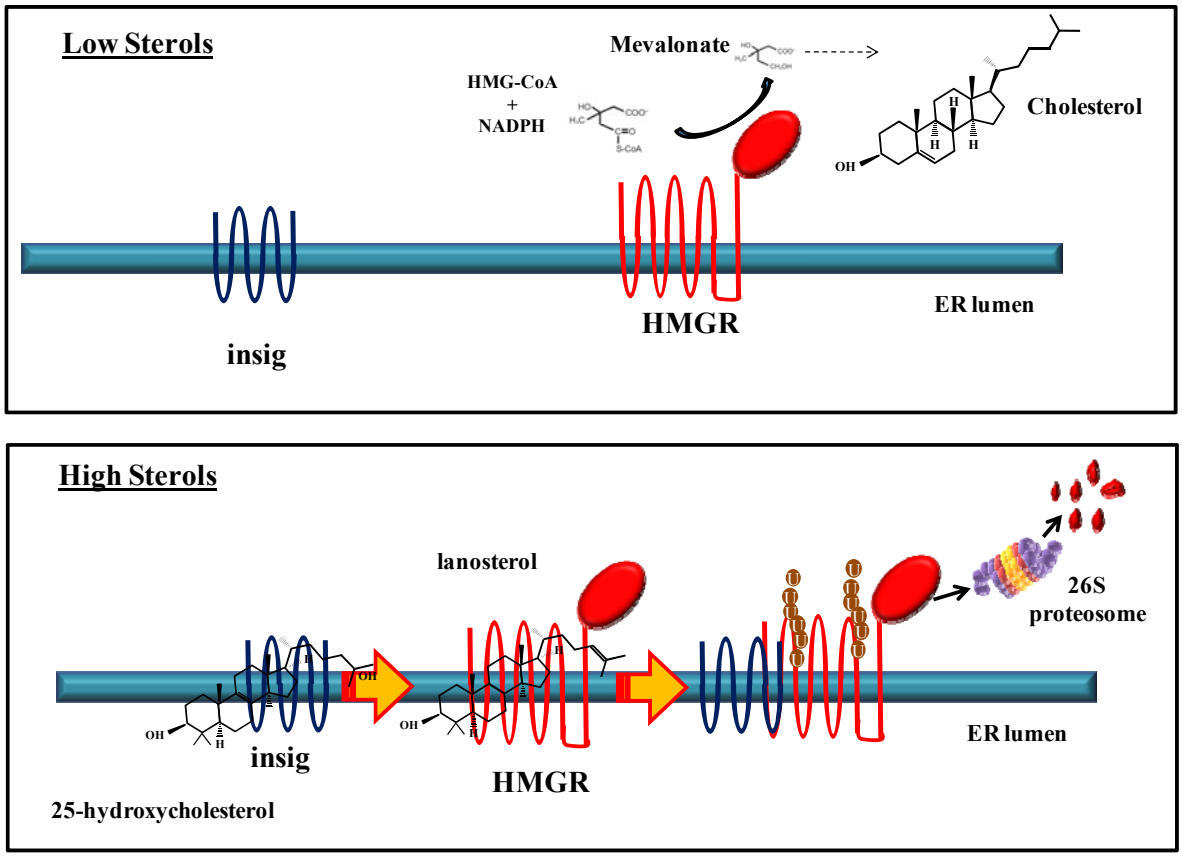


Figure 22. Mechanistic schema showing how sterols and insigs regulate HMGR degradation

10 Effect of hexarelin through its interaction with CD36

Extensive studies have been made on hexarelin as a *bona fide* GHRP; therefore, results were interpreted in connection with its binding to GHS-R1a in a GH-dependent or GH –independent manner while mostly disregarding its ability to bind CD36 (Loche *et al.*, 1995;Cella *et al.*, 1996;Desaphy *et al.*, 1998). A limited amount of papers looked at the effect hexarelin on GHS-R1a–negative tissues such as adipocytes, hepatocytes or skeletal muscle cells. Some studies reported effects of hexarelin on skeletal muscle and adipose tissue in aging subjects but as a GH-releasing peptide (Cella *et al.*, 1996;Desaphy *et al.*, 1998). In rat isolated epididymal white adipocytes, hexarelin reduced isoproterenol-induced

lipolysis (β-adrenergic receptor agonist) but no further effects were investigated (Muccioli *et al.*, 2004). Accumulation of fibrillar β-amyloid protein occurs in the brain of patients with Alzheimer's disease and in atherosclerotic plaques. β-amyloid is also a CD36-ligand that initiates a signaling cascade involving Src kinases and Erk1/2 responsible for the downstream inflammatory response (Moore *et al.*, 2002). The effect of hexarelin on inflammatory response was studied in N9 microglia cells, which are specialized macrophages found in the brain, that express CD36 but not GHS-R1a (Bulgarelli *et al.*, 2009). Hexarelin interfered with β-amyloid inflammatory response as seen by the impeded rise in IL-6 mRNA. Independently from the effect of GH release, hexarelin was shown to possess cardioprotective properties by preventing ventricular dysfunction and by protecting the heart from postischemic reperfusion damage (Locatelli *et al.*, 1999;De Gennaro-Colonna *et al.*, 1997). Ghrelin was far less effective at preventing ischemia-reperfusion damage therefore owing the beneficial effects of hexarelin mainly to CD36 (Torsello *et al.*, 2003). Macrophage infiltration and the subsequent inflammatory response play a major role in the damage and necrosis caused during reperfusion after ischemia (Vilahur *et al.*, 2011;Bohle *et al.*, 1991). Considering the impact that CD36 could have on the heart's function and mainly on the development of atherosclerosis, our group set off to determine the mechanism of action of CD36 and hexarelin in macrophages and its impact on atherosclerosis. Huy Ong, in collaboration with André Tremblay and their respective team showed that treatment of ApoE-null mice on a HFD with EP80317, a CD36 ligand derived from the GHRP family, resulted in a marked decrease in lesion areas (Marleau *et al.*, 2005). The interaction with CD36 was suggested to interfere with oxLDL binding on macrophages and resulted in the activation of PPARγ-LXRα-ABC transporters cascade involved in reverse cholesterol pathway. Using hexarelin to study its interaction with GHS-R1a and CD36, our group also observed a significant regression in the size of atherosclerotic lesions in ApoE-null mice maintained on a HFD (Avallone *et al.*, 2006). Treatment of differentiated THP-1 macrophages and mouse peritoneal macrophages with hexarelin resulted in an increase in cholesterol efflux. This increase in efflux correlated with an

increase in the expression of LXRα, ApoE, ABCA1 and ABCG1, the key players involved in the HDL-mediated cholesterol efflux pathway (Figure 23). The expression of LXRα is mediated by PPARγ and considering the potential of CD36 to activate PPARγ via internalization of oxLDL, PPARγ activity in response to hexarelin was analyzed. In PPARγ +/- peritoneal macrophages, the response to hexarelin was strongly impaired implying that the activation of PPARγ was critical. Furthermore, cell reporter assays showed that the interaction of hexarelin with either CD36 or GHS-R1a resulted in the activation PPARγ. The activation of PPARγ did not lead to an increase in CD36 expression as seen with binding to oxLDL and the subsequent positive autoregulatory loop. Surprisingly, the PPARγ LDB was not necessary for its activation by hexarelin suggesting that AF-1 might mediate transcriptional activation in response to intracellular signaling transduction pathways. In support of this, phosphorylation of PPARγ was observed in treated THP-1 cells. In macrophages, hexarelin interferes with the balance between uptake and efflux that usually leads to intracellular cholesterol accumulation and foam cell formation. Through interaction with CD36 and GHS-R1a, hexarelin is capable of (1) activating PPARγ in a ligand-independent manner, (2) breaking the autoregulatory loop leading to an increase in CD36 expression and (3) increasing overall cholesterol efflux from cells.

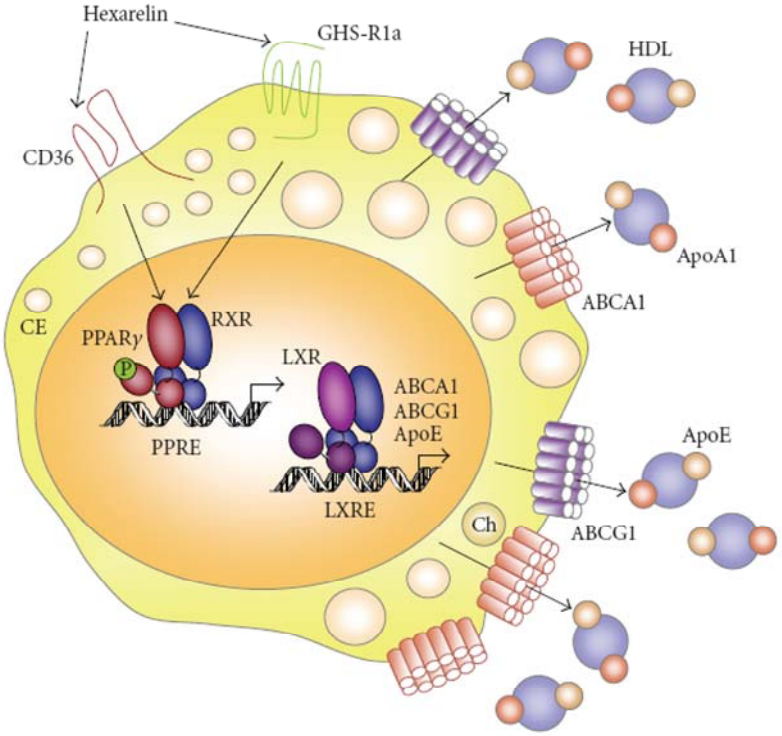


Figure 23. Hexarelin-mediated activation of the PPARγ-LXRα-ABC metabolic pathway in macrophages (Demers et al., 2008)

11 Hypothesis and project objectives

Differing roles and various impact of CD36 on health arise mainly from its ligand selection, tissue type and level of expression. While the role of CD36 in atherosclerosis via oxLDL internalization has been well characterized, emerging studies on the influence of CD36 in fatty acid uptake and in lipid metabolism in muscle demonstrate that lipid metabolism via CD36 remains an interesting avenue to explore but on a different front than its role in atherosclerosis. Beyond its role in cardiac and skeletal muscles, far less is known about the influence of CD36 in adipocytes and in hepatocytes, especially when downstream signaling events and overall effect following ligand binding are concerned.

The selected project for my graduate studies aimed mainly at understanding the role of CD36 in lipid metabolism outside the realm of its impact on atherosclerosis. Considering

the beneficial effect that was uncovered in macrophages, the use of hexarelin solely as a CD36-ligand offered the unique opportunity of looking at the events following its interaction with CD36 in cell types where the function of CD36 is far less known. Since our group has shown that hexarelin activates PPARγ in macrophages and that CD36 is responsible for the uptake of LCFA, we hypothesized that hexarelin through its interaction with CD36 will be capable of modulating PPARγ activity and influencing lipid homeostasis in both adipocytes and hepatocytes.

The objectives of my doctoral studies were:

I. To determine the impact of hexarelin binding to CD36 on lipid metabolism in differentiated adipocytes and adipose tissue by:

a) Establishing, as an investigative measure, a gene expression profile of adipocytes treated with hexarelin using microarray technology to explore known and novel outcomes of CD36 activation

b) Using troglitazone to illustrate possible similarities and differences in gene expression changes and downstream events between hexarelin/CD36 and PPARγ activation in adipocytes

c) Determining total lipid content and potential lipid mobilization pathways in in response to hexarelin

e) Confirming the effect(s) of hexarelin via CD36 *in vivo*

II. Based on the findings that hexarelin via CD36 increases fatty acid oxidation (Part I) and on the fact that CD36 was not known to be a major FA transporter in hepatocytes, the possible impact of hexarelin on cholesterol synthesis was explored by:

a) Determining if LKB1-AMPK is activated and if the downstream HMGR was targeted

b) Measuring total cholesterol and HMGR protein content in hepatocytes

c) Determining the mechanism involved in decrease cholesterol synthesis

c) Exploring the signaling pathways involved in PPARγ activation and its effect on cholesterol synthesis

CHAPTER 2: Results

First author publications

A growth hormone-releasing peptide promotes mitochondrial biogenesis and a fat burning-like phenotype through scavenger receptor CD36 in white adipocytes. **Rodrigue-Way A**, Demers A, Ong H, Tremblay A. **Endocrinology 2007 Mar;148(3):1009-18**.

Scavenger Receptor CD36 mediates inhibition of cholesterol synthesis via activation of the LKB1-AMPK pathway in hepatocytes. **Amélie Rodrigue-Way**, Stéphanie Bilodeau, Meryl Hassan, Véronique Caron, Emile Lévy, Grant A. Mitchell, and André Tremblay. In preparation for Molecular Endocrinology.

Co-author publications

A concerted kinase interplay identifies PPARgamma as a molecular target of ghrelin signaling in macrophages. Demers A, Caron V, **Rodrigue-Way A**, Wahli W, Ong H, Tremblay A. **PLoS One 2009 Nov 4;4(11)e7728**.

A growth hormone-releasing peptide that binds scavenger receptor CD36 and ghrelin receptor up-regulates sterol transporters and cholesterol efflux in macrophages through a peroxisome proliferator-activated receptor gamma-dependent pathway. Avallone R, Demers A, **Rodrigue-Way A**, Bujold K, Harb D, Anghel S, Wahli W, Marleau S, Ong H, Tremblay A. **Molecular Endocrinology 2006 Dec;20(12):3165-78**.

Hexarelin Signaling to PPARgamma in Metabolic Diseases. Demers A**, Rodrigue-Way A**, Tremblay A. **PPAR Res. 2008;2008:364784.** Review

1 Paper 1

A Growth Hormone-Releasing Peptide Promotes Mitochondrial Biogenesis and a Fat Burning-Like Phenotype through Scavenger Receptor CD36 in White Adipocytes

Amélie Rodrigue-Way, Annie Demers, Huy Ong and André Tremblay

Research Center (A.R.-W., A.D., A.T.), Ste-Justine Hospital, and Departments of Biochemistry (A.R.-W., A.T.) and Obstetrics and Gynecology (A.T.), Faculty of Medicine, University of Montréal, Montréal, Québec, Canada H3T 1C5; and Faculty of Pharmacy (A.D., H.O.), Pavillon Jean-Coutu, University of Montréal, Montréal, Québec, Canada H3C 3J7

Short title: Fat Burning via CD36 in White Fat Cells

Disclosure Statement: The authors have nothing to disclose.

Abbreviations: ABC, ATP-binding cassette transporter; aP2, adipocyte- specific fatty acid binding protein; COX, cytochrome c oxidase; CPT, carnitine palmitoyltransferase; FATP, fatty acid transport protein; FBS, fetal bovine serum; GPAT, glycerol-3-phosphate acyltransferase; GHRP, GH-releasing peptide; LXR, liver X receptor; oxLDL, oxidized low-density lipoproteins; PGC-1, PPAR coactivator 1; PPAR, peroxisome proliferator-activated receptor; TIM, translocase of the inner membrane; UCP, uncoupling protein.

Foreword for paper #1

The accumulation of lipid-laden cells in atherosclerotic plaques originates mainly from monocytes/macrophages internalizing oxLDL particles and subsequently differentiating into foam cells. The involvement of CD36 in the development of atherosclerotic plaques was elucidated with the advent of the CD36/apoE-null mice (Febbraio *et al.*, 2000). The absence of CD36 in the atherosclerotic mouse model resulted in a clear decrease in atherosclerotic lesion sizes owing to a reduced internalization of oxLDL particles. The treatment of ApoE-null mice on a HFD with a GHRP was also capable of reducing lesion sizes (Avallone *et al.*, 2006). As presented in Section 10, we had previously shown that hexarelin was capable of activating PPARγ via its binding to CD36 on macrophages. In addition to promoting adipogenesis, PPARγ is crucial in the maintenance of the mature adipocyte's function (Imai *et al.*, 2004). Targeted deletion of PPARγ leads to cell death, loss of fat mass, elevated circulating FFA and TG while activation results in increase FA uptake, TG synthesis but also FAO (He *et al.*, 2003;Wilson-Fritch *et al.*, 2003).

The first part of my project was aimed at studying the effect of hexarelin by looking at expression changes of genes involved in lipid metabolism in mature adipocytes and compare those changes with those stemming from the activation of PPARγ. Since CD36 plays an important role in FA uptake and metabolism, special attention was given to intracellular lipid content in adipocytes. From this approach, we discovered that hexarelin had a transdifferentiation-like effect of white adipocytes into brown adipocytes causing an increase in FAO and a reduction in lipid content in a manner dependent on the presence of CD36.

Contributions of authors:

Amélie Rodrigue-Way: Developed project. Designed, performed experiments and analyzed results. Wrote the first draft of the manuscript.

Annie Demers: Helped with mouse adipose tissue. Participated in project and paper discussion.

Huy Ong: Designed in vivo experiments. Participated in project discussion.

André Tremblay: As my PhD supervisor, supervised and participated in the conception of my project and the design of experiments. Corrected and submitted finished manuscript.

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TIN Number: 73-0531256

Form was submitted by: Amelie Rodrigue-Way
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Journal: Endocrinology
Author Name: A. Rodrigue-Way
Title: A Growth Hormone-Releasing Peptide Promotes Mitochondrial Biogenesis and a Fat Burning-Like Phenotype through Scavenger Receptor CD36 in White Adipocy
Year: 2007
Volume: 148
Page Range: 1009-1018
Figure Reproduction:
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Title: Journal Publications Coordinator
Date: March 24, 2011

Abstract

Whereas the uptake of oxidized lipoproteins by scavenger receptor CD36 in macrophages has been associated with foam cell formation and atherogenesis, little is known about the role of CD36 in regulating lipid metabolism in adipocytes. Here we report that treatment of 3T3-L1 adipocytes with hexarelin, a GH-releasing peptide that interacts with CD36, resulted in a depletion of intracellular lipid content with no significant change in CD36 expression. Microarray analysis revealed an increased pattern in several genes involved in fatty acid mobilization toward the mitochondrial oxidative phosphorylation process in response to hexarelin. Interestingly, many of these up-regulated genes are known targets of peroxisomal proliferator-activated receptor (PPAR)γ, such as FATP, CPT-1, and F_1-ATPase, suggesting that adipocyte response to hexarelin may involve PPAR activation. Expression studies also indicate an increase in thermogenic markers PPAR coactivator 1α and uncoupling protein-1, which are normally expressed in brown adipocytes. Electron microscopy of hexarelin-treated 3T3-L1 adipocytes showed an intense and highly organized cristae formation that spans the entire width of mitochondria, compared with untreated cells, and cytochrome c oxidase activity was enhanced by hexarelin, two features characteristic of highly oxidative tissues. A similar mitochondrial phenotype was detected in epididymal white fat of mice treated with hexarelin, along with an increased expression of thermogenic markers that was lost in treated CD36-null mice, suggesting that the ability of hexarelin to promote a brown fat-like phenotype also occurs in vivo and is dependent on CD36. These results provide a potential role for CD36 to impact the overall metabolic activity of fat usage and mitochondrial biogenesis in adipocytes.

Introduction

THE MAJOR ROLE of the adipose tissue is to store energy in the form of triglycerides and release it as fatty acids in response to an increase in energy demand, such as during fasting or exercise. Peripheral tissues such as skeletal muscle and heart oxidize fatty acids in mitochondria to produce ATP. However, when energy storage is in excess, such lipid accumulation in adipose tissue can result in many pathological states associated with the metabolic syndrome, including central obesity, type 2 diabetes, and insulin resistance (1, 2).

The scavenger receptor CD36, also known as fatty acid translocase, is expressed in adipocytes to mediate the uptake of long chain fatty acids (3), but much of the characterization of the role of CD36 has focused on its scavenging ability to interact and mediate the internalization of oxidized low-density lipoproteins (oxLDL) in macrophages. The selective uptake of oxLDL by CD36 is considered a critical step in the atherogenic formation of foam cells in the extracellular matrix of lesion-prone sites of the arterial wall (4). In addition to initiating a proinflammatory response by monocytes/macrophages, such internalization of oxLDL by CD36 provides a source of oxidized fatty acids and oxysterols that serve as endogenous ligands for the activation of the nuclear receptors peroxisomal proliferator-activated receptor (PPAR)γ and liver X receptor (LXR)α, and subsequent up-regulation of downstream targets involved in reverse cholesterol transport, such as ATP-binding cassette transporters ABCA1 and ABCG1, and apolipoprotein E (5, 6).

Our recent work has identified hexarelin and other analogs of the GH-releasing peptide (GHRP) family as high affinity ligands of CD36 (7, 8). GHRPs were originally described to stimulate central GH release through binding of the GH secretagogue-receptor-1a, a G protein-coupled receptor later defined as the receptor for ghrelin and expressed predominantly in the hypothalamic-pituitary region (9, 10). In recent studies, we observed that GHRPs markedly decreased plaque formation in a mouse model of atherosclerosis, an effect that was shown to require CD36 expression (11, 12). These beneficial effects of GHRPs on cholesterol metabolism were dependent on PPAR and the activation of the

PPAR-LXR-ABC metabolic cascade in macrophages, leading to cholesterol efflux into the high-density lipoprotein reverse pathway (12).

Whereas the role of CD36 in mediating cholesterol and fatty acid uptake by macrophages is well characterized, little is known about how CD36 may impact the overall metabolic activity of fat storage and mobilization by adipocytes. Here we report on the changes in expression of genes related to fatty acid import and oxidation as well as in morphological changes of mitochondria in adipocytes induced by GHRP hexarelin. The resulting increase in expression of F_1-ATP synthase, coactivator PPARγ coactivator (PGC)-1α and uncoupling protein (UCP)-1, all normally found in brown adipocytes, suggests that fatty acids are ushered toward mitochondria oxidative phosphorylation and biogenesis, rather than being converted to triglycerides for their subsequent storage in lipid vesicles. The overall effect is a decrease in total lipid content in fat cells, which provides a functional role of CD36 to modulate fatty acid metabolism and mitochondrial functions.

Materials and Methods

Cell culture and treatments

Mouse 3T3-L1 preadipocytes were grown in DMEM supplemented with 10% fetal calf serum. Two days after confluence (d 0), adipocyte differentiation was initiated with the addition of 115 μg/ml 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, and 0.167 μM insulin in DMEM supplemented with 10% fetal bovine serum (FBS) for 2 d. On d 2, the media was replaced with DMEM/10% FBS containing insulin for 2 more d and then maintained in DMEM/10% FBS until d 8. Treatments with hexarelin (10–7 to 10–5 M) and troglitazone (8 μM) were done for 48–72 h with fresh medium replacement at intervals of 24 h.

Lipid staining

3T3-L1 cells were fixed with 3.7% formaldehyde/PBS and stained with oil red O (Sigma, St. Louis, MO). Quantification of lipid accumulation was achieved by extracting

oil red O from stained cells with isopropyl alcohol and measuring the OD of the extracts at 510 nm.

Triglyceride measurement

Lipids from differentiated 3T3-L1 adipocytes were extracted with Folch solution consisting of a mixture of 2:1 (vol/vol) chloroform/methanol and resuspended in 20% Thesit (Sigma) in Folch solution before evaporation under nitrogen gas. Triglyceride content was determined using a colorimetric assay kit (Zen-bio, Research Triangle Park, NC) and normalized against total protein from each sample determined by Bradford reagent (Sigma).

Microarray analysis

Differentiated 3T3-L1 adipocytes were treated with 10–5 M hexarelin or 8 μM troglitazone for 48 h. Total RNA was isolated from 3T3-L1 cells using TRIzol reagent (Invitrogen, Burlington, Ontario, Canada), according to the manufacturer's protocol. Biotinylated cRNA was generated from 10 μg of total RNA, and hybridized onto mouse 430.2 oligonucleotide arrays. All procedures were followed according to Affymetrix protocols (Santa Clara, CA). Data were analyzed and compared with a second set of hybridization experiments using the Gene-Chip analysis suite software (Affymetrix) and representative results were generated with TM4 software (TiGR, The Institute for Genomic Research, Rockville, MD).

RT-PCR analysis

3T3-L1 cells were treated as above and cDNA was synthesized from 400 ng of total RNA using oligo(dT) primers and RevertAid H minus M-MuLV reverse transcriptase (Fermentas, Burlington, Ontario, Canada). PCR amplification was usually performed in a volume of 20 μl with 0.5–1 μl of reverse transcription reaction for 25–35 cycles. Sequences of the murine primers used in PCR are available upon request. The PCR products were separated on a 2% agarose gel, stained with ethidium bromide, and the relative signal intensity was analyzed (Alpha Innotech, San Leandro, CA) from at least three separate experiments.

Cytochrome c oxidase (COX) activity

COX activity was measured on isolated mitochondria from treated and untreated 3T3-L1 adipocytes. Briefly, adipocytes were collected and resuspended in mitochondrial buffer [0.2 mM EDTA, 0.25 M sucrose, and 0.1 mg/ml digitonin in 10 mM Tris (pH 7.8)]. Cells were ruptured using a glass-Teflon Potter-Elvehjem homogenizer, and the homogenates were centrifuged at 1000 x g for 10 min. Mitochondria were then pelleted at 12,000 x g spin for 15 min and resuspended in mitochondrial buffer supplemented with protease inhibitor cocktail (Roche, Laval, Québec, Canada). Protein content was determined by the Bradford method (Bio-Rad, Mississauga, Ontario, Canada). COX activity was determined from 10 µg of mitochondrial proteins from each treatment according to the manufacturer's protocol (Sigma). The activity was calculated from the rate of decrease in absorbance of ferrocytochrome c at 550 nm (ε= 21.84 $mM^{-1}cm^{-1}$), added to the assay at a final concentration of 10 µM, and represented as milliunits per milligram of protein per minute where 1 U is the amount of enzyme needed to oxidize 1 µmol of ferrocytochrome c per minute (pH 7.0) at room temperature. To assure total permeabilization of mitochondrial membrane, the assay was performed in the presence of 2.5 mM n-dodecyl ß-D-maltoside (Sigma). No significant COX activity was detected in the 12,000 x g spin supernatants.

Fluorescence microscopy

Eight-day differentiated adipocytes seeded in Lab-Tek coverglass chambers (Nalge Nunc, Rochester, NY) were treated for 72 h with either hexarelin or troglitazone. Live cells are then rinsed with PBS and labeled at 37 C for 15 min with 1 mg/ml rhodamine-123, a mitochondrial-specific fluorochrome (Sigma), as described by the manufacturer. Mitochondria are visualized by fluorescence microscopy (TE-2000; Nikon, Melville, NY) with an excitation at 488 nm and emission at 525 nm. Photobleaching is reduced with 1 mg/ml ascorbic acid.

Antibodies and immunoblotting analysis

Antibodies to PPARγ, ATP synthase (F_1 subunit), PGC-1α, adipocyte- specific fatty acid binding protein (aP2) and ß-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and the anti-UCP1 and -UCP2 antibodies were purchased from Calbiochem (San Diego, CA). The antibody against CD36 has been described (8). Immunoblotting analysis was performed as described (13). Briefly, cells were lysed in PBS buffer containing 1% Triton X-100, 0.5% deoxycholate acid, 0.1% sodium dodecyl sulfate, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitors (Roche). Proteins were then resolved by SDS-PAGE and transferred to nitrocellulose for immunoblotting. Membranes were blocked at 4 C with blocking reagent (Roche) in Tris-buffered saline, probed with selected antibodies, and signals revealed by enhanced chemiluminescence using appropriate horseradish peroxidase-conjuguated secondary antibodies. For fat tissue, proteins were isolated using Trizol standard procedure and resuspended in 1% sodium dodecyl sulfate for immunoblot analysis.

In vivo experiments

Wild-type C57BL/6 and CD36-deficient mice were previously described (11) and maintained in a 12-h dark, 12-h light cycle with a standard pelleted diet and water ad libitum. At 12 wk of age, male mice were fed a 60% kcal/60% fat diet (Research Diets Inc., New Brunswick, NJ) and treated with sc injection of 100 μg/kg·d hexarelin, a dose known not to promote GH release (14), or 0.9% NaCl (control) for 12 wk, as previously described (12). Fat tissues were collected from the epididymal fat pads of control and treated mice and rapidly frozen at –80 C. All experimental procedures were done in accordance with the Institutional Animal Ethics Committee of the University of Montreal and the Canadian Council on Animal Care guidelines for use of experimental animals.

Electron microscopy

3T3-L1 cells and mouse fat tissue were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3). Samples were postfixed with 4% OsO4 and dehydrated with ethanol. Before sectioning, tissues were embedded in epoxide resin (Epon 812; Sigma). Ultrathin cryosections were collected on metal grids and poststained with electron-dense

uranyl acetate and lead citrate solutions and electron micrographs were recorded with an electron microscope (model 208S; Philips Medical Systems, Andover, MA). Mitochondria size and cristae formation were determined using an image analyzer (Alpha Innotech).

Results

CD36 ligand hexarelin decreases total lipid content in mature 3T3-L1 adipocytes

Our recent studies using cultured THP-1 macrophages have shown that hexarelin caused a significant decrease in total lipid accumulation via CD36, resulting in an augmentation of cholesterol efflux from cells (11, 12). Because adipocytes are known to express CD36 and not the other known hexarelin receptor-1a (Refs. 15 and 16 and data not shown), we evaluated the effect of hexarelin on lipid content in cultured 3T3-L1 adipocytes. 3T3-L1 cells were differentiated to mature adipocytes for 8 d with insulin/dexamethasone/3-isobutyl-1-methylxanthine, and treated with 10^{-7} and 10^{-5} M hexarelin for 48 h with a media change at 24 h. After treatment with hexarelin, a marked decrease in total cellular lipid and in the size of the lipid droplets was observed, compared with untreated cells (Fig. 1A). Whereas differentiation of 3T3-L1 preadipocytes into adipocytes resulted in a strong accumulation of lipids in vesicles, mature adipocytes treated with hexarelin showed a significant decrease in total lipid amount, compared with untreated cells (Fig. 1B). Such decrease was comparable with cells treated with troglitazone, a specific PPARγ ligand known to deplete lipid content in adipocytes (17). The decrease in lipid staining is associated with a significant reduction in intracellular triglyceride levels in adipocytes treated with hexarelin (Fig. 1C).

Microarray analysis of genes regulated by hexarelin in 3T3-L1 adipocytes

To address the overall effect of hexarelin on genes involved in lipid metabolism in adipocytes, we performed microarray analysis on differentiated 3T3-L1 adipocytes treated with hexarelin and compared the expression profile with cells treated with troglitazone, relative to untreated cells. Total RNA was harvested from each sample treatment and probed against Affymetrix mouse 430.2 oligonucleotide chip. Probe sets that were

identified as absent calls across all samples and experiments were removed from analysis. The relative gene expression levels in each treated sample were compared with untreated controls to determine significant changes. Selected genes were listed according to their known function (Fig. 2A).

Of the entire probe sets analyzed, 1119 were up-regulated in cells treated with hexarelin, suggesting that interaction with CD36 induces profound changes in the expression profile of adipocytes. Interestingly, many of these genes were shared with troglitazone treatment, indicating that PPARγ may be considered as a common regulator in both responses. Consistent with this, among the genes up-regulated by hexarelin, we found many established PPARγ targets, such as nuclear receptor LXRα, fatty acid transport protein (FATP)-1, and ATP synthase (Fig. 2A). However, the response to hexarelin was not totally mimicked by troglitazone as other described PPARγ targets, such as adipocyte fatty acid binding protein-4 (also referred to as aP2), and lipid droplet-associated protein adipophilin remained mostly unchanged upon treatment with hexarelin (Fig. 2, A–C). In addition, troglitazone treatment led to a decrease in PPARγ expression in adipocytes (0.6-fold in protein levels, compared with untreated cells), a finding also observed by others (18), whereas hexarelin did not significantly modify PPARγ expression (Fig. 2). We also reported a similar response in PPARγ expression in macrophages, indicating that this regulation is not cell specific (12).

Given the decrease in triglyceride content in cells treated with hexarelin (Fig. 1), we looked at several genes involved in various aspects of fatty acid metabolism including those involved in entry, transport, synthesis, and mobilization. Of those, hormone-sensitive lipase, GDSL motif-containing lipase, fatty acid synthase, acetyl-CoA synthase, and FATP1 were all up-regulated by hexarelin (Fig. 2A). In contrast, glycerol-3-phosphate acyltransferase (GPAT), which catalyzes the initial and committed step in glycerolipid biosynthesis, was down-regulated by hexarelin. This type of profile is strongly suggestive of an increase in the cellular mobilization of free fatty acids in response to hexarelin.

Hexarelin up-regulates genes involved in fatty acid oxidation and oxidative phosphorylation

What seemed more striking from the microarray experiments was the expression changes of mitochondrial genes involved in fatty acid metabolism. Several genes required for fatty acid transport into mitochondria, such as mitochondrial acyl carrier protein, acyl-CoA binding protein, and carnitine/acylcarnitine carrier protein were up-regulated by hexarelin (Fig. 2A). Similarly, many genes involved in fatty acid oxidation and oxidative phosphorylation were also up-regulated by hexarelin, such as acetyl-CoA acyltransferase 1 and 2, hydroxyacyl-CoA dehydrogenase, and several subunits of the ATP synthase complex (Fig. 2, A and B). These changes may reflect an increased activity of mitochondrial processes toward oxidation of fatty acids and oxidative phosphorylation, two features closely related to mitochondrial thermogenic activity and biogenesis.

One of the key enzymes involved in ß-oxidation of long-chain fatty acids for energy production is the carnitine palmitoyltransferase (CPT). The muscle isoform M-CPT I, also known as CPT1b, is not normally expressed in mouse adipose tissue (19). Interestingly, we observed by RT-PCR a strong induction of the expression of CPT1b in 3T3-L1 adipocytes treated with hexarelin and with troglitazone (Fig. 2B). Because mitochondrial proteins that process fatty acids through entry and oxidation in mitochondria are often associated with energy production, we next analyzed the expression of genes involved in ATP production. Mitochondrial F_1-ATPase is responsible for the synthesis of ATP during oxidative phosphorylation to generate energy. Interestingly, the expression of F_1-ATP synthase was increased by hexarelin to levels slightly lower than those obtained with troglitazone when compared with untreated cells (Fig. 2, B and C). Protein levels of F_1-ATPase were increased by 3.1- and 3.4-fold in response to, respectively, 10^{-7} and 10^{-5} M hexarelin, whereas troglitazone induced a 4.4-fold increase, compared with controls (Fig. 2C). These results correlate with the microarray data showing many of the catalytic subunits of F_1-ATPase up-regulated in response to hexarelin. These results therefore link the response of adipocytes to hexarelin with the production of energy.

Hexarelin promotes the expression of thermogenic markers in 3T3-L1 adipocytes

The increase we observed in the expression of genes involved in ß-oxidation of fatty acids and oxidative phosphorylation in response to hexarelin suggests that these cells may generate more ATP. Several studies have shown that such metabolic needs for energy requires the PPARγ coactivator PGC-1, which by inducing the expression of UCP1, a biological uncoupler of mitochondrial oxidative phosphorylation, initiates a broad program of thermogenesis in brown fat and muscle tissues (20, 21, 22, 23, 24). Although both proteins are poorly expressed, if not absent in white adipocytes, we found a remarkable increase in the expression of PGC-1α and UCP1 in 3T3-L1 adipocytes treated with increasing doses of hexarelin (Fig. 2, B and C). Protein levels of PGC-1α and UCP1 reached, respectively, a 5.1- and 4.2-fold increase in response to 10–5 M hexarelin. Similar increases were also observed in cells treated with troglitazone, suggesting that the response to troglitazone and hexarelin may converge at some point with PPARγ activation. UCP2 was detected in 3T3-L1 adipocytes but was not substantially modulated by hexarelin.

Mitochondrial cytochrome c oxidase activity is increased by hexarelin in 3T3-L1 adipocytes

In view of the above results indicating a marked increase in genes related to energy production, we measured the activity of COX, which catalyzes the terminal and rate-limiting step of the energy-transducing respiratory chain in mitochondria leading to ATP production. We found that treatment of 3T3-L1 adipocytes with hexarelin for 72 h significantly induced COX activity in isolated mitochondria, compared with untreated cells (Fig. 2D). Similarly, COX activity was also augmented in response to troglitazone using the same conditions. These changes in COX activity are consistent with the increases in expression levels of subunits forming COX enzymatic complex and other components of the respiratory chain in cells treated with hexarelin (Fig. 2A) and therefore support the ability of hexarelin to induce mitochondrial activity in adipocytes.

Hexarelin induces ultrastructural changes indicative of increased mitochondrial activity and biogenesis

Expression of PGC-1α is known to stimulate mitochondrial energy-producing capacity and biogenesis in tissues with high oxidative potential, such as heart, muscle, and brown fat (24, 25). First, we determined whether the changes in mitochondrial gene expression correlated with changes in mitochondrial morphology by staining differentiated 3T3-L1 adipocytes with rhodamine-123, a nontoxic mitochondrial fluorescent dye. Mitochondria of untreated cells were seen as a dense interconnected reticular motif (Fig. 3A), a pattern also reported by others (26). However, when treated with hexarelin for 72 h, the mitochondrial appearance was remodeled into individual densely packed structures, highly similar to the mitochondrial shape observed in cells treated with troglitazone (Fig. 3A).

The ultrastructure of the mitochondria was further defined using electron microscopy. Mitochondria of 3T3-L1 adipocytes treated with hexarelin were characterized by an increase in size and intense formation of lamellar cristae, compared with untreated cells (Fig. 3B). In addition, the cristae membrane of mitochondria from cells treated with hexarelin was highly organized and linearly displayed across the entire width of the organelle, compared with controls. The average mitochondrial size and percentage of mitochondrial matrix occupied by cristae were calculated and showed that mitochondrial size was increased by more than 2-fold ($P < 0.001$), and the percentage of surface within the mitochondrial matrix occupied by cristae membrane increased from 32% to almost 45% ($P < 0.001$) in adipocytes treated with hexarelin, compared with control cells (Fig. 3, C and D). This particular phenotype depicts a condition to maximize the intramitochondrial spanning of cristae, a pattern highly characteristic of mitochondria from tissues with high energy production rate, such as brown adipose tissue, heart, and skeletal muscle (27).

Consistent with enhanced *de novo* mitochondrial synthesis, we found that hexarelin increased the expression of several translocases of the outer and inner membrane (TIM) of mitochondria (Fig. 3E). The translocases of the outer membrane and TIMs are responsible for the import of mitochondrial proteins encoded by the nuclear genome into the matrix and the intermembrane space of mitochondria (28). Among these family translocases, TIM17b

reached a 3.8-fold increase in response to hexarelin, compared with control cells. Interestingly, TIM17b is ubiquitously expressed in humans and mice with a higher expression pattern in tissues with high oxidative potential, such as heart and skeletal muscle (29). Also up-regulated by hexarelin were several of the mitochondrial ribosomal proteins or MRPs involved in the translation of many proteins of the respiratory chain (30), indicating that mitochondrial transcription and translation was increased in response to hexarelin (Fig. 3E). In addition, both subunits of prohibitin, which form a large complex in the mitochondrial inner membrane to stabilize newly synthesized subunits of the respiratory chain (31), were up-regulated by hexarelin (Fig. 3E). It was reported that impaired function of these subunits resulted in a decreased number and mass of mitochondria and was associated with deficient mitochondrial biogenesis (32).

Induction of thermogenic markers and mitochondrial biogenesis by hexarelin occurs in vivo and is dependent on CD36

To address whether the phenotypic changes we observed in cultured adipocytes in response to hexarelin could also occur *in vivo*, we treated C57BL/6 mice with saline (control) or 100 μg/kg·d hexarelin for 12 wk. The concentration of hexarelin used in this study was reported not to elicit GH release and therefore prevented any undesired effects of GH (11, 12, and 14). No adverse health problems were noticed throughout the treatment. The epididymal white fat was collected from treated mice and saline controls and analyzed by electron microscopy. Electronic images of fat tissues of hexarelin-treated mice showed an intense cristae formation in mitochondria, compared with controls, and more noticeably, the size in mitochondria was increased by 55% in these conditions (Fig. 4, A and B). In addition, we performed Western blot analysis on epididymal tissue that showed that protein levels of F_1-ATPase and thermogenic markers PGC-1α and UCP1 were increased in response to hexarelin (Fig. 4C). In contrast, there were no apparent changes in steady-state levels of these proteins in epididymal fat tissue derived from CD36-null mice treated as above with hexarelin, compared with saline-treated CD36-null mice (Fig. 4C). Invalidation of CD36 also induced an increase in F_1-ATPase levels in epididymal fat, whereas those of

PGC-1α and UCP1 remained mostly unchanged. These results therefore suggest that the ability of hexarelin to promote mitochondrial metabolic changes in cultured adipocytes can be transposed in vivo and are dependent on CD36.

Discussion

One of the critical regulators of fatty acid metabolism in fat is PPARγ, which controls the expression of a broad range of genes involved in fatty acid and glucose uptake, ß-oxidation, and lipid storage (33). Based on our previous reports that ligands of the GHRP family interact with scavenger receptor CD36 to promote PPARγ activation and downstream effects on cholesterol metabolism in macrophages (11, 12), we hypothesized that GHRP hexarelin might have an impact on adipocytes that express CD36. In this article, we described profound changes in the gene expression profile and mitochondria morphology in white fat cells treated with hexarelin correlating with a fat burning-like phenotype characteristic of brown adipocytes.

Interestingly, many of the genes up-regulated by hexarelin were shared with troglitazone treatment, indicating that PPARγ activation is likely to be involved in the response of adipocytes to hexarelin. Among the PPARγ target genes up-regulated by hexarelin, we found nuclear receptor LXR, FATP1, FATP4, CPT1b, and F_1-ATP synthase. Otherwise, not all established PPAR genes were regulated in the same manner as with troglitazone. Genes such as aP2 and adipophilin remained unaffected in hexarelin-treated cells, suggesting that the response to hexarelin is likely to be more complex than the sole activation of the PPARγ pathway. Consistent with this, CD36 gene expression was modestly increased by hexarelin with no change in protein levels, whereas troglitazone significantly induced both in treated adipocytes. Similar results were found in macrophages in which CD36 expression remains mostly unaffected by GHRPs, whereas troglitazone significantly up-regulated CD36 (11, 12). Such regulation was associated with a differential CD36 promoter occupancy by PPARγ as determined by chromatin immunoprecipitation assay (12). Additionally, PPARγ expression seems to be regulated differently in response to

GHRPs than PPARγ ligands. We found that treating adipocytes with troglitazone lead to a decrease in PPARγ expression, a finding that was not associated with a decrease in target gene expression in mature adipocytes (18, 34) and that is generally observed for many nuclear receptors in response to ligands. However, the PPARγ mRNA level was slightly increased in response to hexarelin, whereas no obvious change was noticed for its protein level. A similar observation was also obtained in macrophages, suggesting that GHRPs contribute to maintain steady-state levels of PPARγ (11, 12). The mechanism by which hexarelin stimulates PPARγ activity but not down-regulating its expression deserves further investigation.

The white adipose tissue is the major site for triglyceride storage in the body and plays a critical role in maintaining homeostatic levels of circulating fatty acids and energy balance by promoting triglyceride breakdown and fatty acid release. Our results indicate that adipocytes respond to hexarelin with an increased mobilization of fatty acids rather than triglyceride synthesis. The depletion in lipid content in cells treated with hexarelin correlates with an increase in expression level of hormone-sensitive lipase, the enzyme involved in lipolysis. Genes involved in fatty acid synthesis and import were also augmented, such as fatty acid synthase and transporters FATP1 and FATP4. Interestingly, the expression of mitochondrial GPAT was decreased in adipocytes treated with hexarelin. It was recently shown that mitochondrial GPAT1, which catalyzes the initial and rate-controlling step in glycerolipid synthesis, partitions acyl-CoAs toward triacylglycerol synthesis and its deficiency in mice resulted in a redirection of fatty acids into the oxidation pathway in liver (35).

Such apparent mobilization of fatty acids induced by hexarelin seems to be unexpectedly directed toward the ß-oxidation pathway in treated mature white adipocytes. Adipose tissue functions normally to release fatty acids in the circulation to be used by peripheral tissues of high oxidative potential, such as heart and muscle to produce ATP in response to energy expenditure. Brown adipocytes also use fatty acid oxidation to burn fat necessary for adaptive thermogenesis. We found that cultured 3T3-L1 adipocytes treated

with hexarelin exhibit an increased expression profile of mitochondrial genes related to long-chain fatty acid oxidation. The expression of CPT1b, a key enzyme for fatty acid oxidation in the heart, skeletal muscle and brown adipose tissue in human and rat, but normally absent in mouse white adipocytes or in 3T3-L1 cells (19), was strongly induced by hexarelin. Induction in CPT1b was described to be responsible for the dramatic increase in fatty acid oxidation that occurs in the heart after birth in which energy production switches from glucose to fatty acid use (36). Compared with its related isoform CPT1a, mainly expressed in liver, kidney, and intestine, CPT1b is more sensitive to the inhibition by malonyl-CoA (37). The expression of malonyl-CoA decarboxylase, which catalyzes the conversion of malonyl-CoA to acetyl-CoA, was up-regulated by hexarelin. Such an increase would potentially result in CPT1 activation by relieving the inhibitory effect of malonyl-CoA, and therefore increasing fatty acid oxidation.

Studies using genetic approaches and PPAR ligands have described the gene for CPT1 as regulated by PPAR isoforms, including PPARα and PPARß (38, 39, 40). Although the precise role of PPARß on adipocyte functions remains to be determined, PPARα plays a pivotal role in fatty acid metabolism by regulating the expression of genes involved in mitochondrial and peroxisomal ß-oxidation pathways (41, 42). This raises the possibility that, in response to hexarelin, the increase of the expression of genes related to fatty acid oxidation in adipocytes might also depend on PPARα activation. Consistent with this, we found that hexarelin contributed to activate all three PPAR subtypes using a cell reporter assay, suggesting that cellular signaling induced by CD36 might influence PPAR activity (12). However, because the PPARs can all be activated to various degrees by low micromolar concentrations of unsaturated fatty acids (43), we cannot exclude the possibility that the mobilization of free fatty acids in cells due to hexarelin could provide endogenous ligands to selectively activate the PPARs and therefore fatty acid oxidation. Nevertheless, using genetically ablated PPARγ ± macrophages, we showed that the activation of PPARγ target genes such as LXRα in response to hexarelin was impaired, suggesting that PPAR

activation is a major determinant of the response to GHRPs (12). Further studies are required to determine the exact contribution of hexarelin to PPARγ activation in adipocytes. Genes involved in oxidative phosphorylation and ATP synthesis were also strongly up-regulated by hexarelin, supporting the redirection of fatty acids toward mitochondrial oxidation rather than their release or their conversion into triacylglycerol. This profile was supported by a significant increase in F_1-ATP synthase expression and mitochondrial COX activity and a noticeable change in mitochondrial morphology in either treated adipocytes or mouse white adipose tissue. Electronic microscopy showed a significant increase in the intramitochondrial matrix surface and an intense cristae formation that spans the entire width of the organelle in response to hexarelin. Microarray analysis indicated an increase in the expression of many catalytic subunits of the ATPase and COX multimeric complexes, which both reside within the cristae membrane. Such phenotypic organization of mitochondria is typical of tissues with high oxidative potential, including muscle and brown fat, to support an enhanced activity in ATP production by the ATP synthase complex and mitochondrial respiration process (27). Most strikingly were the enhanced mRNA and protein levels of PGC-1α and UCP1 in response to hexarelin, which rose from low detectable levels normally found in white adipocytes to those mainly characteristic of brown fat. Under the same conditions, UCP2, a more ubiquitously expressed protein than UCP1 but for which its role is normally less related to the thermogenic response (44, 45, 46), appeared not to be regulated by hexarelin, indicating that the effects of hexarelin in promoting mitochondrial metabolic activity are more dependent on UCP1 up-regulation.

PGC-1α is highly expressed in brown fat and plays a critical role in initiating a broad program of thermogenesis that includes enhanced oxidative metabolism and mitochondrial biogenesis (22). Interestingly, the transgenic expression of PGC-1α in white fat cells was shown to induce UCP1 expression and mitochondrial biogenesis, indicative that uncoupling of mitochondrial respiration is an important component of energy expenditure in vivo (47). Such metabolic need for energy supported by PGC-1α and UCP1 expression and mitochondrial morphological changes also occurred in white fat of treated mice, indicating

that the ability of hexarelin to promote a fat burning-like phenotype was maintained in vivo. PGC-1α also controls critical aspects of energy metabolism in other tissues such as heart and muscle and largely contributes to the expression of genes of gluconeogenesis in liver (21, 23, 48, 49). Thus, modulating the relative activity of PGC-1 within a particular tissue may lead to fine-tuning of mitochondrial function in fatty acid oxidation and energy balance. Whether hexarelin may promote similar effects in other tissues expressing PGC-1α remains to be determined. In addition, with the propensity of PGC-1α to coactivate other nuclear receptors besides PPARγ, such as thyroid hormone receptor-, retinoic acid receptor-, estrogen-related receptor, and PPARα, and to result in enhanced UCP1 expression (47), it is expected that these pathways may also be affected by hexarelin.

Although the exact mechanisms by which GHRPs exert their effects through CD36 are not fully understood, it becomes clear that interacting with CD36 induces profound changes in metabolic activities of target tissues, especially regarding PPARγ-regulated events. In macrophages, GHRPs induced the PPARγ-LXRα-ABC pathway, leading to cholesterol efflux and reduction of atherosclerosis (11, 12). Here we report that hexarelin promotes the expression of key regulatory genes in fat metabolism, many of which are controlled by PPARγ, resulting in the mobilization of fatty acids toward mitochondria oxidative phosphorylation and biogenesis in white fat cells. These results therefore implicate CD36 in the regulation of the overall metabolic activity of mitochondria in adipocytes. With the emerging evidence that mitochondria dysfunction is associated with metabolic defects such as insulin resistance and type 2 diabetes (50), one can expect that modulating CD36 function might be potentially beneficial.

Acknowledgments

We acknowledge the expert assistance of Hugues Bernard for microarray analysis. We are grateful to Ksenia Scoulsky for skillful technical assistance in electron microscopy and Lynda Abdel for expert tissue analysis. We also acknowledge the technical assistance of Eve-Marie Charbonneau, Carole Garofalo, and Petra Pohankova. We are grateful to Susan Acton and Grant Mitchell for critical examination of the manuscript.

Footnotes

This work was supported by the Canadian Institutes of Health Research (CIHR) and the Canadian Foundation for Innovation. A.R.-W. holds a doctoral award from the Natural Sciences and Engineering Research Council of Canada. A.D. holds a doctoral award from the CIHR. A.T. is a New Investigator of the CIHR.

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Figure legends

Figure 1. Hexarelin reduced lipid content in mouse 3T3-L1 adipocytes. A, Representative images of differentiated 3T3-L1 adipocytes untreated (Diff) or treated with 10–5 M hexarelin for 48 h. Lipids were stained with oil red O and examined by microscopy. B, Photometric measurement of lipids stained with oil red O from

undifferentiated (–) or differentiated 3T3-L1 cells treated with hexarelin or troglitazone (Tro) or left untreated (Diff). Data are presented as mean ± SEM of at least six separate experiments. C, Intracellular triglyceride content in 3T3-L1 adipocytes treated with hexarelin or troglitazone, compared with untreated cells as in A. Data are presented as mean ± SEM of at least six separate experiments. *, $P < 0.05$ and **, $P < 0.01$ vs. untreated differentiated cells.

Figure 2. Hexarelin induced the expression of genes associated with fatty acid oxidation and brown adipocyte phenotype. A, DNA microarray analysis of differentiated 3T3-L1 adipocytes treated with troglitazone, or hexarelin. Shown are selected PPAR target genes and genes associated with fatty acid metabolism. Results are presented as fold changes compared with control cells set at 1.0. B, RT-PCR analysis of selected markers from differentiated 3T3-L1 cells treated with troglitazone (Tro) or hexarelin (Hexa) or left untreated for 48 h before RNA isolation. Representative images are shown from at least three separate experiments. 36B4 expression was used to normalize samples. C, Western analysis of 3T3-L1 adipocytes treated as above. Samples were normalized for protein loading with ß-actin. D, Hexarelin (Hexa) induces COX activity in 3T3-L1 adipocytes. Differentiated adipocytes were treated with hexarelin or troglitazone (Tro) or left untreated (Diff) for 72 h, and COX activity was measured on isolated mitochondria and normalized to protein content. Data are presented as mean ± SEM of at least six separate experiments. *, $P < 0.005$ vs. untreated differentiated cells.

Figure 3. Hexarelin induces morphological changes in mitochondrial ultrastructure. A, Representative images of 3T3-L1 adipocytes stained with mitochondria-specific rhodamine-123 dye. Cells were untreated (Diff) or treated with 10–5 M hexarelin (Hexa) or 8 μM troglitazone (Tro) for 72 h before staining and microscopic analysis. Magnification, x100. B, 3T3-L1 cells were treated with 10–5 M hexarelin for 72 h or left untreated and visualized by electron microscopy. Representative images show an increase in

mitochondrial size and cristae formation in response to hexarelin. C, Quantification of the average mitochondrial size in hexarelin-treated 3T3-L1 adipocytes, compared with untreated cells. Sizes are depicted as the mean of calculated surface area ± SEM of more than 70 mitochondria per group. *, $P < 0.001$. D, Relative surface area occupied by the cristae membrane within mitochondria expressed as % of total surface area. Data are presented as mean ± SEM of more than 25 mitochondria per group. *, $P < 0.001$. E, DNA microarray analysis of selected genes involved in mitochondrial biogenesis and found to be up-regulated by hexarelin, compared with untreated 3T3-L1 cells. Results with troglitazone are also shown. Fold changes are presented as in Fig. 2A.

Figure 4. Hexarelin induced expression of thermogenic markers and mitochondrial biogenesis in vivo. A, C57BL/6 mice were treated with hexarelin or saline (control) for 12 wk, and epididymal adipose tissue was analyzed by electron microscopy. Representative images from both samples are shown. B, Quantification of the average mitochondrial size in adipose tissue from mice treated as in A. Sizes are depicted as the mean of calculated surface area ± SEM of more than 50 mitochondria per group. *, $P < 0.001$. C, Western analysis of epididymal fat isolated from C57BL/6 wild-type and CD36-null mice treated with hexarelin (Hexa) or saline for 12 wk. Shown are samples obtained for each treatment from two separate experiments.

FIGURES

Figure 1

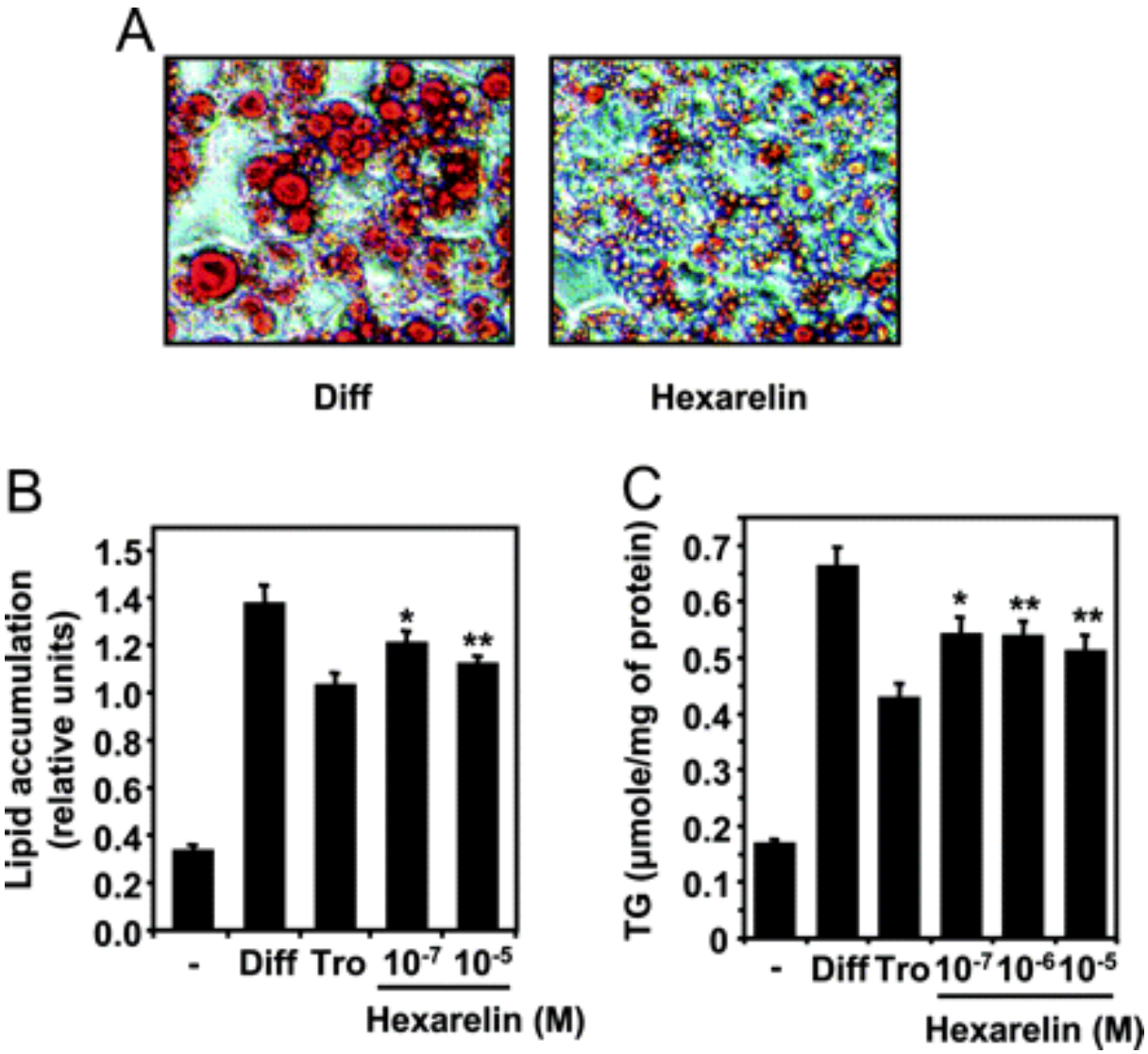
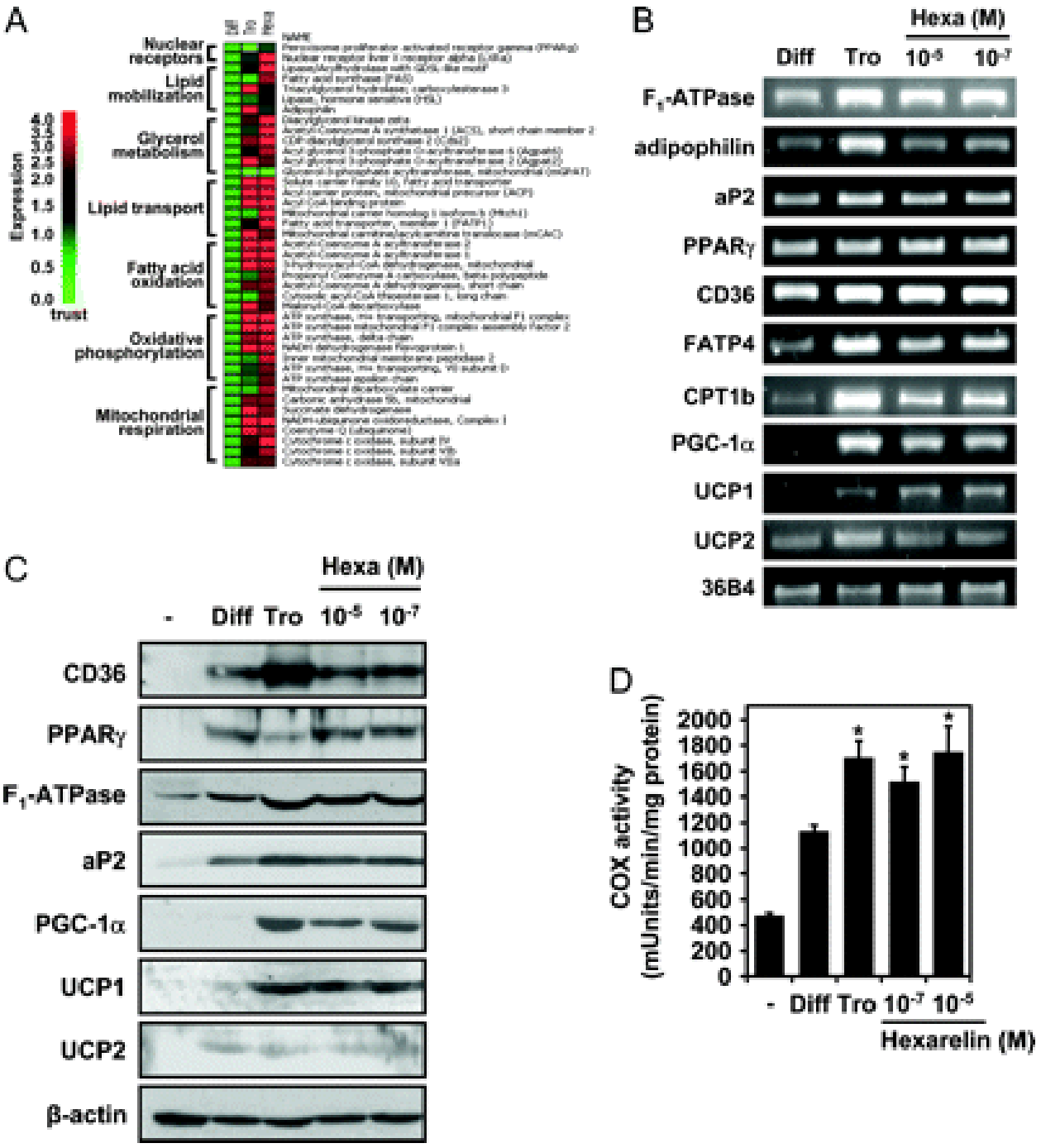


Figure 2


A
Nuclear receptors
Lipid mobilization
Glycerol metabolism
Lipid transport
Fatty acid oxidation
Oxidative phosphorylation
Mitochondrial respiration
Expression
trust
B
Hexa (M)
Diff
Tro
10-5
10-7
F1-ATPase
adipophilin
aP2
PPARγ
CD36
FATP4
CPT1b
PGC-1α
UCP1
UCP2
36B4
C
Hexa (M)
-
Diff
Tro
10-5
10-7
CD36
PPARγ
F1-ATPase
aP2
PGC-1α
UCP1
UCP2
β-actin
D
COX activity
(mUnits/min/mg protein)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
-
Diff
Tro
10-7
10-5
Hexarelin (M)

Zoom on Figure 2A

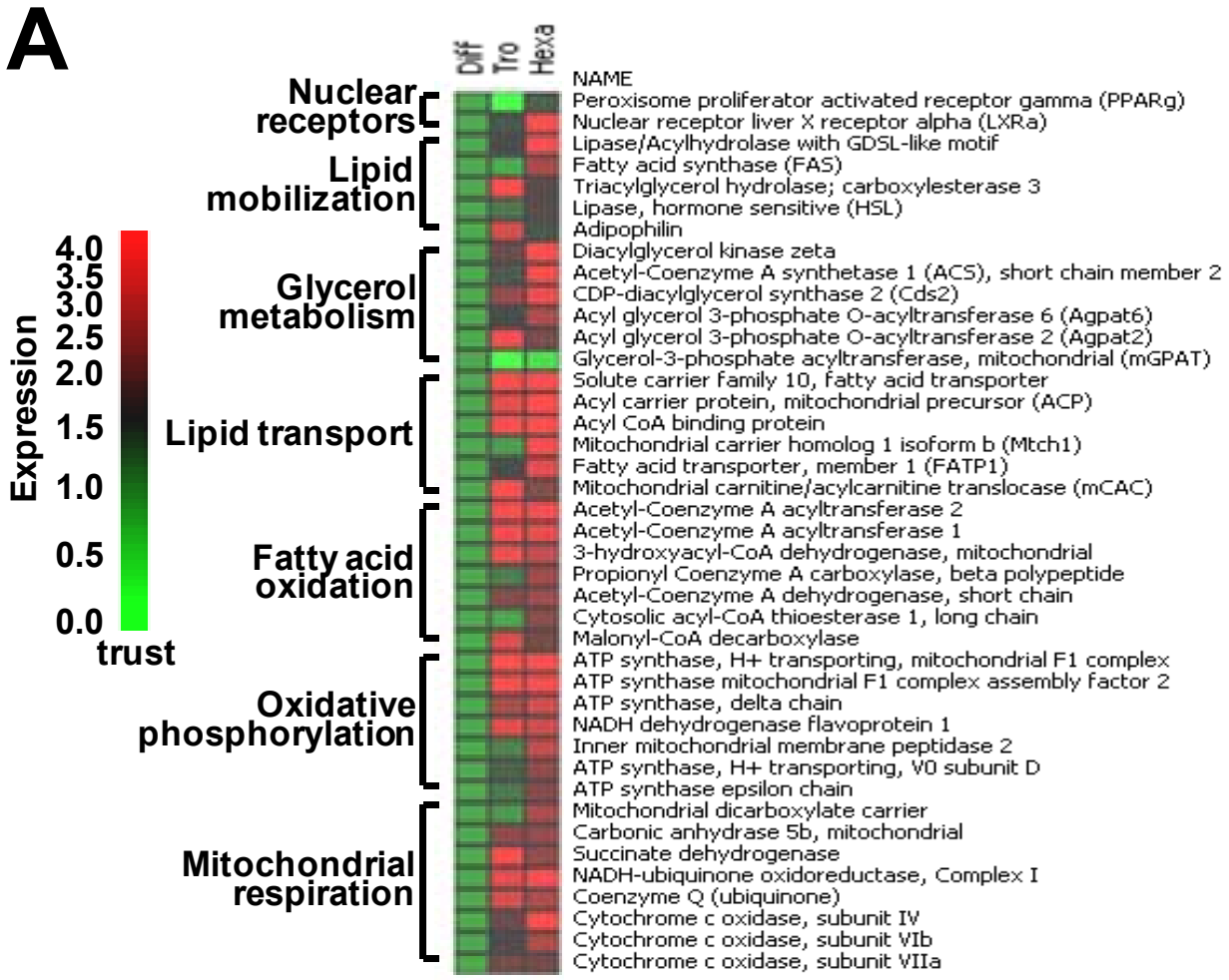


Figure 3

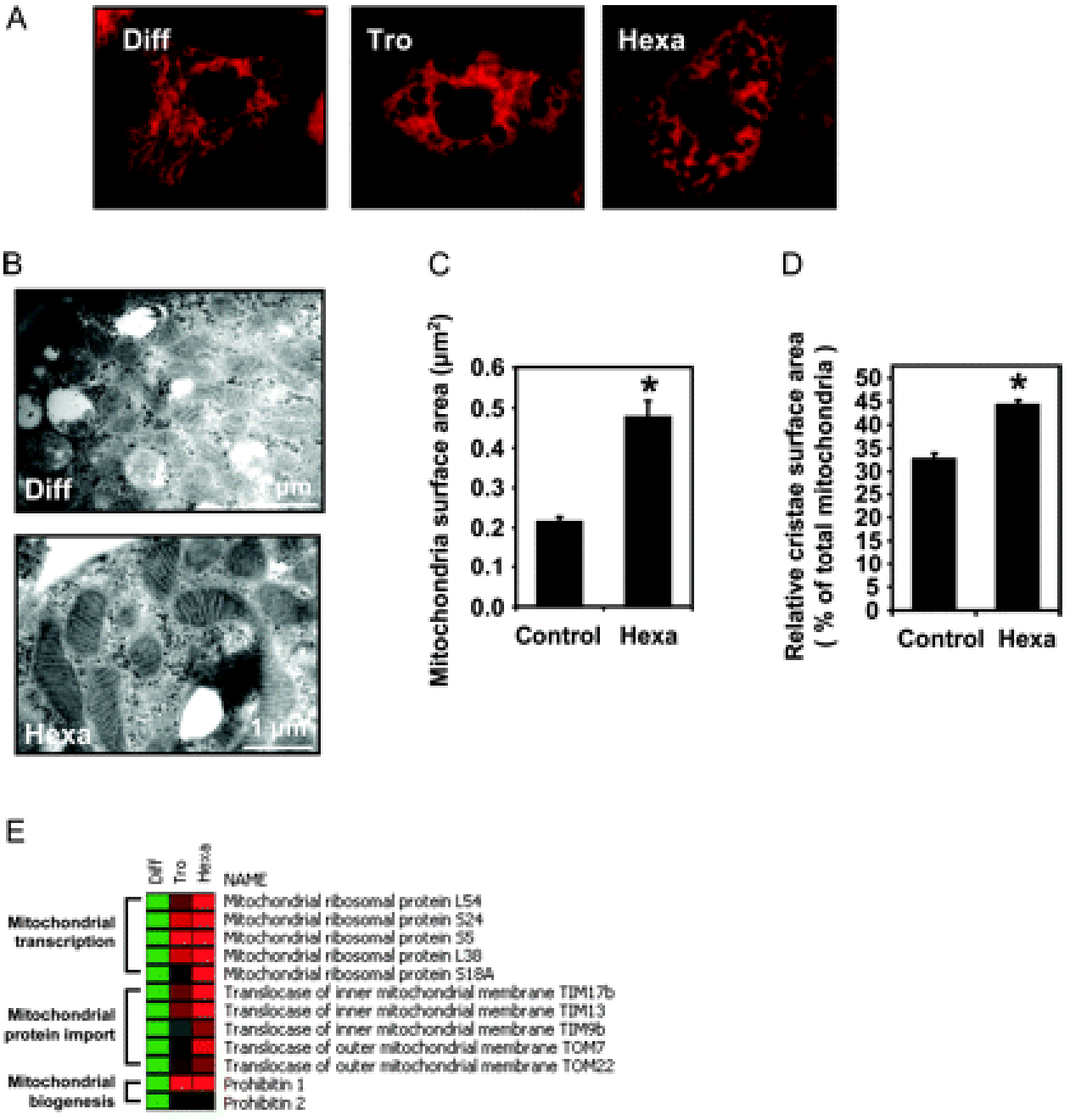
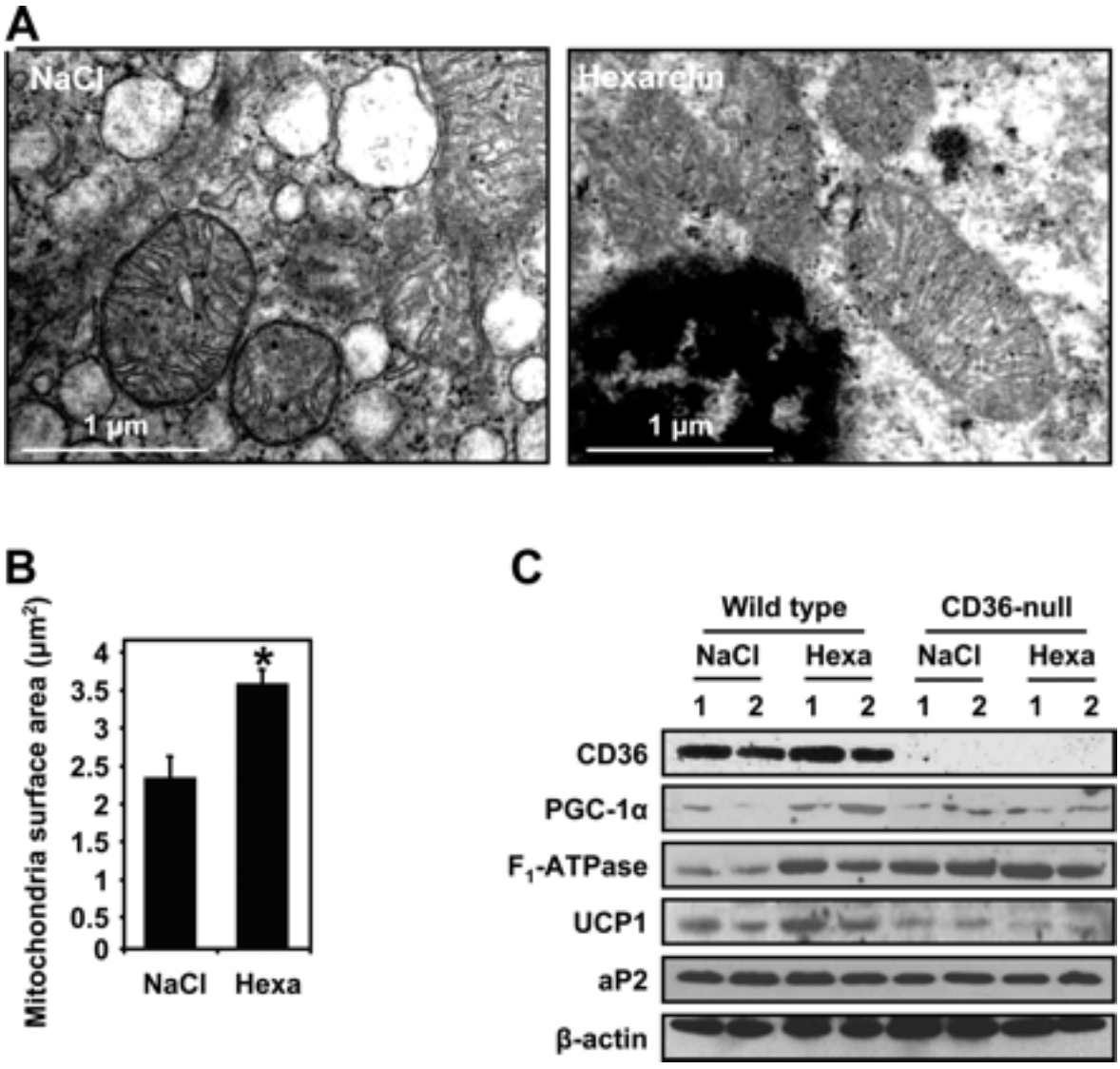

A
Diff
Tro
Hexa
B
Diff
1 µm
Hexa
1 µm
C
Mitochondria surface area (µm²)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
Control
Hexa
D
Relative cristae surface area
(% of total mitochondria)
50
45
40
35
30
25
20
15
10
5
0
*
Control
Hexa
E
Diff
Tro
Hexa
NAME
Mitochondrial transcription
Mitochondrial ribosomal protein L54
Mitochondrial ribosomal protein S24
Mitochondrial ribosomal protein S5
Mitochondrial ribosomal protein L38
Mitochondrial ribosomal protein S18A
Mitochondrial protein import
Translocase of inner mitochondrial membrane TIM17b
Translocase of inner mitochondrial membrane TIM13
Translocase of inner mitochondrial membrane TIM9b
Translocase of outer mitochondrial membrane TOM7
Translocase of outer mitochondrial membrane TOM22
Mitochondrial biogenesis
Prohibitin 1
Prohibitin 2

Figure 4


A
NaCl
Hexarelin
1 μm
1 μm
B
Mitochondria surface area (μm²)
4
3.5
3
2.5
2
1.5
1
0.5
0
*
NaCl
Hexa
C
Wild type
CD36-null
NaCl
Hexa
NaCl
Hexa
1 2 1 2 1 2 1 2
CD36
PGC-1α
F1-ATPase
UCP1
aP2
β-actin

2 Paper 2

Scavenger Receptor CD36 mediates inhibition of cholesterol synthesis via activation of the LKB1-AMPK pathway in hepatocytes

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Short title: CD36 triggers HMG-CoA reductase degradation

DISCLOSURE STATEMENT: The authors have nothing to disclose.

Keywords: Scavenger receptor CD36; hexarelin; growth hormone releasing peptides; GHRP; AMP-activated kinase; PPARγ; HMG-CoA reductase; Insig-1; Insig-2; SCAP; cholesterol synthesis; HepG2 hepatocytes; ubiquitin; 26S proteasome

Foreword for paper #2

Despite an ongoing interest in both CD36 and PPARγ, their role in liver or hepatocytes remains somewhat less well defined. Previous studies demonstrated that administration of hexarelin in rodents reduced total plasma cholesterol, increased HDL-c, but decreased LDL-c (De Gennaro-Colonna *et al.*, 2000;Pang *et al.*, 2010). We wanted to establish aside from its role in the activation of the reverse cholesterol transport pathway (in macrophages), if a decrease in *de novo* cholesterol synthesis could be factored into the effect of hexarelin via CD36. Based on the results generated in Paper #1, we set out to determine if in hepatocytes, hexarelin's binding to CD36 would result in the induction of FAO. We looked at the cell's response following activation of the key regulators of FAO, LKB1 and AMPK. Since cholesterol synthesis in hepatocytes is particularly important and AMPK is known to target HMGR, special attention was given to the impact of AMPK activation on cholesterol synthesis. In addition, the downstream effect on PPARγ activity was also evaluated.

Contributions of authors:

Amélie Rodrigue-Way: Developed project. Designed, performed experiments and analyzed results. Wrote the first draft of the manuscript.

Stéphanie Bilodeau: Performed PCRs and participated in paper discussion.

Méryl Hassan: Constructed HA-PGC-1α and participated in project discussion.

Véronique Caron: Participated in project discussion.

Emile Levy: Design intracellular cholesterol experiment.

Grant A Mitchell: Participated in project and paper discussion.

André Tremblay: As my PhD supervisor, supervised and participated in the conception of my project and the design of experiments. Corrected and submitted finished manuscript.

Abstract

Scavenger receptor CD36 is known to play a central role in lipid metabolism through at least the uptake of oxidized LDL in macrophages and its ability to internalize long chain fatty acids in adipocytes. Here, we demonstrate that CD36 activity impacts cholesterol synthesis in hepatocytes. Using hexarelin, a growth hormone-releasing peptide that interacts with CD36, we found a rapid phosphorylation of LKB1 which led to subsequent AMPK phosphorylation in treated HepG2 cells. HMG-CoA reductase, which catalyzes the rate-limiting step in cholesterol synthesis, was phosphorylated and inactivated by AMPK following hexarelin treatment. This was accompanied with a significant degradation of HMG-CoA reductase by the ubiquitin-proteosome system in response to CD36 activation through an enhanced recruitment of the escort protein Insig-2 in hepatocytes. We also determined that hexarelin lifted the exerted inhibitory effect of Erk on nuclear receptor PPARγ activity through a rapid dephosphorylation of Erk, and promoted the recruitment of AMPK to PPARγ coactivator PGC-1α, suggesting an enhanced transcriptional potential of PPARγ. Several genes of the oxysterol-binding protein (OSBP) family were found upregulated by hexarelin and PPARγ ligand troglitazone in treated cells, supporting an enhanced shuttling of cholesterol from endoplasmic reticulum and identifying OSBPs as PPARγ-regulated genes. These results provide a mechanistic basis by which CD36 modulates HMG-CoA reductase degradation and PPARγ coactivation through the LKB1/AMPK pathway, providing a novel role of CD36 to regulate cholesterol synthesis in hepatocytes.

Introduction

The pathogenesis of the metabolic syndrome is thought to involve a complex interaction of multiple factors, which include central obesity, insulin resistance, inflammation, hypertension, atherogenic dyslipidemia, and prothrombotic states. Restoration of these individual metabolic abnormalities currently involves numerous treatment options with various efficacies and development of novel approaches is of intense interest. Inherent to its direct impact on glucose and lipid metabolism, the peroxisome-proliferator activating receptor PPARγ is a therapeutic target of the thiazolidinedione family of compounds currently used to improve insulin sensitivity. PPARγ is a transcription factor that upon ligand activation regulates the expression of a number of gluco/lipogenic genes in tissues such as heart, muscle, liver and fat.

By providing fatty acid derivatives which serve as endogenous ligands for PPARγ through the selective uptake of long chain fatty acids or internalization of oxidized LDL particles, the scavenger receptor CD36 has been associated with PPARγ activation. In macrophages, this CD36-PPARγ pathway has been linked to pro-atherogenic events by contributing to a positive feedback loop in which activation of PPARγ results in enhanced expression of CD36, itself a target gene of PPARγ, thereby delivering more ligands to PPARγ. To escape this atherogenic loop, macrophage-internalized oxidized LDL also provides oxysterols which can activate nuclear receptor LXRα, leading to enhanced cholesterol efflux through expression of ABC sterol transporters and apolipoprotein E. In particular, the role of CD36 in macrophages is best known for the uptake of oxLDL particles and the subsequent formation of foam cells contributing to atherosclerotic plaque formation (1-3).

Hexarelin was originally described to stimulate central GH release through its binding to the GH secretagogue-receptor-1a (GHS-R1a), a G protein-coupled receptor now recognized as the ghrelin receptor (4;5). Hexarelin and other synthetic GH-releasing peptide (GHRP) were also shown to interact with CD36 in myocardium (6;7). The

peripheral distribution of CD36 and GHS-R1a receptors in tissues such as heart, adrenals, fat and bone has supported physiological roles of hexarelin not exclusively linked to GH release. For instance, we have shown that hexarelin impacted fatty acid mobilization through CD36 in white fat by increasing fatty acid oxidation and mitochondrial biogenesis with an enhanced expression of PPARγ target genes, resulting in a thermogenic-like profile (8). In addition, hexarelin exerted an enhanced cholesterol removal from macrophages through the activation of the PPARγ-LXRα-ABC metabolic pathway, resulting in beneficial reduction in plaque formation in atherosclerotic mice (9). These studies have provided a potential for hexarelin to regulate peripheral fatty acid and cholesterol metabolism through CD36.

The ubiquitously expressed 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) is the rate-limiting enzyme in the cholesterol synthesis pathway. HMGR enzyme is tightly regulated by cellular cholesterol and this at various levels including gene expression regulation, inactivation via enzyme phosphorylation and protein degradation (10). The expression of HMGR as well as several other genes involved in cholesterol synthesis are regulated by sterol regulatory element-binding proteins SREBP-1 and -2, which are endoplasmic reticulum (ER) membrane-embedded transcription factors that are released to translocate to the nucleus upon sterol depletion (11-15) . When sterols accumulate in cells, HMGR is rapidly degraded in a process involving binding to the insulin-inducible genes Insig-1 and -2. Such recruitment of Insigs results in a rapid ubiquitination and degradation of HMGR through the ubiquitin-proteosome system (16;17). Insigs are also know to inhibit SREBP processing and activation by binding to SREBP cleavage-activating protein Scap also embedded in the ER membrane, therefore sequestering the Scap/SREBP complex in the ER and subsequently decreasing the expression on SREBP target genes when high cellular sterol levels are achieved (18-20). The Insig-regulated swift degradation of HMGR protein and retention of SREBP in the ER prevent the accumulation of sterols to toxic levels within the cell. Insig-1 and -2 are highly expressed in liver (18), and despite their functional similarities, they conceal important

differences. Unlike Insig-2, Insig-1 is a target of SREBP and therefore its expression is high in sterol-depleted cells, whereas like HMGR, it is rapidly ubiquitinated and degraded in the presence of sterols (21). With its longer life span and insensitivity to sterols, Insig-2 appears as an interesting target in modifying cholesterol homeostasis outside of the tightly regulated mechanism observed with Insig-1 (21;22).

AMP-activated kinase (AMPK) is activated under energy deprivation conditions typically inhibiting anabolic reactions such as fatty acid and protein synthesis and enhancing catabolic processes such as fatty acid oxidation with the overall goal to produce more ATP (23). In a state of energy expenditure, AMPK was shown to phosphorylate HMGR at Serine 872 leading to enzyme inhibition and therefore providing more acetyl-CoA substrate for oxidation (24). All these actions are aimed at reducing energy expenditure and favoring ATP production, which defines AMPK as a cellular energy sensor.

Here, we demonstrate that hexarelin inhibits cholesterol synthesis in hepatocytes through the activation of the LKB1/AMPK pathway and HMGR phosphorylation, supporting a role of CD36 in regulating AMPK activity and downstream targets. In addition to enzyme inhibition, HMGR protein ubiquitination and degradation was also favored, with a concomitant increase in the expression of Insig-2, resulting in an increased interaction between Insig-2 and HMGR in a CD36-dependent manner. We also show that hexarelin caused a rapid dephosphorylation of Erk which, in conjunction with AMPK activation of PGC-1α, results in the downstream activation of PPARγ and increased expression of several genes of the oxysterol-binding protein family, highlighting the potential of CD36 to regulate intracellular cholesterol trafficking in hepatocytes.

Material and Methods

Cell Culture, Reagents and Treatments

The human liver hepatocellular carcinoma cell line HepG2 were grown in DMEM supplemented with 10% fetal bovine serum (FBS). At 70-80% confluence, media of

HepG2 cells was changed either to DMEM supplemented with 10%FBS or without serum for 16 hours prior to treatment with hexarelin (ProSpec-Tany TechnoGene, Ltd., Rehovot, Israel) for the determined time and concentration. Treatment with either lanosterol (2.5 uM) or 25-hydroxysterol (2.5 uM) (Sigma) was done for 5 hours unless otherwise stated. Troglitazone (8 uM) and Rosiglitazone (1 uM) were purchased from Cayman Chemicals (Ann Arbor, Michigan, USA). Lanosterol (2.5 uM), 25-hydroxyxholesterol (2.5 uM), the PPAR gamma antagonist GW9662 (10 uM), the AMPK agonist AICAR, 5-amino-4-imidazolecarboxamide riboside (0.2-2 mM) and its inhibitor Compound C (20 uM) were purchased from Sigma and used from the indicated times. PD98059 (50 uM) and SB203580 (10 uM) were purchased from BioMOL Research Labs and LY294002 (10 nM) from Enzo Life Sciences Plymouth Meeting, US).

RNA isolation and RT-PCR analysis

Total RNA was isolated from HepG2 cells using TRIzol reagent (Invitrogen, Burlington, Ontario, Canada) and RT-PCR analysis was done as described (8;9;25). PCR products were analyzed on gel (Alpha Innotech, San Leandro, CA) from at least 3 separate experiments. All values were normalized against 36B4 expression. Primers were designed for human HMG-CoA reductase, mevalonate kinase, CD36, SREBP-2, insig-1 and insig-2, PPARγ, ABCA1, lanosterol synthase, oxysterol-binding protein, OSBP-related proteins and 36B4.

RNA Interference

To silence CD36 expression, small hairpin RNA duplexes targeting the sequence AGGTCAACATATTGGTCAA of human CD36 (shCD36) were inserted into the pSuper lentiviral vector for small interfering RNA production. A shRNA containing the sequence of luciferase (shLuc) was used as a negative control. Viral particles were produced in 293T cells as described previously (26), and used to infect HepG2. CD36 efficient knockdown was monitored by RT-PCR and Western analysis (data not shown).

Total Cellular Cholesterol Measurement

Total lipid content was extracted using the method of Folch (8;27) and total cholesterol measurement was determined using a detection kit by Roche as described previously (28). Briefly, lipids HepG2 cells were extracted with Folch solution consisting of a mixture of 2:1 (vol/vol) chloroform/methanol and resuspended in 20% Thesit (Sigma) in Folch solution before evaporation under nitrogen gas. Total cellular cholesterol is determined by the means of cholesterol esterase and cholesterol oxidase and the amount of H_2O_2 produced is measured by colorimetric assay involving peroxidase. The amount of cholesterol is normalized against total protein from each sample which was determined by Bradford reagent (Sigma).

Stable cell line

pCMV-Insig-2-Myc was purchased from ATCC (Manassas, VA, USA). Plasmid construct was introduced into HepG2 cells using the calcium phosphate precipitation method as described as previously (29). Stable transfectants of Insig-2/Myc were obtained following selection with G418 at 1000 ug/ml for 6 weeks. For a lack of a suitable antibody against insig-2, cells that expressed stable Insig-2-Myc were monitored by Western using anti-myc antibody (9E10 hybridoma) as described as previously (19;30) prior to co-immunoprecipitation experiments.

Plasmid Constructs, DNA Transfection and Luciferase Assay

The pHMGR-bLuc reporter plasmid was generated by cloning the 247-bp portion of the promoter region of HMG-CoA reductase (positions -243 to +4 of human gene) containing the characterized SREBP-responsive element (SRE) into the b-luciferase reporter gene (31). Typically, for luciferase assay, HepG2 cells were seeded into 24-well plate and transfected with 500 ng pHMGR-bLuc construct and 200 ng pCMX-βgal in a total of 1.5 ug DNA per well. After 16 hours, the medium was changed. Forty-eight hours after transfection, medium was changed to DMEM without serum overnight. Cells were treated with 10^{-5} M hexarelin or with 2.5 uM 25-hydroxycholesterol. Cells were then harvested in potassium phosphate buffer containing 1% Triton X-100 and lysates were analyzed for luciferase activity using a luminometer (Wallac, Turku, Finland). Luciferase

values were normalized for transfection efficiency to β-galactosidase activity and expressed as relative fold response compared to controls. Luciferase assays were performed in triplicates from at least three independent experiments. The Gal4-PPARγ1 and Gal4-PPARγ1 serine-84 to alanine mutant (Gal4-PPARγ1S84A) have been described previously (25). HepG2 cells were transfected with either PPARγ1 construct and UAStkLuc reporter as described above. Typically, 500 ng of reporter plasmid, 100 ng PPARγ1 expression vector and 200 ng pCMX-βgal in a total of 1.5 ug DNA were added per well. Cells were treated with hexarelin or rosiglitazone at the indicated concentration. pSV-SPORT-PGC-1α was a generous gift from Bruce M. Spiegelman. PGC-1α was extracted and subcloned into pCMX-HA vector using the Bam HI sites. HepG2 cells were transfected and co-immunoprecipitation experiments were performed as described in the cell lysates, immunoprecipitation and immunoblotting analysis section to analyze the HA-PGC-1α and AMPK interaction in response to hexarelin.

Antibodies

Antibodies to phospho-AMPK (Thr172), phospho-LKB (Ser428), LKB, phospho-AKT (Ser473) and total AKT were obtained from Cell Signaling Technologies (Beverly, MA). Antibodies to AMPK, PPAR gamma, PGC-1, HMGR, anti-ubi, SCAP and F1-ATP synthase were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to phospho-ERK1/2 (Thr202/Tyr204) and ERK1/2 were obtained from Invitrogen (Carlsbad, CA). Antibodies to HMGR and phospho- PPARγ (Ser84/Ser112) were obtained from Upstate (Lake Placid, NY). Antibodies to HMGR, phospho-HMGR (Ser871) and anti-β-actin were purchased from Abcam (Cambridge, MA). Antibodies to c-myc have been described previously (32). HA antibody was purchased from Roche (Laval, Quebec, Canada).

Cell Lysates, Immunoprecipitation and Immunoblotting Analysis

Immunoblotting analysis was performed as described (8). Briefly, cells were lysed in RIPA, consisting of PBS buffer containing 1% Triton X-100, 0.5% deoxycholate acid, 0.1% sodium dodecyl sulphate (SDS), 1 mM sodium fluoride, 1 mM sodium

orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitors (Roche, Laval, Qc). Proteins were then resolved by SDS-PAGE and transferred to nitrocellulose for immunoblotting. Membranes were blocked with blocking reagent (Roche) in Tris-buffered saline, probed with selected antibodies, and signals revealed by Enhanced Chemiluminescence or ECL Plus Western Blotting Detection System (Amersham) using appropriate horseradish peroxidase-conjuguated secondary antibodies. Co-immunoprecipitation of insig-2-Myc with HMGR was performed as follow. HepG2 stable insig-2-Myc transfectants were incubated overnight in DMEM without serum at 70% confluence. MG132 (10nM) was added to100-mm dishes 1 hour prior to treatments and treatments were done at the indicated concentration and time. Cells were collected in ice-cold PBS 1x and spun at 8000xg, 5min. Cells were lysed in modified Nonidet P-40 buffer (50 mM Hepes-KOH (pH 7.4), 100mM NaCl, 1.5 mM $MgCl_2$, 0.5% NP-40, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM PMSF, and protease inhibitors) as previously described (33;34). Briefly, cell lysates were passed through 26-gauge needle 15 times and then extracted for 1.5 hours at 4 C. For co-immunoprecipitation, cell lysates were precleared prior to incubation with anti-myc antibody (9E10) overnight at 4 C with gentle agitation. Immune complexes were recovered with protein A/G-PLUS agarose (Santa Cruz Biotechnology), washed four times in lysis buffer and subjected to SDS-PAGE and immunoblotting with indicated antibody. Two different antibodies were used to confirmed presence of HMGR in insig-2-myc precipitates. Immunoprecipitation of PPARγ was done essentially as described (25). Cells were lysed in modified RIPA consisting of 1% Nonidet P-40, 0.25% deoxycholate acid and 0.7M NaCl. Immunoprecipitation of HMGR was done as described above with addition of N-ethylmaleimide (25mM) in modified RIPA for the detection of Ubi-HMGR.

Results

Activation of CD36 by hexarelin promotes the LKB1-AMPK pathway in hepatocytes

We previously showed that activation of CD36 by hexarelin induced an increase in fatty acid oxidation and metabolic regulation of mitochondrial energy pathway in adipocytes (8). Based on these findings and on the prominent role of AMPK in cellular energy sensing mechanism, we wanted to analyze the effect of hexarelin on AMPK activity in HepG2 cells. Cultured hepatocarcinoma HepG2 cells exhibit high oxidative capacity and express CD36 at high levels while GHS-R1a expression was not detected (35;36) (and data not shown). We found that treatment of HepG2 cells with 10^{-5}M hexarelin induced a rapid time-dependent increase in AMPK phosphorylation at Thr-172, a required activating site of AMPK catalytic α-subunit (Fig.1). The upstream LKB1 kinase, known to phosphorylate AMPKα Thr-172, was also activated by hexarelin in a manner preceding AMPK phosphorylation (Fig.1). These results indicate that hexarelin activates the LKB1-AMPK pathway in HepG2 cells, which suggests a role in modulating energy metabolism in these cells.

Hexarelin decreases HMG-CoA reductase and total cholesterol content in HepG2

AMPK is considered a metabolic sensor of cellular energy depletion state which upon its activation, acts on different targets to arrest energy expenditure and produce ATP. HMG-CoA reductase (HMGR), which catalyzes the rate-limiting step in cholesterol synthesis, is amongst the proteins targeted by AMPK. We thus examine by Western analysis the steady-state levels of HMGR and found that hexarelin treatment of HepG2 cells for 6 hours reduced HMGR protein to levels comparable with sterols 25-hydroxycholesterol and lanosterol, both known to regulate sterol-dependent inhibition and degradation of HMGR (Fig. 2A). To correlate with the reduction of HMGR, we measured total cellular cholesterol content in cells following treatment with hexarelin for up to 24 hours. We observed a time-dependent decrease in total cholesterol that reached a significant 2.4-fold reduction at 12hrs of treatment compared to control cells, and that was maintained with a 1.7-fold decrease after 24 hours (Fig.2B). These results suggest that the reduction in HMGR protein following hexarelin treatment results in total cholesterol depletion in hepatocytes.

Sterol depletion caused by hexarelin induced a rapid and transient increase in expression of SREBP-regulated genes

Gene expression of HMGR and other enzymes involved in cholesterol synthesis and uptake is tightly regulated by SREBPs, which upon proteolytic release from the Golgi, translocate to the nucleus to regulate transcription (10). We thus measured the transcriptional activity of SREBPs in HepG2 using a luciferase reporter gene under the control of the proximal promoter of HMGR which contains a SREBP-responsive element (SRE). We found that hexarelin treatment of HepG2 cells induced a modest and transient increase in SREBP activity over a 24hr period (Fig.3A). As a control, treating cells with 25-OH cholesterol resulted in the expected sterol-mediated inhibition of SREBP. We then measured the expression of HMGR and other components of cholesterol metabolism in HepG2 cells and found that hexarelin exerted a rapid increase of HMGR expression, and of mevalonate kinase (MVK) and lanosterol synthase (LSS), also involved in key steps of cholesterol biosynthesis and under SREBP regulation (Fig.3B). SREBP-2 was itself upregulated by hexarelin and maintained over the 6hr period of treatment. Despite a similar decrease in HMGR protein content as with hexarelin (Fig.2A), exposing HepG2 cells to 25-OH cholesterol did not result in increased expression of SREBP and target genes as opposed to hexarelin, indicating a different mechanism of action of hexarelin in comparison with sterol loading. In line with the reduced HMGR and cholesterol content exerted by hexarelin in HepG2 cells (Fig.3), the transient increase of genes directly involved in *de novo* cholesterol synthesis we observed might represent a compensatory mechanism intended to counteract the decrease in cholesterol.

Phosphorylation of HMG-CoA reductase by AMPK

Our results suggest that CD36 activation with hexarelin might impact HMGR function at the protein level. We thus tested the possibility that by promoting AMPK activity, hexarelin can direct HMGR phosphorylation at Ser-872, described to be targeted by AMPK and inactivate the enzyme to halt cholesterol synthesis (24;37). Treatment of HepG2 cells with increasing amounts of hexarelin (10^{-7}M to 10^{-5}M) and over a period of

30min to 24hrs resulted in enhanced specific phosphorylation of HMGR Ser-872 as determined by Western analysis (Fig.4A and B). Such increase in HMGR phosphorylation was paralleled with a corresponding phosphorylation of AMPK Thr-172, demonstrating the concomitant activation of AMPK. As Ser-872 phosphorylation is involved in HMGR inhibition and that HMGR activity is highly controlled through degradation by the ubiquitin-proteasome system, we measured HMGR ubiquination in HepG2 cells in response to hexarelin. Cells were first serum-depleted to reduce the rate of degradation of HMGR, and then treated for 1-24hrs and subjected to immunoprecipitation of HMGR prior to Western analysis with anti-ubiquitin antibody. We found that in these conditions, hexarelin promoted the ubiquitination of HMGR in a time-dependent manner, with a peak at 6hrs of treatment, corresponding to also maximal HMGR phosphorylation (Fig.4C). This suggests that hexarelin has a direct impact on HMGR degradation.

CD36-dependent regulation of HMG-CoA reductase degradation is mediated by Insig-2

In order to further investigate the molecular events responsible for HMGR ubiquitination and degradation; we next analyzed the contribution of Insigs in the response to hexarelin. In response to sterols, endoplasmic reticulum (ER)-embedded Insig-1 and -2 were described to directly interact with HMGR and Scap, an another ER protein, which results in respective HMGR ubiquitination and SREBP sequestration in order to limit cholesterol synthesis (10). Interestingly, gene expression analysis revealed that expression of Insig-2 was increased in hexarelin-treated HepG2 cells, while Insig-1 remained unaffected (Fig.5A), suggesting an isoform-specific effect of hexarelin. These changes did not correlate with the expected decrease in Insig-1 and no change in Insig-2 expression following treatment with 25-OH cholesterol (38), again indicating a different mechanism triggered by hexarelin as opposed to sterol loading (Fig.5A). To address the functional role of Insig-2 in the response to hexarelin and to ensure unregulated constitutive expression in respect with hexarelin effect on Insig-2 expression, we generated stable transfectants of HepG2 cells expressing myc-tagged Insig-2 to perform co-immunoprecipitation studies of

Insig-2 with HMGR. Treatment of the Insig-2/HepG2 cells with hexarelin promoted the interaction of Insig-2 with HMGR both in a time- and concentration-dependent manner, with a peak at 5hrs of treatment (Fig.5B and C). Although less pronounced, a similar recruitment of Scap with Insig-2 was noticed under the same conditions, indicating that hexarelin also promotes to some extent the retention of SREBP in the ER through Insig-2 and Scap (Fig.5B). These effects possibly prevent a more pronounced increase in expression of target genes in order to compensate for the reduced total cholesterol in treated cells (Fig.2B). In addition, Figure 5D shows that the hexarelin-induced recruitment of HMGR to Insig-2 was completely abrogated using a lentiviral knockdown strategy with a shCD36 as previously described (25). These results indicate that CD36 is essential in mediating the effects of hexarelin on Insig-2/HMGR assembly

The recruitment of Insig-2 to HMGR is triggered by the activation of AMPK

In order to establish whether phosphorylation of HMGR is involved in Insig-2 recruitment, therefore linking phosphorylation of HMGR with its degradation process, Insig-2/HepG2 cells were treated with the AMPK agonist, 5-amino-4-imidazolecarboxamide riboside (AICAR) in presence or absence of hexarelin. Figure 6A shows a potent increase in the interaction of Insig-2 with HMGR in AICAR-treated cells compared to untreated cells. The Insig-2/HMGR recruitment was further enhanced when hexarelin was combined with AICAR, suggesting an additive recruiting effect of both compounds (Fig.6A). To further demonstrate the contribution of AMPK, hexarelin-induced Insig-2/HMGR interaction was abrogated in the presence of the AMPK inhibitor Compound C, suggesting an obligatory role of AMPK in mediating the response to hexarelin on HMGR recruitment to Insig-2 (Fig.6B).

PPARγ mediates the effect of CD36 on HMGR and Insig-2 interaction

Our previous studies have shown that hexarelin induces the transcriptional activity of nuclear receptor PPARγ in macrophages and adipocytes, establishing a CD36-PPARγ pathway that regulates expression of target genes involved respectively in cholesterol removal and lipid metabolism, including PPARγ itself but without affecting CD36

expression (8;9). In order to determine whether such pathway is functional in hepatocytes and might translate the effects of hexarelin on HMGR degradation, we observed a rapid increase in PPARγ expression in HepG2 cells treated with hexarelin, while CD36 remained unaffected (Fig.7A), suggesting a similar situation as in macrophages and adipocytes. Interestingly, HepG2 cells treated with troglitazone, a synthetic agonist of PPARγ of the thiazolidinedione family, exhibit an increase in AMPK activity (Fig.7B), which support previous findings in skeletal muscle and fibroblasts that ascribed such effect to a rise in AMP levels (39;40). In our conditions, troglitazone was even more potent in activating AMPK compared to hexarelin or AICAR, providing an important contribution of PPARγ. Troglitazone was also as effective as hexarelin in promoting the recruitment of HMGR to Insig-2 in stable HepG2 cells (Fig.7C). In order to determine the involvement of PPARγ in translating the effect of hexarelin, we found that treating Insig-2 stable cells with the PPARγ inhibitor GW9662 abolished the interaction of Insig-2 with HMGR in the presence of hexarelin (Fig.7D), suggesting that the elicited degradation of HMGR by hexarelin required activation of PPARγ.

Hexarelin relieves the inhibitory effect of Erk on PPARγ in HepG2 cells

In order to clarify the role of PPARγ in HMGR degradation, we sought to determine by which intracellular pathway hexarelin can modulate PPARγ activity in hepatocytes. Not much is known about the signalling events induced by CD36, especially regarding those triggered by hexarelin. These issues have been mostly covered with the reported activation of the ghrelin GHS-R1a receptor by hexarelin. In particular, we have shown that GHS-R1a activation by hexarelin induced PPARγ phosphorylation and subsequent activation in macrophages involving a concerted action of the Erk and Akt kinase pathways (9). We thus analyze the effect of hexarelin on both pathways in HepG2 cells. We found a rapid and prolonged decrease in Erk activity over a period of 60min of treatment with hexarelin, while Akt was activated under the same conditions in HepG2 cells (Fig.7E). In respect with this differential effect in kinase activity and based on previous work that reported an inhibitory role of Erk-mediated phosphorylation of PPARγ1 Ser-84 and corresponding Ser-

112 of fat specific-PPARγ2 isoform (41-43), we analyzed PPARγ1 (isoform present in hepatocytes) Ser-84 specific phosphorylation in HepG2 cells. We observed a marked and rapid de-phosphorylation of PPARγ Ser-84 by hexarelin, which was maintained from 15min to 24hrs of treatment (Fig.7F), suggesting that the inhibitory effect of Ser-84 phosphorylation was relieved in hexarelin-treated HepG2 cells. To directly address the effects of hexarelin on PPARγ activity, we performed luciferase assay on HepG2 cells transfected with a Gal4-PPARγ construct along with a UAStkLuc reporter, and observed a significant increase in PPARγ activity in response to hexarelin (Fig.7G). Altogether, these results demonstrate the ability of hexarelin to signal to and activate PPARγ in HepG2 cells through the release of the inhibitory action of the Erk kinase pathway. These results also suggest a role of Akt to act as an activating signal leading to PPARγ Ser-84 independent phosphorylation and activation, as it was demonstrated in other cell systems (9;44;45).

CD36 promotes activation and recruitment of coactivator PGC-1α to PPARγ

Based on the critical role of PPARγ in regulating glucose and lipid metabolism, and on the ability of CD36 to promote PPARγ-dependent expression of mitochondrial proteins involved in fatty acid oxidation in adipocytes (8), we tested whether a similar outcome was also taking place in hepatocytes. We observed that protein levels of F_1-ATP synthase, a target of PPARγ which catalyzes mitochondrial ATP production during oxidative phosphorylation, and of PGC-1α, a nuclear receptor transcriptional coactivator involved in energy metabolism and mitochondrial biogenesis, were both increased in HepG2 cells treated with hexarelin (Fig.8A). The increase in PGC-1α prompted us to examine its potential role in the response to hexarelin. Using co-immunoprecipitation assay, we found that hexarelin caused a rapid and prolonged increase in the recruitment of PGC-1α to PPARγ in HepG2 cells (Fig.8B). Such interaction correlated with a time-dependent increase in AMPKα recruitment to PGC-1α following hexarelin treatment (Fig.8C). AMPKα was described to directly phosphorylate and activate PGC-1α (46). Therefore, our results provide a molecular mechanism by which hexarelin promotes PGC-1α phosphorylation and

activation, resulting in enhanced coactivation of PPARγ and target gene expression in hepatocytes.

Expression of oxysterol-binding proteins (OSBPs) is upregulated by hexarelin

Newly synthesized cholesterol is rapidly transferred out of the endoplasmic reticulum by lipid transfer proteins (LTP), in order to maintain a low ER sterol content. The oxysterol-binding proteins (OSBP) and OSBP-related proteins (ORP) are members of the LTP family which bind sterol derivatives to achieve various cellular processes including cholesterol/oxysterol transfer, sterol sensing, cell signaling, lipid metabolism, vesicular trafficking and SREBP regulation (47). In view with our observation that hexarelin contributes to lower cholesterol levels while increasing genes involved in cholesterol synthesis, we addressed whether expression of several OSBP family members was regulated in these conditions. We found that expression levels of OSBP1 and ORP2, ORP3, ORP9 and to a lesser extent ORP11 were all increased in a time-dependent manner by hexarelin in HepG2 cells (Fig.9). Interestingly, all these genes were also upregulated to some extent by troglitazone, indicating a shared mechanism of regulation between hexarelin and PPARγ. However, treating cells with AICAR did not achieve a similar upregulation of OSBP1 and related ORPs, suggesting that AMPK activation is not sufficient in promoting a maximal response of these genes. These results provide a link between a possible redistribution of cholesterol to and from the ER with the sterol-sensing mechanism that directs HMGR degradation, and also identify OSBP members as novel PPARγ-regulated genes in hepatocytes.

Discussion

Unquestionably, AMPK plays an imperative role in energy balance. Most commonly, upon activation by an increase in AMP/ATP ratio following an increase in ATP spending, AMPK targets a plethora of pathways for the main purpose of reducing energy usage and produce readily available fuel for the cell. Thanks to AMPK, fatty acid oxidation is increased to produce acetyl-CoA which will then be shuttle towards the TCA cycle and oxidative phosphorylation to produce ATP. Cell proliferation, lipid synthesis and protein

synthesis are reduced as well as gluconeogenesis depending on the cell type and function (48). Hepatocytes have a high rate of fatty acid oxidation and AMPK activity, and are a major site for cholesterol synthesis. Our previous work with hexarelin in adipocytes has shown a marked increase in fatty acid oxidation and activation of PPARγ in a CD36-dependent manner (8). Based on these results, we hypothesized that hexarelin would have a similar effect in hepatocytes and that this effect would also have a direct impact on cholesterol synthesis by means of the activation of AMPK. In this article, in addition to showing an increase in phospho-AMPK and a propensity towards fatty acid oxidation, we show that AMPK also inactivates HMGR through phosphorylation. More importantly, we described for the first time the unexpected finding that hexarelin caused HMG-CoA reductase degradation in hepatocytes causing a marked decrease in total cellular cholesterol.

In hepatocytes, we established that hexarelin promoted a rapid increase in the phosphorylation of LKB and its downstream target AMPK. LKB1 mediates AMPK activation through the AMP-dependent pathway suggesting that hexarelin binding to CD36 in hepatocytes might cause an increase in AMP/ATP ratio and therefore activating LKB1 and AMPK. In addition, we determined that protein level of known factors involved in fatty acid oxidation such as PGC-1α and ATP synthase subunit F1 were increased in treated HepG2 cells conveying in this manner our previous findings in adipocytes to hepatocytes as well. What was surprising was the rapidity of the response to hexarelin. We saw an increase in pLKB and pAMPK within 5-10 minutes of treatment with hexarelin in HepG2 cells. Given one of the roles of CD36 in internalizing long chain fatty acids, it still remains interesting that the interaction of hexarelin with CD36 is capable of rapidly activating AMPK and signaling to the cell a need to conserve energy in hepatocytes. A reverse phenomenon is observed in cardiomyocytes, in which contractions decrease AMP levels and stimulate LKB1/AMPK pathways resulting in the translocation of CD36 to the cell membrane to increase LCFA uptake suggesting a crosstalk between CD36 and AMPK that can be triggered (49).

We wanted to extend our research beyond the realm of AMPK activation by hexarelin and look at its downstream impact on cholesterol synthesis in hepatocytes. We determined that AMPK rapidly targeted HMG-CoA reductase by inactivating it through phosphorylation at Ser-872. We also found that there was a marked decrease in total cellular cholesterol at 12 hours and was still maintained at lower levels even after 24 hours compared to untreated. The short time point suggested a rapid effect of hexarelin on cholesterol metabolism and therefore our analysis was focused mainly on the early events occurring in hepatocytes that would lead to cholesterol depletion. Acetyl-CoA can be produced from glycolysis or from fatty acid oxidation. Fatty acid oxidation generates a much larger quantity of acetyl-CoA depending on the length of fatty acid chain than glucose which generates only two molecules of acetyl-CoA demonstrating the more efficient pathway of FAO to produce large quantities of ATP from fat rather than sugar. Acetyl-CoA is associated with various pathways and its usage is closely monitored to provide cells with the capacity to respond to various conditions such as proliferation, energy consumption and production, lipid metabolism and distribution. In a sense, AMPK finds itself at the center of many of these processes to control energy consumption. Since the synthesis of HMG-CoA requires 3 acetyl-CoA molecules, it is of no surprise that the pathway to cholesterol synthesis would be inhibited or decreased if more energy is required. We therefore show that hexarelin caused a shift in energy balance towards preservation of energy expenditure and burning of fat to produce ATP by means of AMPK activation and inhibition of HMGR.

Unexpectedly, we observed that total HMGR protein level was decreased with hexarelin treatment following a short time period of 5 hours coinciding with the shortened half-life of HMGR exposed to high level of sterols (16). Along with an increase in HMGR phosphorylation by AMPK, hexarelin caused a decrease in protein level and an increase in ubiquitination of HMGR suggesting that HMGR underwent degradation. Association of insigs with HMGR is an absolute requirement and non-reversible step prior to ubiquitination and degradation of HMGR. Given the rapidity of the response, we looked at

earlier expression changes of genes involved in cholesterol metabolism. Genes involved in cholesterol synthesis such as HMGR and mevalonate kinase that are under the control of SREBPs were rapidly upregulated; however, the expression decreased roughly 6 hours despite a decrease in intracellular cholesterol. We also found that insig-2 but not insig-1 expression changed in response to hexarelin. Insig-1 expression is inversely correlated with its protein level while the expression of insig-2 is not; this is mainly due to differences in their degree of stability. Despite their functional similarities in retaining SCAP in the ER and accelerating degradation of HMGR, the differences observed in their protein level versus their expression level are due to the presence of ubiquitination sites in insig-1 (50). In the absence of sterols, SREBPs are translocated to the Golgi for processing and activation leading to an increase in the expression of insig-1. Its protein is no longer associated with SCAP and is susceptible to ubiquitination and proteosomal degradation making its expression level high while its protein level low. The expression of insig-2 is not controlled by SREBPs and has been shown to be upregulated by PPARα and γ agonists in hepatocytes and downregulated by insulin while its protein not susceptible to degradation has a longer half-life than insig-1 (51;52). To favor a strong detection of an interaction between HMGR and insig-2, we stably transfected insig-2 coupled to 6 copies of Myc (18). In insig-2 stably transfected cells treated with hexarelin, we observed an increase in the binding of insig-2 and HMGR. Therefore, the degradation of HMGR was not solely dependent on the increase in insig-2 expression but also on other events regulating the degradation of HMGR. In addition, the lack of more prominent increase in the expression of genes involved in cholesterol synthesis was explained by the fact that insig-2 also bound to SCAP, retaining SCAP/SREBP complex in the ER and preventing activation of SREBPs. Given the coincidence between AMPK activation and HMGR degradation by hexarelin, we wondered if the latter was influenced by AMPK activation. Indeed, we did see that activation of AMPK through the use of AICAR was contributing to HMGR degradation indirectly while the use of the inhibitor of AMPK, compound C abrogated the effects of hexarelin.

In the present study, we discovered a new mechanism via CD36 capable of triggering the degradation of HMGR through increased insig-2 expression and activation of insig-2/HMGR/ubiquitination without a rise in cholesterol or oxy/sterols. In an attempt to understand the events that led to the degradation of HMGR by hexarelin, we discovered that binding of hexarelin to CD36 resulted in a rapid dephosphorylation of Erk1/2. OxLDL activates Erk in THP-1 macrophages contributing to migration and foam cell formation (53). Interestingly, oxLDL and hexarelin share the same binding region on CD36 (6). In macrophages expressing both GHS-R1a and CD36, binding to either receptor can have opposite effects. Indeed, in a recent study, we demonstrated that treating macrophages with ghrelin caused an increase in phospho-Erk1/2 and while this activation can result in inactivation of PPARγ, we found that Dok-1 associated to GHS-R1a restrains the inhibitory potential of Erk (25). In the current study, we demonstrate that in the absence of GHS-R1a, the binding of hexarelin to CD36 in hepatocytes causes a decrease in phospho-Erk which can no longer phosphorylate PPARγ at Ser84. Therefore, hexarelin lifts the exerted inhibitory effect of Erk on PPARγ activity. To add to this effect, we found that hexarelin caused an increase in the interaction between AMPK and its known target, PGC-1α. Surprisingly, both AMPK and PPARγ seem to trigger HMGR degradation as both use of AICAR (known to increase PPARγ expression) or troglitazones can induce interaction of insig-2 with HMGR. Insig-2 is much more stable than insig-1; therefore, its effect on lipid metabolism can be sustained by an increase in its expression (52). What is surprising is that HMGR degradation is thought to only be triggered by sterols; which brings us to consider potential mechanisms capable of bypassing this tightly regulated pathway. Although, insigs, HMGR and SCAP have determinant roles in the fate of HMGR half-life and SREBP activity, it seems logical to deduce that the amount of sterol in the ER is the predominant factor affecting cholesterol metabolism. The ER is the main site for cholesterol synthesis; however cholesterol is rapidly transported to other organelles, making the cholesterol content in the ER approximately 5 mol% (54). Sterol concentrations are highly controlled and monitored to maintain cholesterol at this level in the ER (55;56). Recent studies have

emerged demonstrating that a concentration below that point triggers the SCAP/SREBP complex to be translocated to the Golgi for subsequent activation of SREBPs and target genes (55;56). On the other hand, the cholesterol concentration in plasma membrane is around 30 mol% (54). Once synthesized, the cholesterol in the ER is rapidly transported to other organelles such as the plasma membrane and the Golgi (54). The search for oxysterol-binding protein (OSBP) and OSBP-related proteins (ORPs) was prompt by the notion that cholesterol and other lipids in order to be relocated to and from various biological membranes would require transporters (47). OSBP/ORPs are part of a large family of proteins encoded by 12 genes and which result in 16 protein products that have cholesterol and oxysterol-binding motifs along with regulatory and membrane targeting domain functions and are involved in sterol signaling and sterol transport functions between lipid rafts, ER, Golgi, possibly having distinct effects in cholesterol metabolism (47;57). The affinity of OSBP/ORPs for specific subcellular membranes and how their membrane-association is regulated still remains elusive. However, studies on specific family members are emerging and clearly demonstrate that individual members seem to have a specific role and preference in lipid transport and cell signaling. For example, depletion of membrane cholesterol has been shown to increase the level of phospho-Erk in caveolea membranes and cytosolic fractions and OSBP was found to indirectly control Erk through its association with cholesterol and Erk phosphatases (58). Another study showed that ORP2 overexpression increased HMGR activity by increasing export of cholesterol from ER by ORP-2 (59). ORP9 was recently shown to mediate the transport of sterols between the ER and *trans*-Golgi/*trans*-Golgi network (60). New studies show that OSBP/ORPs are also targets of kinases; for instance, ORP3 was shown to be phosphorylated when macrophages lost their adhesion contact while OSBP was recently shown to be phosphorylated by protein kinase D (61;62). A recent study demonstrated the association of the regulation of cholesterol synthesis with the mobilization of cholesterol to the ER without modifying exposure of cells to sterols (63). For instance, treatment of human fibroblasts with bacterial SMase C, which degrades the sphingomyelin (component of cell membrane known to

interact with cholesterol) into ceramide caused a rapid translocation of plasma cholesterol to the ER. This change in ER cholesterol content resulted in a 90% inactivation of HMGR represented by an increase in its phosphorylation. However, in this study the authors failed to show the steady state of HMGR and therefore degradation of HMGR was not analyzed. No studies have reported a role of AMPK in the phosphorylation of OSBP or ORP; however, given our results demonstrating a role of AMPK in HMGR degradation, it would be interesting to determine if OSBP/ORPs are indeed targets of AMPK. We were able to determine that hexarelin and troglitazone induced expression changes of OSBP and several ORPs indicating a disruption in the transport of cholesterol between different compartments and these changes in expression may represent a direct effect of hexarelin on HMGR degradation. These results offer a potential explanation for changes of cholesterol content triggering HMGR degradation. These studies demonstrate the capability of hexarelin to change the expression of OSBP and ORPs which control the trafficking of sterols to and from the ER and other organelles. Therefore, we speculate that the increase in HMGR degradation due to hexarelin might result from the involvement of one or more oxysterol-binding proteins influenced by the activity of PPARγ and possibly AMPK. It is possible that in response to hexarelin binding to CD36, a deregulation in the expression and/or the activity of one or more OSBPs occurs, causing an increase in cholesterol in the ER, an indirect inactivation of Erk by OSBP and degradation of HMGR. Moreover, the failed increase in SREBP activity in cholesterol depleted cells treated with hexarelin can also be linked to Erk and OSBP since that in addition to its effect on PPARγ, Erk is known to target SREBPs and that OSBP expression has a repercussion on the activity of SREBP-1c (64;65). Evidently, given the emerging studies on OSBPs, it will be important to determine the precise role of AMPK, PPARγ and possibly OSBPs in HMGR degradation.

As depicted in Figure 10, the present study demonstrates that the binding of hexarelin to CD36 causes the activation of AMPK which in turn inactivated HMGR and activated PGC-1 thus promoting fatty acid oxidation. We also observed a decrease in phospho-Erk1/2 and an increase in PPARγ activity in a ligand-independent manner causing

a modification in the expression of several key genes involved in cholesterol metabolism such as insig-2, and OSBP/ORPs. The interaction of hexarelin with CD36 caused the degradation of HMGR following its interaction with insig-2. We extrapolate our findings by suggesting that OSBP/ORPs might be the binding factors between AMPK and PPARγ by causing a deregulation in cholesterol trafficking in the ER and thereby triggering the degradation of HMGR.

Although the precise mechanisms by which hexarelin exerts its effect through CD36 is not fully known, it had become clear that interacting with CD36 induces profound changes in lipid metabolism. We have shown previously that hexarelin induces gene expression changes of key regulators in fat metabolism under the control of PPARγ resulting in the mobilization of fatty acids toward mitochondrial oxidation phosphorylation in white adipocytes. In the present study, we demonstrated that a similar phenomenon occurs in hepatocytes, an important site for fatty acid oxidation. In addition, along with an activation of PPARγ, we observed an activation of AMPK and a degradation of HMGR. These results implicate for the first time CD36 in the regulation of cholesterol synthesis via an AMPK- and/or PPARγ-dependent pathway(s) through its interaction with hexarelin. Therefore, modulation of CD36 function might be an interesting approach to treating hypercholesterolemia.

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Figure Legends

Figure 1. Hexarelin induces phosphorylation of LKB1 and AMPKα in human hepatoma cell line, HepG2. Phosphorylation of AMPKα and LKB1 in HepG2 cells treated with hexarelin 10^{-5}M for 5 to 60 minutes. Cell lysates were analyzed by immunoblot

using a phospho-AMPKα (Thr172) or a phospho-LKB1 (Ser473) antibodies and normalized to anti-AMPK and anti–LKB1 antibodies. Protein loading was normalized using anti-β-actin antibody.

Figure 2. Hexarelin decreases HMG-CoA reductase and induces a net decrease in total intracellular cholesterol in HepG2. (A) Hexarelin causes a decrease in HMGR protein level. Western analysis of serum-deprived HepG2 cells treated with either hexarelin 10^{-5}M and 10^{-6}M, with lanosterol (2.5uM) or with 25-hydroxycholesterol (2.5uM) for 5 hours was performed and compared to untreated. HMGR protein level was determined using an anti-HMGR antibody. Samples were normalized for protein loading with β-actin. (B) Measure of the total intracellular cholesterol in HepG2 cells treated with hexarelin at 10^{-5}M between 1 and 24 hours, with 25-hydroxycholesterol (25-HC) at 2.5 uM for 24 hours or left untreated. Cells were serum-deprived for 24 hours prior to treatment. Total lipid content was extracted and total cholesterol in cells was measured as described. The amount of cholesterol was normalized against total protein.

Figure 3. Hexarelin induces a rapid and transient increase in SREBP activity. (A) Measurement of endogenous activity of SREBPs in response to hexarelin in hepatocytes. HepG2 cells were transfected with pHMGR-bLuc reporter plasmid containing a portion of the promoter of HMGR containing a SREBP-responsive element (SRE). Cells were then treated with hexarelin 10^{-5}M between 3 and 24 hours in absence or in presence of serum or with 25-hydroxycholesterol 2.5uM (25-HC) for 6 hours. Results are expressed as fold response compared to untreated (set at 1.0) from at least 3 separate experiments. (B) Hexarelin causes a rapid but transient increase in the expression of genes involved in cholesterol synthesis. RT-PCR analysis of selected cholesterol metabolism markers in HepG2 cells treated with hexarelin 10-5M for the indicated times (between 1 and 6 hours), with 25-hydroxycholesterol for 6 hours or left untreated prior to RNA isolation.

Representative images are shown from at least 3 separate experiments. 36B4 expression was used to normalize samples.

Figure 4. Hexarelin induces the phosphorylation of AMPK and HMGR, and impacts HMGR ubiquitination.

(A) Phosphorylation of HMGR and AMPK in HepG2 cells treated with hexarelin 10^{-5}M, 10^{-6}M or 10^{-7}M or left untreated for 30 minutes. Western analysis was performed on whole cell extract using specific phospho-HMGR (Ser871) or phospho-AMPKα (Thr172) antibodies. Samples were normalized for total HMGR using anti-HMGR antibody or for protein loading with anti-β-actin antibody. (B) Phosphorylation of HMGR and AMPK in HepG2 treated with hexarelin 10^{-5}M in function of time (1 to 24 hours). Western analysis performed as described above. (C) Hexarelin causes an increase in the ubiquitination of HMGR. HepG2 cells were treated between 1 and 24 hours with hexarelin 10^{-5}M. Cell extracts were immunoprecipitation with an anti-HMGR antibody and ubiquitination of HMGR was determined by immunoblot using an antibody against ubiquitin. Samples were normalized using HMGR antibody.

Figure 5. Hexarelin causes the recruitment of insig-2 to HMGR in a CD36-dependent manner. (A) RT-PCR analysis of insig-1 and insig-2 in HepG2 cells treated with hexarelin 10-5M for the indicated times (between 1 and 6 hours), with 25-hydroxycholesterol for 6 hours or left untreated prior to RNA isolation. Representative images are shown. 36B4 expression was used to normalize samples. (B) Increased association between HMGR and insig-2 in HepG2 cells due to hexarelin. Stable transfected HepG2 cells expressing Insig-2/Myc were serum-deprived overnight, pre-treated with proteasome inhibitor MG132 (1uM) for 1 hour then treated between 1 and 24 hours with hexarelin 10^{-5}M, or with lanosterol (2.5uM) or 25-hydroxycholesterol (2.5 uM) for 5 hours. Cell extracts were subjected to co-immunoprecipitation using anti-myc antibody. Protein complex was analyzed by immunoblotting with specific anti-HMGR, anti-SCAP and anti-myc

antibodies. (C) Co-immunoprecipitation was performed as described in (B) in insig-2/Myc stable HepG2 cells treated with hexarelin 10^{-5}M and 10^{-6}M. (D) CD36 is required for HMGR degradation by hexarelin. CD36 expression was silenced by infecting cells with lentivirus carrying a shCD36 plasmid or a negative control shLuc plasmid. Infected cells were treated at the indicated time with hexarelin 10^{-5}M. Co-immunoprecipitation was carried out as described above using anti-myc antibody and analyzed by immunoblot using anti-HMGR and anti-myc antibodies.

Figure 6. The recruitment of Insig-2 to HMGR is dependent on AMPK activation. (A) The AMPK agonist, AICAR increases the interaction between HMGR and insig-2. Stable transfected HepG2 cells expressing insig-2 were serum-deprived overnight, pre-treated with proteasome inhibitor MG132 (1uM) for 1 hour prior to treatment with AICAR (0.2mM) and/or hexarelin 10^{-5}M for 5 hours. Co-immunoprecipitation was performed as described in Fig 4. The protein complex was analyzed by immunoblotting with specific anti-HMGR and anti-myc antibodies. (B) AMPK inhibitor, Compound C abrogates the effect of hexarelin. Stable insig-2 cells were treated as in (A) with the exception of samples pre-treated with CC (20uM) 1 hour before addition of hexarelin (10^{-5}M). HMGR/Insig-2/myc complex was analyzed by Western blot.

Figure 7. Hexarelin relieves the inhibitory effect of Erk on PPARγ which then mediates the effect of CD36 on the recruitment of insig-2 to HMGR. (A) RT-PCR analysis of PPARγ and CD36 in HepG2 cells treated with hexarelin 10-5M for the indicated times (between 1 and 6 hours), with 25-hydroxycholesterol for 6 hours or left untreated prior to RNA isolation. Representative images are shown. 36B4 expression was used to normalize samples. (B) The PPARγ agonist, troglitazone (Tro) induces the phosphorylation of AMPK in cultured hepatocytes. HepG2 cells were treated either with hexarelin (10^{-5}M), Tro (8uM) or AICAR (2mM) at the indicated times. Whole cell extracts were analyzed by immunoblot using a phospho-AMPKα (Thr172) antibody and protein loading was

normalized using β-actin. (C) Troglitazone induces similar effect to hexarelin and AICAR on HMGR interaction with insig-2. Treatments and co-immunoprecipitation were carried out as described in Figure 6. Insig-2 stable cells were treated with Tro (8 uM), hexarelin (10^{-5}M) or left untreated for 5 hours. (D) The inhibitor of PPARγ, GW9662 abrogated the effect of hexarelin on HMGR/insig-2 interaction. Treatments and co-immunoprecipitation were carried out as described in Figure 6. Insig-2 stable cells were treated with hexarelin (10^{-5}M), GW9662 (10^{-6}M), a combination of both or left untreated for 5 hours. (E) Hexarelin decreases Erk1/2 activity in overnight serum-deprived HepG2 cells when treated between 5 and 60 minutes at 10^{-5}M. Cell lysates were analyzed by immunoblot using specific antibodies to phospho-Erk, total Erk, phospho-Akt and total Akt. (F) Phosphorylation of PPARγ Ser-84 is decreased following inhibition of Erk by hexarelin. Cells were treated as in (E) at the indicated times, and lysates were immunoprecipitated with a PPARγ-specific antibody, and analyzed by immunoblot using phospho-PPARγ (Ser84), total PPARγ antibodies. (G) The activation of PPARγ by hexarelin is dependent on its Erk phosphorylation site at Ser-84 in hepatocytes. HepG2 cells were transfected with UAStkLuc, and with Gal4-PPARγ or Gal4-PPARγS85A plasmids. Transfected cells were then treated with hexarelin 10-5M or with Rosi (1uM) for 24 hours. Normalized values are presented as relative luciferase units (RLU).

Figure 8. CD36 promotes the activation and the recruitment of PGC-1α to PPARγ in HepG2. (A) Effect of hexarelin (10^{-5}M) on protein level of PGC-1α and F_1-ATP synthase in HepG2 treated for 24 hours. Western blot analysis was performed on total cell lysates using anti-PGC-1α and F1-ATP synthase antibodies. Protein level of each marker in 24-hour treatment was compared to untreated samples and loading was normalized using anti-β-actin antibody. (B) Hexarelin increases the interaction between the co-activator PGC-1 and PPARγ. HepG2 cells were treated with hexarelin (10^{-5}M) ranging from 5 minutes to 24 hours and cell extracts were subjected to co-immunoprecipitation using a PPARγ antibody.

Protein complex was analyzed by immunoblot using PGC-1 and PPARγ antibodies. (C) Hexarelin increases the interaction between PGC-1 and AMPK. HepG2 cells were transfected or not (Ctl) with HA-PGC-1 construct and treated as indicated in (B) at the indicated time points. Samples were subjected to co-immunoprecipitation using an HA antibody and protein complex was analyzed by immunoblot using AMPK and PGC-1antibodies.

Figure 9. **Gene expression changes of oxysterol-binding protein (OSBP) and OSBP-related proteins (ORPs) by hexarelin.** RT-PCR analysis of selected OSBP and ORP genes in HepG2 cells treated with hexarelin 10^{-5}M, with troglitazone 8 uM for the indicated times or left untreated prior to RNA isolation. Representative images are shown. 36B4 expression was used to normalize samples.

Figure 10. Schematic representation of the proposed effect of hexarelin in hepatocytes through its interaction with CD36. Binding of hexarelin to CD36 promotes a rapid phosphorylation/activation of AMPK and Akt, and dephosphorylation/inactivation of Erk1/2. In turn, AMPK phosphorylates/inactivates HMG-CoA reductase (HMGR) rapidly inhibiting cholesterol synthesis. AMPK also activates PGC-1α which then associates with PPARγ to increase expression of fatty acid oxidation marker and OSBP/ORPs genes. Binding of hexarelin to CD36 also leads to degradation of HMGR via its association with insig-2 possibly through a pathway involving AMPK and OSBP/ORPs. Insig-2 also prevents the translocation of SREBP to the Golgi by retaining the insig-2/SCAP/SREBP complex in the ER. The multi-level signaling pathway activation by hexarelin results in a decrease in cholesterol synthesis in hepatocytes.

FIGURES

Figure 1

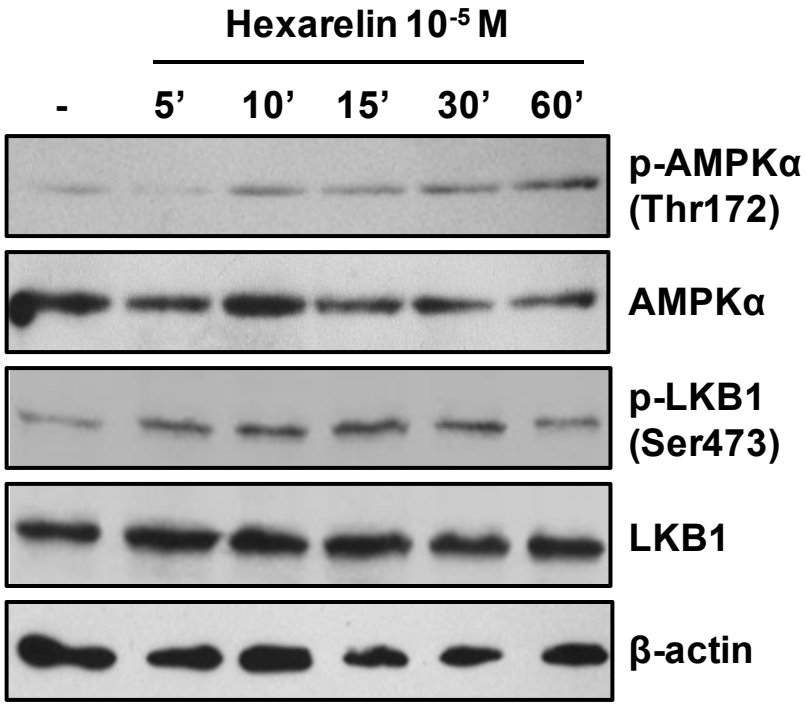


Figure 2

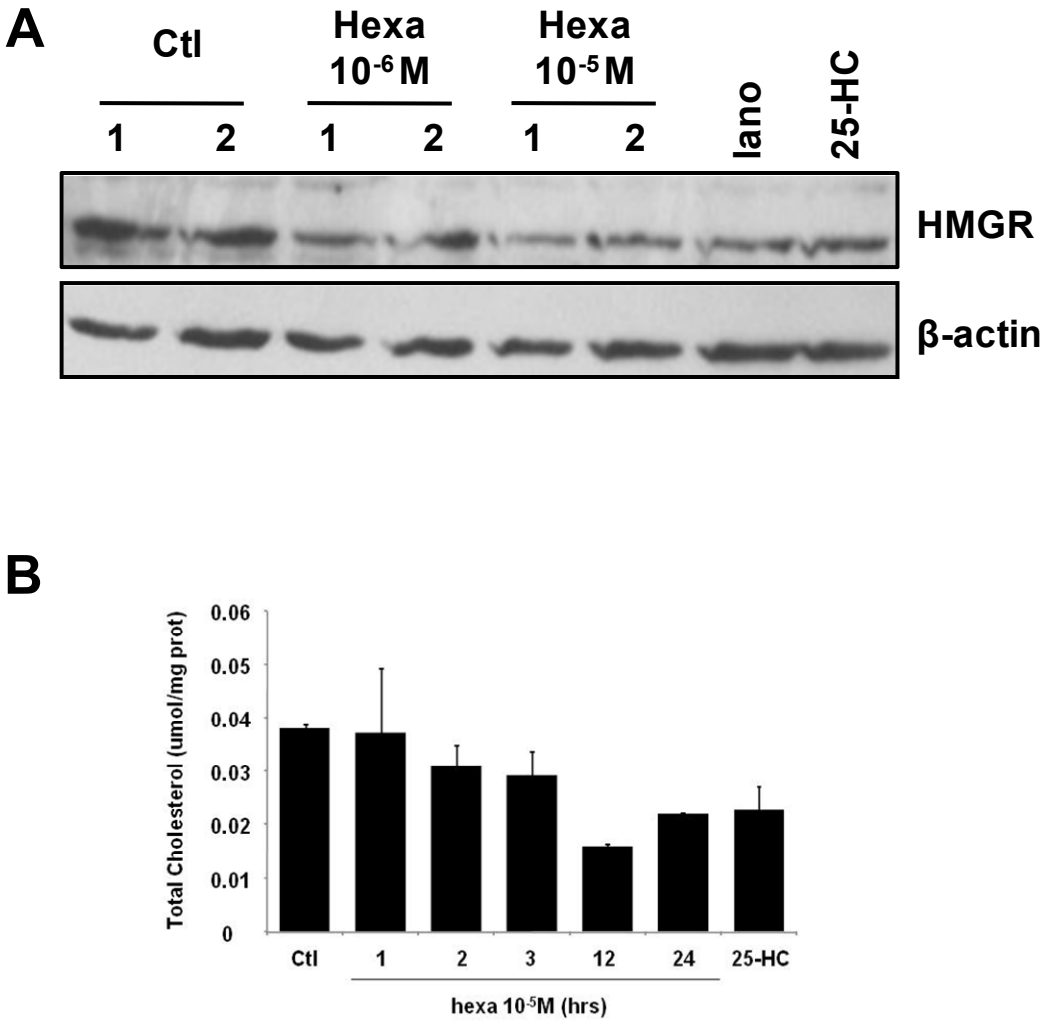

A
Ctl
Hexa 10-6 M
Hexa 10-5 M
1
2
1
2
1
2
lano
25-HC
HMGR
β-actin
B
Total Cholesterol (umol/mg prot)
0.06
0.05
0.04
0.03
0.02
0.01
0
Ctl
1
2
3
12
24
25-HC
hexa 10-5M (hrs)

Figure 3

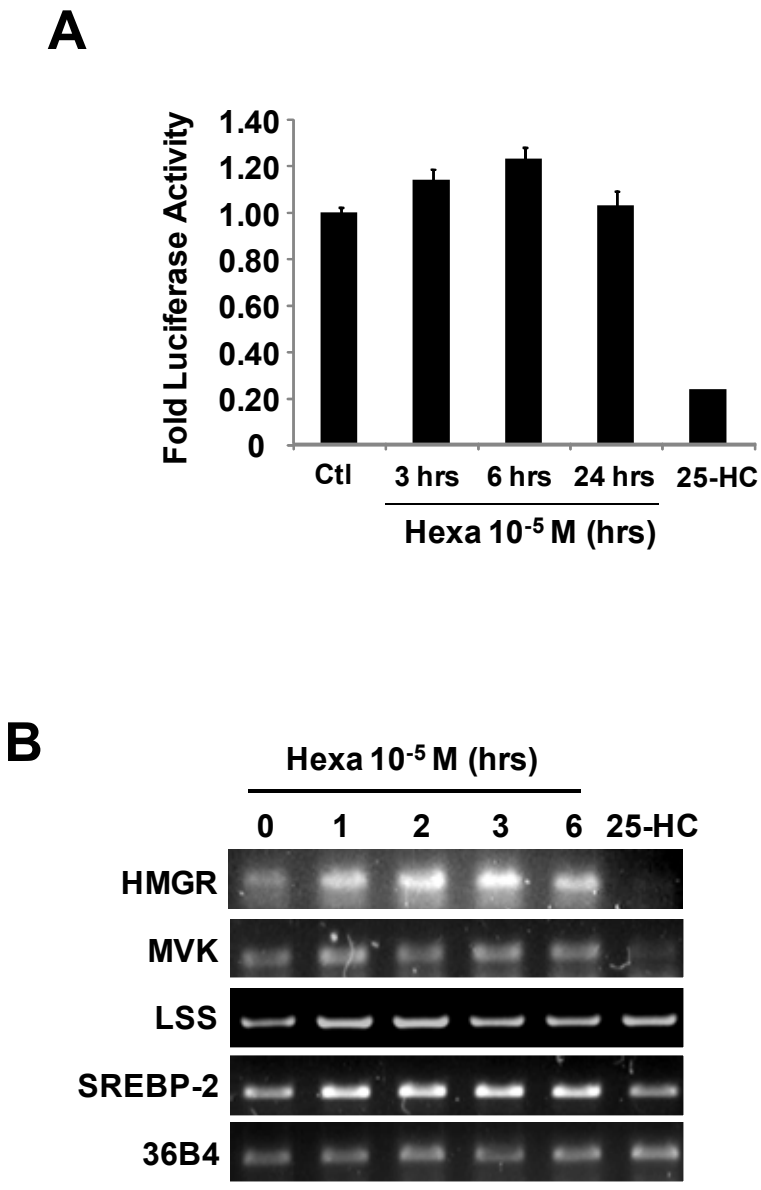

A
Fold Luciferase Activity
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0
Ctl
3 hrs
6 hrs
24 hrs
25-HC
Hexa 10-5 M (hrs)
B
Hexa 10-5 M (hrs)
0
1
2
3
6
25-HC
HMGR
MVK
LSS
SREBP-2
36B4

Figure 4

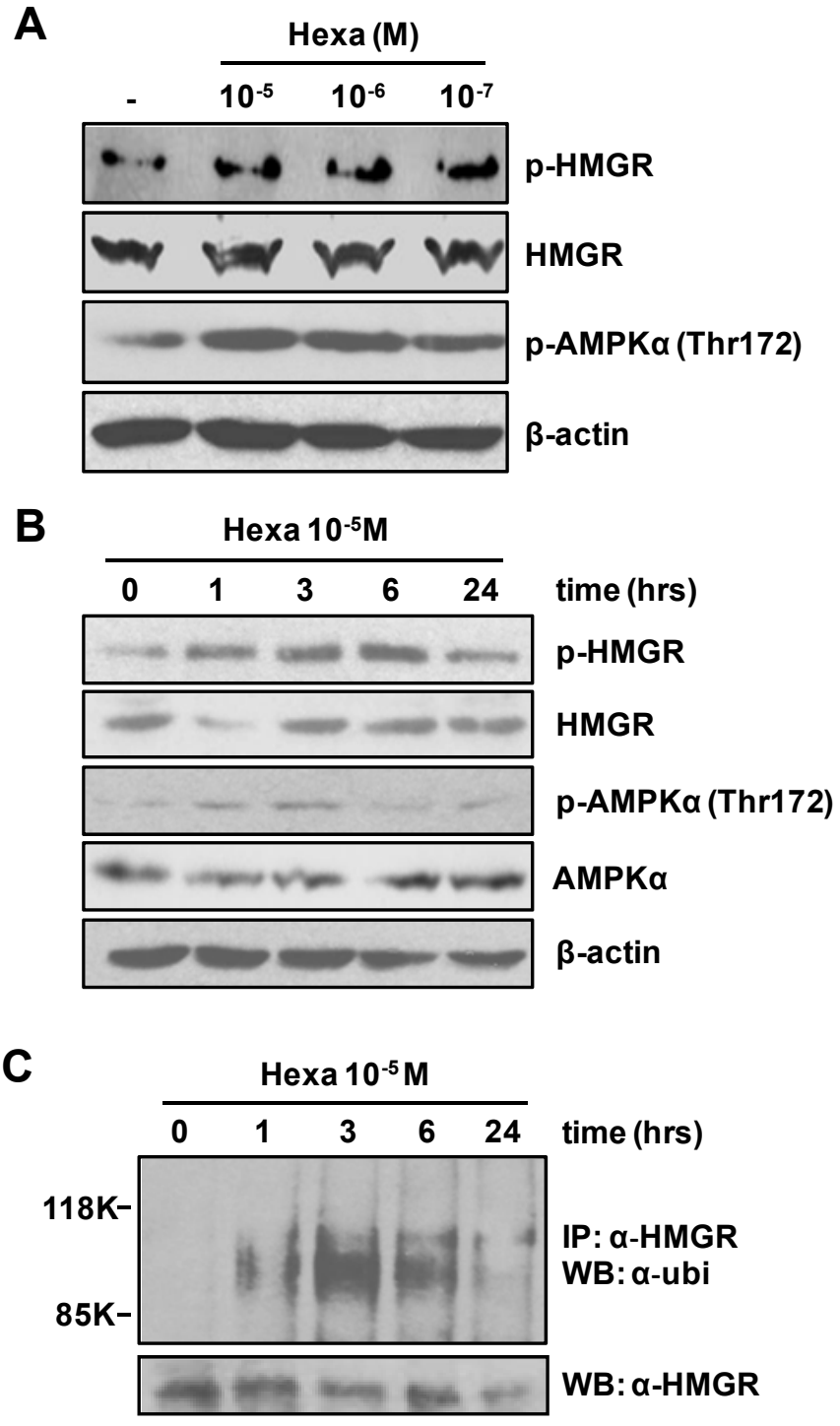

A
Hexa (M)
-
10-5
10-6
10-7
p-HMGR
HMGR
p-AMPKα (Thr172)
β-actin
B
Hexa 10-5M
0
1
3
6
24
time (hrs)
p-HMGR
HMGR
p-AMPKα (Thr172)
AMPKα
β-actin
C
Hexa 10-5 M
0
1
3
6
24
time (hrs)
118K–
85K–
IP: α-HMGR
WB: α-ubi
WB: α-HMGR

Figure 5

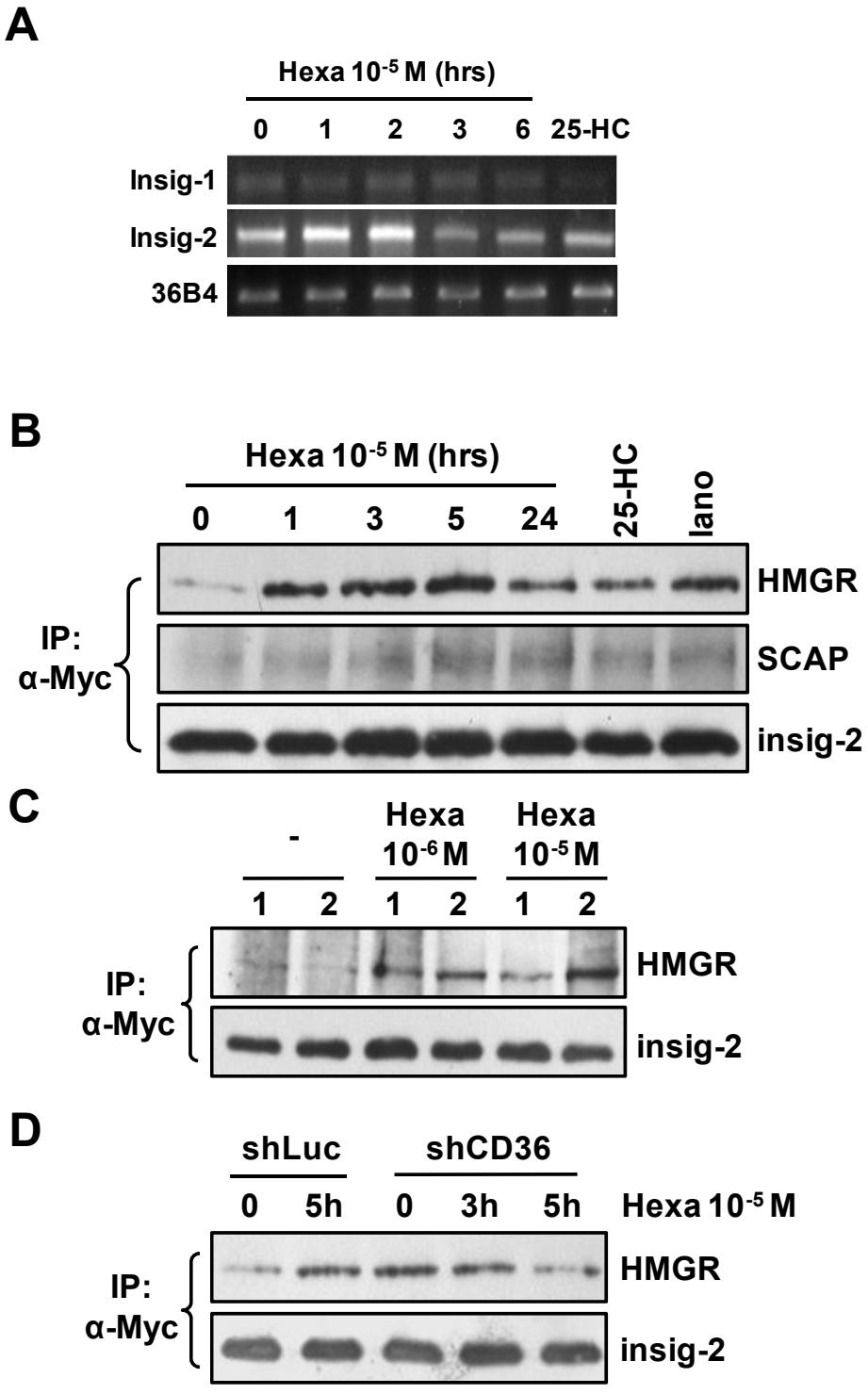

A
Hexa 10-5 M (hrs)
0 1 2 3 6 25-HC
Insig-1
Insig-2
36B4
B
Hexa 10-5 M (hrs)
0 1 3 5 24 25-HC lano
IP: α-Myc
HMGR
SCAP
insig-2
C
- Hexa 10-6 M Hexa 10-5 M
1 2 1 2 1 2
IP: α-Myc
HMGR
insig-2
D
shLuc shCD36
0 5h 0 3h 5h Hexa 10-5 M
IP: α-Myc
HMGR
insig-2

Figure 6

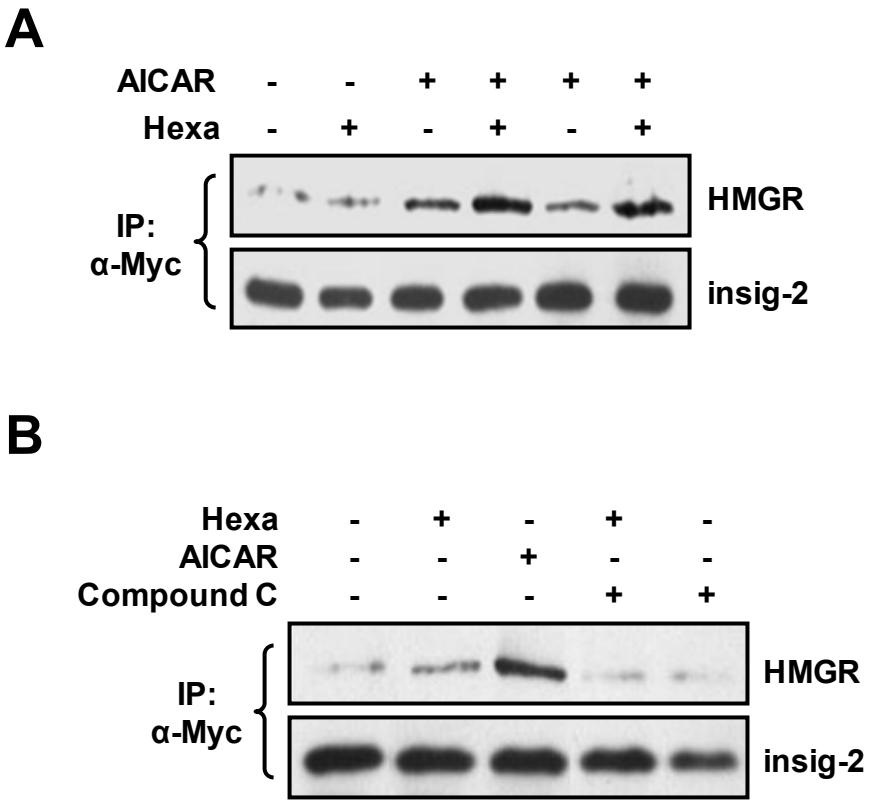

A
AICAR - - + + + +
Hexa - + - + - +
IP:
α-Myc
HMGR
insig-2
B
Hexa - + - + -
AICAR - - + - -
Compound C - - - + +
IP:
α-Myc
HMGR
insig-2

Figure 7

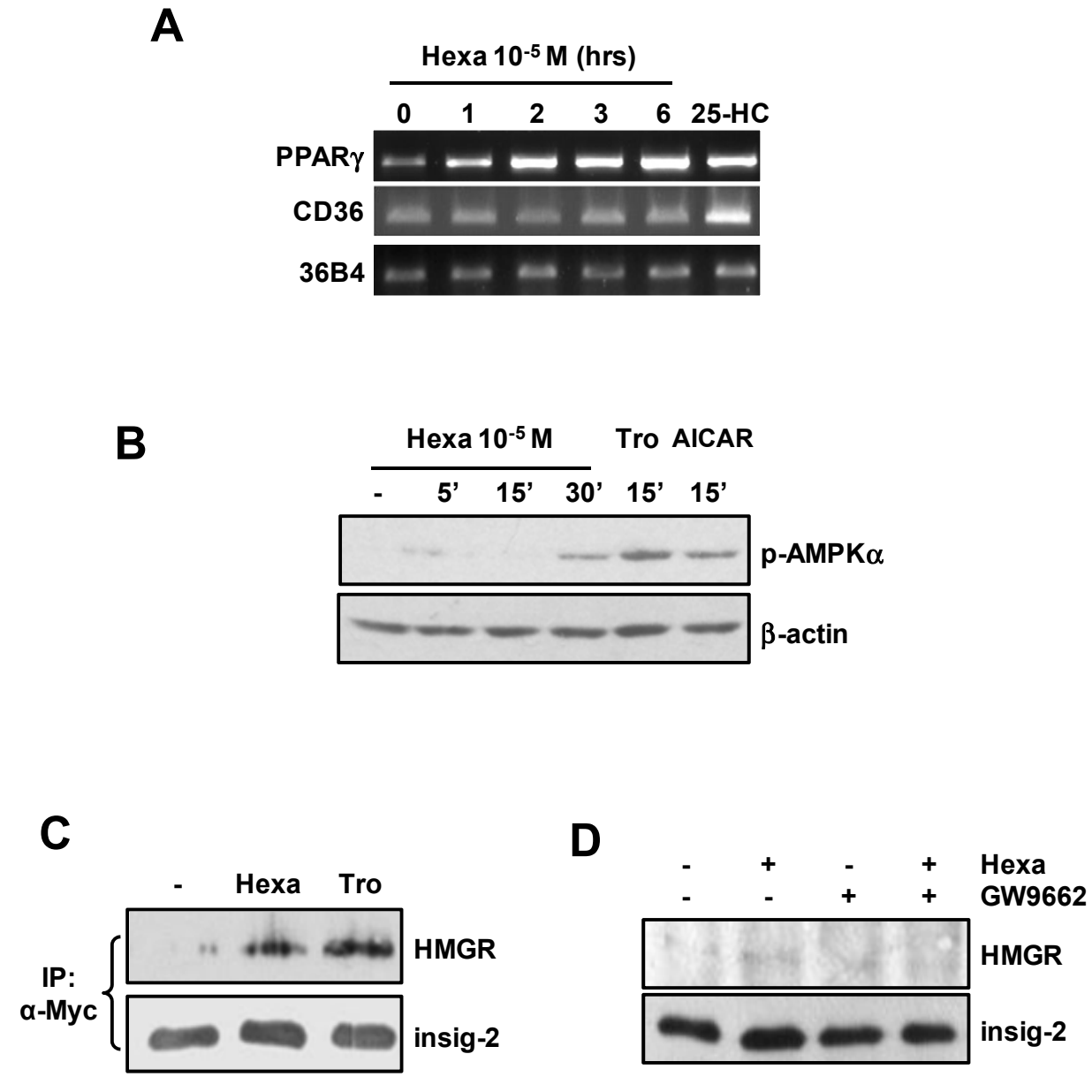

A
Hexa 10-5 M (hrs)
0 1 2 3 6 25-HC
PPARγ
CD36
36B4
B
Hexa 10-5 M
Tro AICAR
- 5' 15' 30' 15' 15'
p-AMPKα
β-actin
C
- Hexa Tro
IP:
α-Myc
HMGR
insig-2
D
- + - + Hexa
- - + + GW9662
HMGR
insig-2

Figure 7 (Continued)

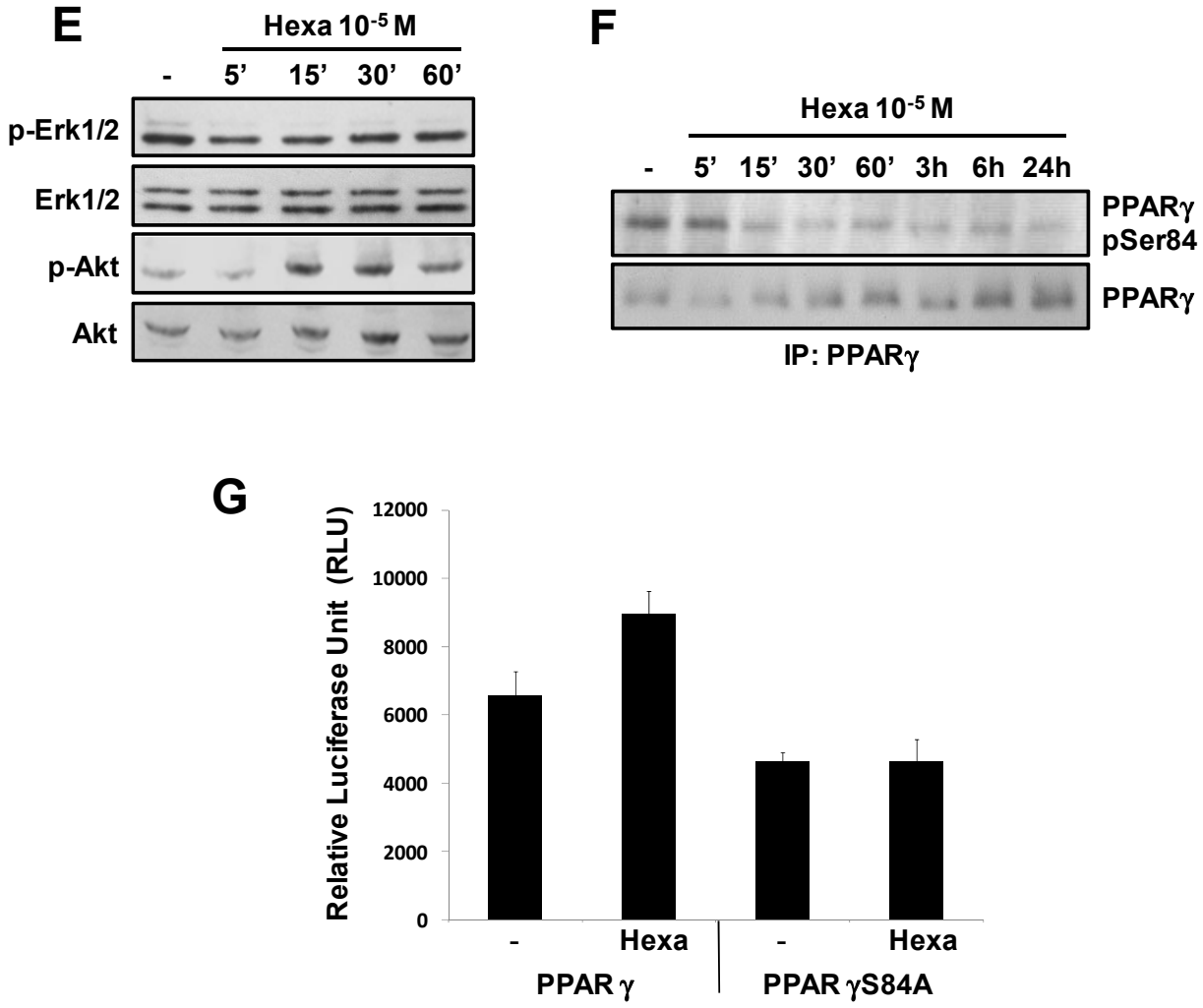


Figure 8

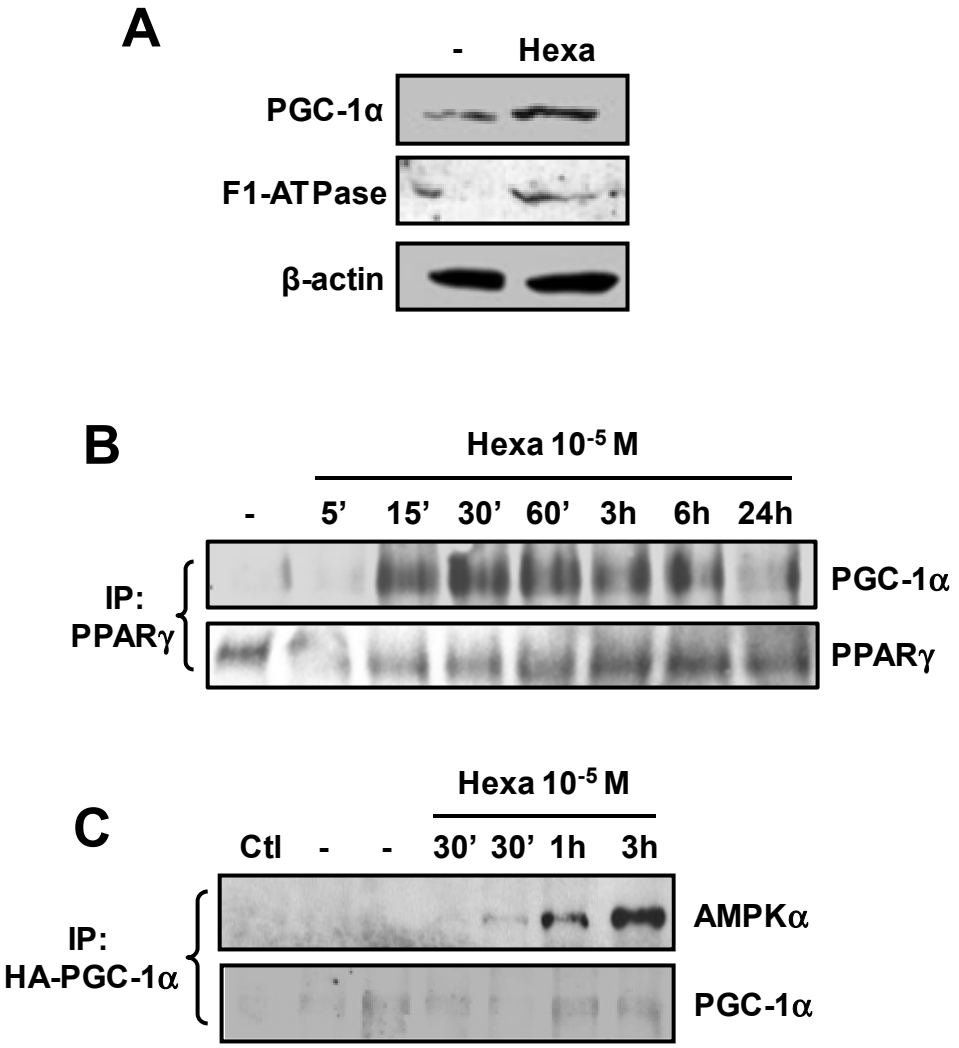

A
-
Hexa
PGC-1α
F1-ATPase
β-actin
B
Hexa 10-5 M
-
5'
15'
30'
60'
3h
6h
24h
IP:
PPARγ
PGC-1α
PPARγ
C
Hexa 10-5 M
Ctl
-
-
30'
30'
1h
3h
IP:
HA-PGC-1α
AMPKα
PGC-1α

Figure 9

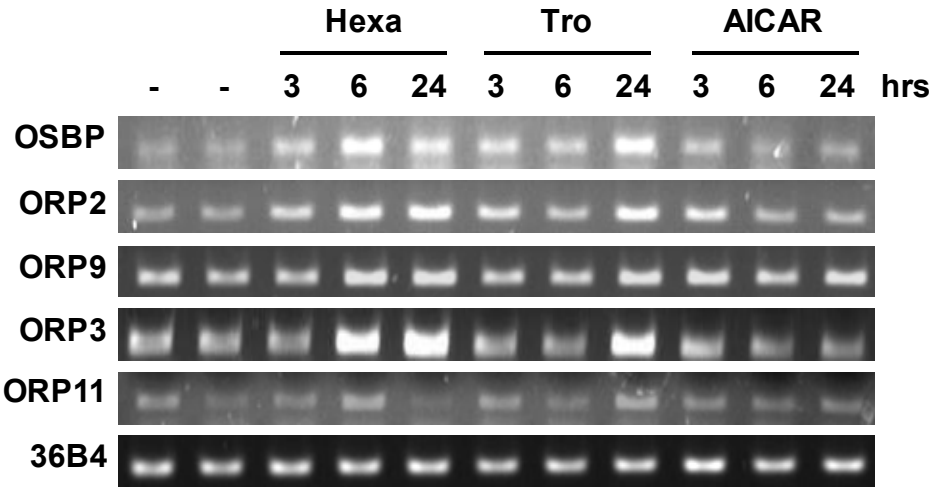
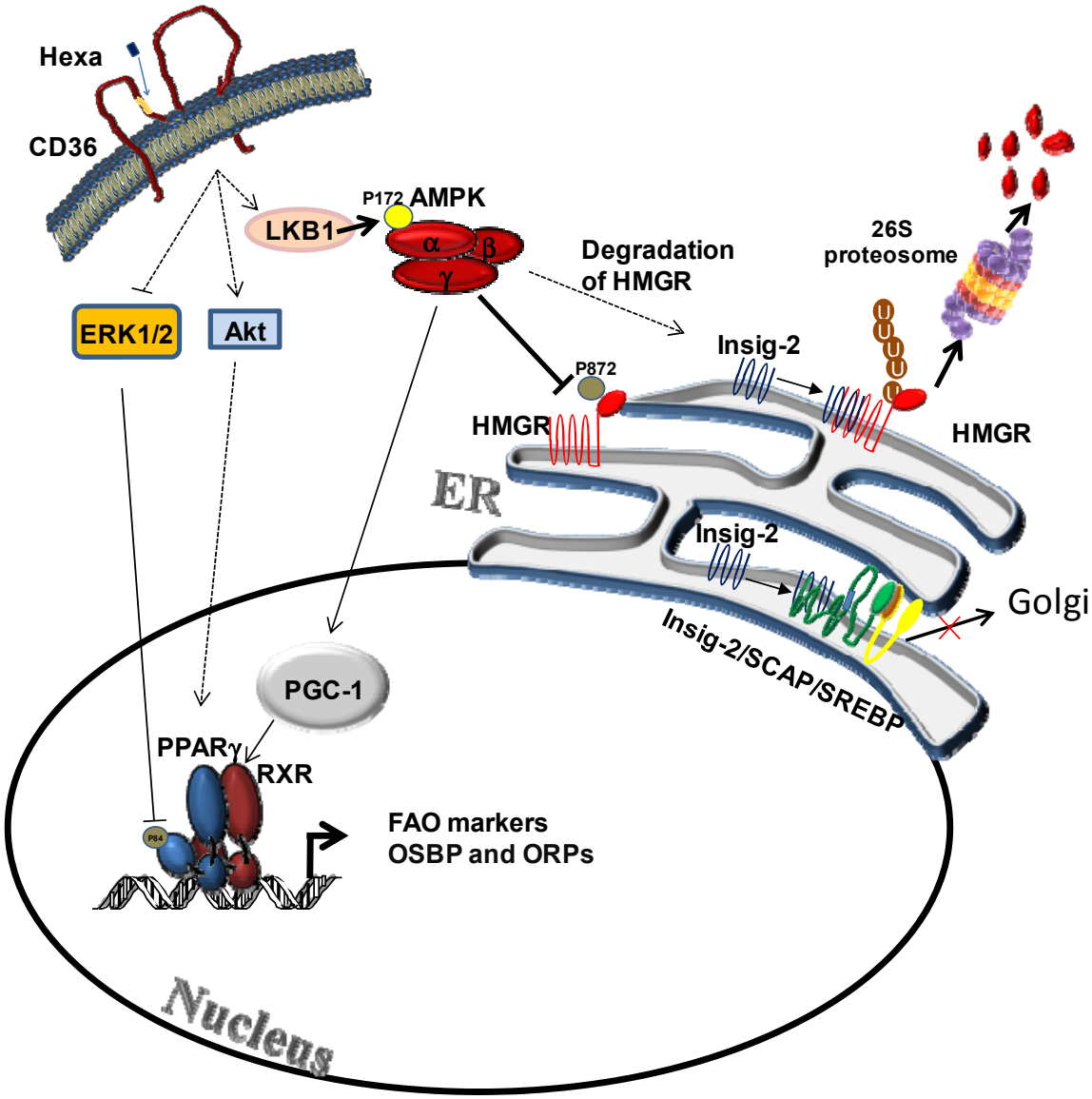

Hexa
Tro
AICAR
- - 3 6 24 3 6 24 3 6 24 hrs
OSBP
ORP2
ORP9
ORP3
ORP11
36B4

Figure 10


Hexa
CD36
LKB1
P172 AMPK
α
β
γ
ERK1/2
Akt
Degradation
of HMGR
26S
proteosome
Insig-2
P872
HMGR
HMGR
ER
Insig-2
Golgi
Insig-2/SCAP/SREBP
PGC-1
PPARγ
RXR
FAO markers
OSBP and ORPs
Nucleus

CHAPTER 3: General discussion, perspectives and conclusions

1 Discussion

1.1 Elucidation of the impact of CD36 on lipid metabolism

The major contribution of the work presented in this thesis was the demonstration of the profound impact that CD36 has on lipid metabolism through its interaction with hexarelin. Two different cell types were used to study (1) its impact on the fate of intracellular lipids (adipocytes) and (2) its control exerted on cholesterol synthesis (hepatocytes). Given its multi-ligand receptor properties, it is not difficult to appreciate the challenges involved in the task of elucidating the role(s) of CD36 in different tissues. CD36 is not considered an inert protein that simply imports lipoprotein particles and LCFA into the cell. CD36 is rather viewed as a protein that dynamically interacts with specific ligands and that is capable of triggering important signaling cascades which results in important changes in cellular function. The use of hexarelin brought a unique approach to studying CD36 since it does not entirely mimic binding of ligands but nonetheless elicited interesting and unforeseen cellular changes. We set out to decipher the effect of hexarelin interaction with CD36 in cell types solely expressing CD36 and not GHS-R1a. In addition, the role of CD36 in these cell types was somewhat elusive and therefore provided the opportunity to further our knowledge of the impact of CD36 on lipid metabolism.

1.1.1 CD36 in Adipocytes

The role of CD36 in adipocytes is correctly associated with the uptake of LCFA and their storage in the form of TG. With the finding that the absence of CD36 in adipocytes resulted in a 60-70% reduction of the cell's FA uptake potential, this places CD36 as an

essential transporter of FA (Coburn *et al.*, 2000). Since GHS-R1a is not expressed in adipocytes, the interaction of hexarelin with CD36 could only but have an important effect on the outcome of intracellular lipids.

1.1.1.1 Hexarelin reduces lipid content in adipocytes

Given the role of CD36 in FA uptake, the first rational effect to examine was the intracellular lipid content. We looked at both lipid staining and TG measurement to confirm our finding that hexarelin caused a net decrease in intracellular lipid content in adipocytes. As expected, treatment of differentiated 3T3-L1 adipocytes with troglitazone also showed a similar decrease in intracellular lipids (Okuno *et al.*, 1998). Since we have previously seen that hexarelin increased the activity of PPARγ through its interaction with CD36, troglitazone was used throughout this study as a comparitive tool to evaluate the role of PPARγ in the cell's response to hexarelin. Previous studies of acute administration of hexarelin in young dogs and in rats have shown a marked increase in food intake similarly to ghrelin through direct action on hypothalamic GHS-R1a (Rigamonti *et al.*, 1999;Torsello *et al.*, 1998). Since hexarelin also binds to GHS-R1a, it would be expected that similarly to ghrelin, administration of hexarelin would results in increase adiposity and weight gain. Long-term effect of hexarelin therapy on GH release was performed in humans and while changes in food intake were never addressed, total body fat was unchanged by hexarelin after 16 weeks (Rahim *et al.*, 1998). More recently, Antonio Torsello and colleagues have further investigated the orexigenic potential of hexarelin in young and old rats (Bresciani *et al.*, 2008). Hexarelin, at a minimal level to induce maximal stimulation of food intake, was chronically administered during a period of 8 weeks. A persistent orexigenic effect was observed in both young and old rats due to hexarelin; however, this increase in food intake was not accompanied by weight gain. We had therefore shown an unexpected and direct effect of hexarelin on adipocyte fat content. To ascertain whether hexarelin is capable of reducing adipocyte lipid content in vivo, further studies would be required; however it is

possible that an increase in food intake induced by hexarelin via GHS-R1a could counterbalance its effect on reducing adiposity via CD36.

The potential region of interaction of LCFA on CD36 is relatively large and has not been fully characterized. The putative region is based on the comparison of CD36 ectodomain with FA binding region of M-FABP (Baillie *et al.*, 1996). Since hexarelin inhibits the binding and internalization of oxLDL owing in part to its anti-atherogenic properties (Demers *et al.*, 2004), the question remains whether hexarelin blocks in fact FA uptake in adipocytes which could result in a decrease in intracellular lipid. If we assume that hexarelin blocks FA uptake, hexarelin would somewhat mimic the absence of CD36. In CD36-deficient humans and in CD36-null animal models, the absence of CD36 results in marked elevation in circulating FFA but also TG plasma level (Febbraio *et al.*, 1999;Moore *et al.*, 2005;Yamashita *et al.*, 2007). However, chronic administration of hexarelin in both lean and obese Zucker rats significantly decreased plasma cholesterol concentration in obese rats while having no effect on TG levels in both lean and obese rats (De Gennaro-Colonna *et al.*, 2000). In addition, 30-day treatment with hexarelin in atherosclerotic rats showed a marked improvement in their cholesterol profile but had no effect on the TG levels (Pang *et al.*, 2010). Moreover, treating rats with hexarelin would result in an increase in food intake combined with a decreased capacity in FA uptake which would ultimately lead to an increase in TG plasma level. Given the reported effect of hexarelin on TG, an inhibition of FA uptake seems to be an unlikely event in the reduction in adipocyte lipid content following hexarelin treatment. It is possible, however, that the inhibition of FA uptake through CD36 triggers a compensatory mechanism to re-establish FA uptake (see FATP1, next section).

1.1.1.2 Gene expression profiling: an indication of important changes in lipid metabolism

One of the first approaches to determine the effect of hexarelin on adipocytes was to establish a profile of genes changed by hexarelin which were then categorized according to their function. We chose to look at gene changes after 48 hours of treatment, at which point

the response and adaptation of adipocytes to hexarelin would give time for any phenotypic changes to occur. More than a thousand probesets were upregulated demonstrating an important cellular response to hexarelin; however, only those indicative of a role in lipid metabolism were discussed. Many gene changes due to hexarelin were paralleled to those in troglitazone-treated cells. However, certain interesting genes were found to be upregulated by hexarelin while unchanged by troglitazone (such as FAS), while troglitazone had a greater effect on certain genes such as aP_2, adipophilin and carboxylesterase 3. Hexarelin increased the expression of HSL and it was shown that in human adipose tissue, HSL expression level was negatively correlated with adiposity (Nagashima *et al.*, 2011). Interestingly, FATP1 was also upregulated by hexarelin but not by troglitazone. Since CD36 and FATP1 (Lobo *et al.*, 2007) are the major LCFA transporters in adipocytes, it is possible that in the event that hexarelin blocks FA uptake by CD36, the upregulation of FATP1 could compensate eventually for the decrease in FA uptake. While interpreting gene expression changes, we were well aware of its limitations. Undoubtedly, the use of microarray technology was an ideal primary approach to elucidate the downstream effect of hexarelin on mature adipocytes. Moreover, microarray experiments were never previously performed on hexarelin-treated cells. However, gene expression changes while often regarded as a causative effect on cellular function, expression changes can also represent a compensatory one. In addition, gene expression upregulation does not always imply an increase in protein level or in its activity; and similarly, a decrease in expression does not necessarily mean a reduction in its protein activity (Schena *et al.*, 1998;Stolovitzky, 2003). For instance, activation of nuclear receptors are often accompanied by a decrease in expression level (as seen in this paper with adipocytes treated with troglitazone and a reduction in PPARγ expression) and a decrease in protein level, the latter due to increased protein degradation (Perrey *et al.*, 2001;Picard *et al.*, 2008). The same is observed for insig-1 for which in a sterol-depleted environment, degradation of insig-1 protein is coupled with increased in its expression (Lee

et al., 2006). Ideally, as a cautionary approach, gene expression changes have to be sustained by enzymatic activity and in our case also by phenotypic changes.

1.1.1.3 The influence of CD36 in the induction of mitochondrial biogenesis and phenotypic changes of 3T3-L1 cells into brown fat cells

Several genes involved in the transport of FA into mitochondria, genes involved in various steps of FAO, respiratory chain as well as oxidative phosphorylation were upregulated by hexarelin namely the muscle isoform of CPT-1 (CPT1b) and several subunits of ATP synthase. Although CPT-1b is also found in adipose tissue, the predominant isoform normally found in 3T3-L1 cells is CPT-1a (Brown *et al.*, 1997). Interestingly, CPT-1b is more sensitive to malonyl-CoA (Saggerson & Carpenter, 1981) and this isoform expression induction might depict a change in the sensitivity to cellular energy supply induced by hexarelin. The observed gene expression changes were supported by equal changes in selected protein level. In addition, to confirm an increase in oxidative phosphorylation, cytochrome c oxidase activity (Complex IV of the respiratory chain) was measured. Adipocytes treated with either troglitazone or hexarelin resulted in a significant increase in the oxidative capacity of mitochondria isolated from treated cells. Along with the increased capacity of hexarelin-treated cells to burn fat, we looked at the expression and protein level of two important markers of brown fat phenotype, PGC-1α and UCP-1. Both markers were induced in adipocytes treated with hexarelin and troglitazone. As previously stated, PGC-1α plays a critical role in the initiation of the thermogenic program to induce oxidative metabolism and mitochondrial biogenesis (Spiegelman & Heinrich, 2004). Following external stimulation, mitochondrial biogenesis begins with induction of nuclear genes encoding mitochondrial proteins. Mitochondrial transcription factors such as Tfam, p43 and mtTFB escorted into the mitochondrion will activate the transcription of the few mitochondrial genes (13 components of the respiratory chain, 2 ribosomal RNAs and 22 transfer RNAs) while the prohibitins stabilize newly synthesized mitochondrial proteins (Nijtmans *et al.*, 2000). And since the vast majority of mitochondrial proteins are encoded

by the nuclear genome, a carefully orchestrated protein import complex takes care of transporting proteins into the mitochondrion (TIMs and TOMs). Several of these key components to protein import (Tims and toms), mitochondrial ribosomal proteins (MRPs) and prohibitin 1 and 2 were found to be upregulated by hexarelin and troglitazone. Prohibitins are associated with mitochondrial biogenesis and stabilization of oxidative phosphorylation complexes, but its expression is markedly reduced in senescence (Coates *et al.*, 2001). Recently, prohibitins were also shown to be essential in cristae morphogenesis (Merkwirth *et al.*, 2008). Many of the listed genes involved in mitochondrial biogenesis such as prohibitins, upregulated by hexarelin or troglitazone could possibly be new and interesting targets of PPARγ and/or PGC-1α. To confirm the gene expression profile that suggested an induction in mitochondrial biogenesis in treated adipocytes, we looked at structural characteristics of mitochondria. By electron microscopy, we were able to determine two important features: (1) an increase in mitochondrial size and (2) an increase in cristae; both indicative of an increased oxidative potential in adipocytes in response to hexarelin.

The possibility of the role of the transcriptional regulator PRDM16 in brown adipocyte transdifferentiation was not addressed in our studies which was published prior to the discovery of PDRM16 participation in this process; but given our results, PRDM16 could potentially play a role in hexarelin's effect since it coactivates with PGC-1α and PPARγ to suppress the expression of white adipocyte markers (Seale *et al.*, 2007;Kajimura *et al.*, 2008).

1.1.1.4 In vivo confirmation of the role of CD36 in the mediation of the effect of hexarelin on adipocytes

In vivo studies demonstrated that hexarelin was capable of triggering GH release from the pituitary gland via its interaction with GHS-R1a (Imbimbo *et al.*, 1994). Because of the opposite effect of GH and hexarelin on lipolysis as well as the various potential anabolic effects of GH, it was important to evaluate the overall influence of hexarelin on

specific mitochondrial protein markers and ultrastructure in treated adipose tissue. In the context of the adipose tissue project, we were neither able to monitor food intake, nor to determine the overall fat mass (such as Dual-Energy X-ray Absorptiometry or DEXA scan) or look at adipocyte volume in treated mice. However, protein level of ATP synthase, UCP-1 and PGC-1a as well as analysis of mitochondrial morphology indicated that in mice, hexarelin was capable of inducing similar phenotypic changes in WAT. More importantly, the effect of hexarelin on those key proteins level was abrogated in absence of CD36 as seen in CD36-null mouse adipose tissue.

So through interaction with CD36, hexarelin was able to induce important phenotypic changes leading to a net decrease in intracellular lipid content by inducing the expression of the master regulator of mitochondrial biogenesis, PGC-1α, and by increasing the expression of several genes involved in various steps of FAO, in the respiratory chain and in oxidative phosphorylation. Although activation of PPARγ by troglitazone was shown to have certain similarities to the effect of hexarelin, this was the first study demonstrating the potential involvement of CD36 in inducing changes that pushed white adipocytes toward a brown-like phenotype. Figure 24 depicts the overall effect of hexarelin on adipocyte lipid metabolism. AMPK presented in this figure is further discussed in section 1.1.1.6.

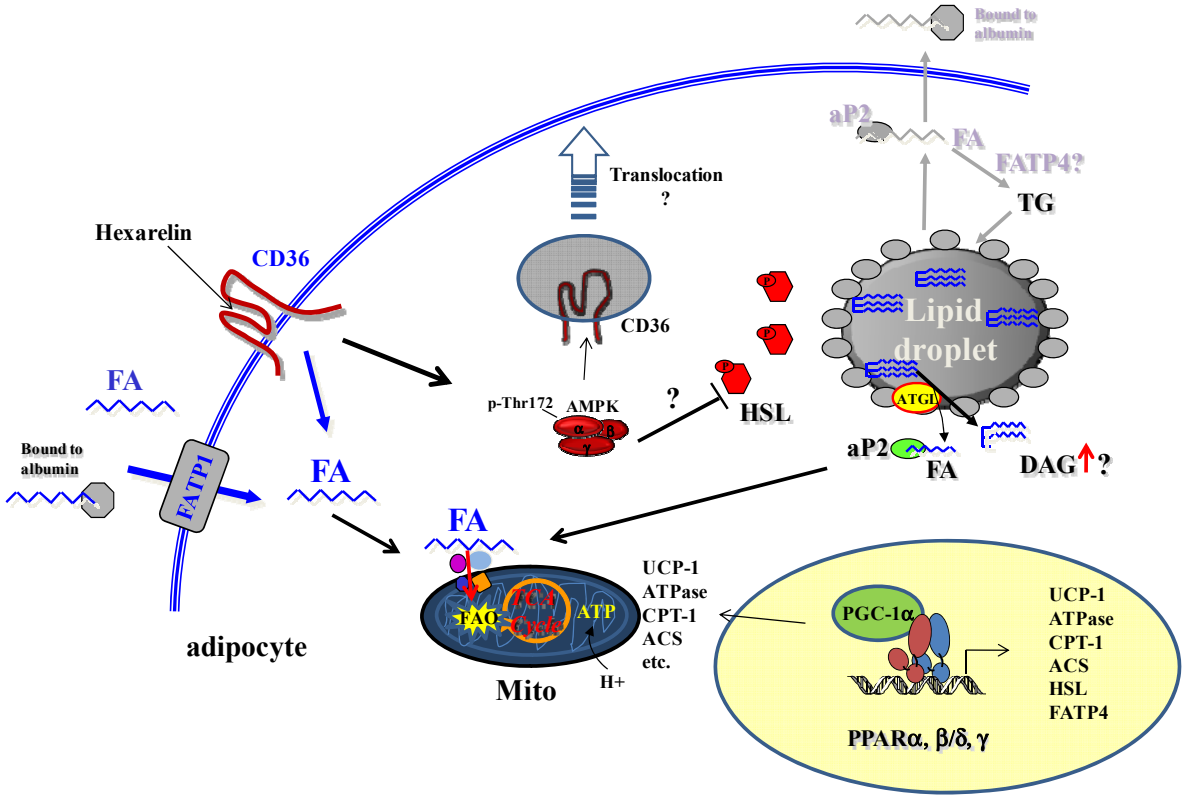


Figure 24. The effect of hexarelin's interaction with CD36 on lipid metabolism in adipocytes

1.1.1.5 Role of PPARγ and PGC-1α in mediating hexarelin`s effect on mitochondrial function

Similar studies using TZDs have shown the induction of mitochondrial biogenesis and increase in FAO (Guan *et al.*, 2002;Wilson-Fritch *et al.*, 2003;Wilson-Fritch *et al.*, 2004). Subtle differences in the gene expression pattern between hexarelin and troglitazone gave rise to the possibility that not all the effects seen with hexarelin are solely explained by the activation of PPARγ. Indeed, using a cell reporter assay, we have previously shown that hexarelin was capable of activating all three subtypes of PPAR (α, β/δ and γ) (Avallone et al., 2006). Both PPARα and β/δ play important roles in fatty acid oxidation

and mitochondrial function and could potentially mediate hexarelin's effect such as an increase in the expression of CPT-1b (Kersten *et al.*, 1999;Brandt *et al.*, 1998;Wang *et al.*, 2003). In addition, PGC-1α is known to associate with several other nuclear receptors that play a role in mitochondrial biogenesis and oxidative phosphorylation. Estrogen-related receptor alpha (ERRα) expression is strongly induced by PGC-1α but also requires PGC-1α for its own activation (Huss *et al.*, 2002;Schreiber *et al.*, 2003). In ERRs-deficient heart, reduced oxidative phosphorylation gene expression and decreased ATP synthesis rate were observed (Huss *et al.*, 2007). The transcription factor, Yin-Yang 1 (YY1) directly activates the expression of mitochondrial genes such as several subunits of ATP synthase while nuclear respiratory factor 1 (NRF-1) controls the expression of mtTFA (Breen *et al.*, 1996;Wu *et al.*, 1999a). White and brown adipocytes share similar transcriptional programs but most notably they share the expression of PPARγ. Specific gene regulatory programs for WAT and BAT involving co-regulators might also contribute to the phenotypic transformation induced by hexarelin via PPARγ. Other than PGC-1α, the involvement of PPARs co-regulators such as co-repressors RIP140, NCoR, SMRT and even PRDM16, and other co-activators such as CREB, CBP/p300, SRC and TRAP220 have not been addressed in this study but could also have played a role in selective activation or inhibition of PPAR target genes. Such an example is the activation of PPARγ by hexarelin without the rise in the expression of aP2 and adipophilin as seen with troglitazone. Various co-regulators for WAT and for BAT have been suggested to play a specific role in either adipocyte type. For example, steroid receptor coactivator-1 (SRC-1) promotes energy expenditure in BAT while SRC-2 (or TIF2 for transcriptional intermediary factor 2) stimulates TG accumulation (Picard *et al.*, 2002). CBP/p300 is mainly functional in WAT and was recently shown to be recruited to A/B-domain of PPARγ2 (Yamauchi *et al.*, 2002;Bugge *et al.*, 2009). Two co-repressors, NCoR and SMRT, are recruited to PPARγ2 and repress the expression of glycerol kinase in white adipocytes (Guan *et al.*, 2005;Yu *et al.*, 2005). To determine the role of these co-regulators in the response of 3T3-L1 to hexarelin, further investigation is required.

1.1.1.6 Potential influence of hexarelin on CD36 translocation and on lipolysis through AMPK activation

Given the results combining an increase in the expression of genes involved in FAO, cytochrome c oxidase activity, changes in mitochondrial ultrastructure and a decrease in TG cellular content, demonstrating an activation of AMPK was not necessary. However, with today's knowledge of the involvement of AMPK in lipolysis and in CD36 mobilization, as well as in other aspects of adipocyte's function, knowing whether AMPK was activated by hexarelin could provide a more complete picture of the events following hexarelin binding to CD36 (Gauthier *et al.*, 2008;Wang *et al.*, 2010;Wang *et al.*, 2009). Indeed, along with treatments of hepatocytes with hexarelin, the level of AMPK phosphorylation was evaluated in adipocytes (data not shown). We detected a rapid phosphorylation of AMPK at its obligatory activation site, Thr-172 in treated adipocytes. This observation adds weight to the induction of FAO but also provided an interesting link with perhaps CD36 mobilization to cell surface (contributing to increasing FA uptake) and inhibition of HSL (linked with resistance to obesity).

In muscle cells, contractions or direct activation of AMPK induces the translocation of CD36 to the cell surface (Bonen *et al.*, 2000;Luiken *et al.*, 1999;Bonen *et al.*, 2007). A recent study showed that translocation of CD36 due to AMPK was also possible in adipocytes (Wang *et al.*, 2010). However, to date, no studies have ever reported an activation of AMPK following ligand binding to CD36. It is possible that in adipocytes, following AMPK activation by hexarelin, more CD36 proteins are translocated to the cell surface to increase FA uptake (hypothetically presented in Figure 24). Lipolysis was not addressed in our study either but given the possibility of AMPK activation by hexarelin, it could represent an additional downstream effect. During lipolysis, AMPK is activated by changes in AMP/ATP ratio due to the energy consuming reesterification process therefore causing AMPK to inhibit HSL (Gauthier *et al.*, 2008). It was previously shown that HSL disruption had a protective effect against HFD-induced obesity (Osuga *et al.*, 2000). The reesterification of FA is an important step in controlling the rate of hydrolysis and

excessive transport of FA outside the cell (Zambell *et al.*, 2001). FATP4, like other members of its family, exhibits acyl-CoA synthetase (ACS) activity and plays an important role in reesterification of FA (Hall *et al.*, 2003;Lobo *et al.*, 2007). HSL hydrolyzes mainly DAG to MAG and to a lesser degree TAG to DAG; therefore, absence of HSL leads to accumulation of DAG (Shen *et al.*, 2011). Interestingly, an increase in DAG intermediates was also observed in adipose tissue of CD36-null mice which could not be explained by a decrease in TG synthesis from DAG by DGAT (Coburn *et al.*, 2000). Perhaps inhibition of HSL by AMPK in absence of CD36 is the key to this reported observation. If this is the case, then the question still remains whether hexarelin mimics an absence (or inhibition) of CD36. In any event, hexarelin through activation of AMPK could potentially inhibit HSL, limiting lipid hydrolysis. In support of this, one of the few studies of the effect of hexarelin in adipose tissue reported a decrease in isoproterenol-induced lipolysis (β-adrenergic receptor) (Muccioli *et al.*, 2004). However, caution must be taken on this interpretation since they measured the release of glycerol from cells. Because hexarelin induce a brown cell-like expression program, it is possible that the expression of glycerol kinase is also induced (as seen with TZDs), re-entering the glycerol into the FA reesterification cycle.

Even if the rate of lipolysis is decreased, based on our results it seems that due to hexarelin, imported or freed FA is redirected toward mitochondria for oxidation resulting in the overall net decrease in cellular lipid content. It is difficult to consolidate earlier events such as AMPK phosphorylation with later actions such as an increase in the expression of HSL and FATP4 which could simply represent compensatory mechanisms to regulate the initial signaling events. It is clear however that interaction of hexarelin with CD36 results in important changes in lipid metabolism and an increased demand in energy production, suggestive of fat burning conditions that could be beneficial to treating obesity.

1.1.2 CD36 in hepatocytes

The biodistribution of BMIPP and IPPA, two LCFA analogs, did not show any difference in their uptake between liver from wild type and from CD36-null mice,

signifying a minimal role by CD36 in hepatic FA uptake (Coburn *et al.*, 2000). However, from the standpoint of gene expression, these studies suggest an important influence of CD36 on hepatic lipid metabolism. Under normal conditions, CD36 does not seem to participate in FA uptake. However, overexpression of hepatic CD36 stimulate LCFA uptake and intracellular TG accumulation similarly to what is observed in obesity and type 2 diabetes (Koonen *et al.*, 2007). Similarly, the expression of CD36 was increased in the liver of mice on a HFD and its expression was positively correlated with LCFA uptake (Ge *et al.*, 2010). In contrast, CD36 accounts for 30% of total HDL uptake in cultured hepatocytes (Brundert *et al.*, 2011). CD36 is also capable of internalizing oxLDL while possibly delaying native LDL clearance (Luangrath *et al.*, 2008;Truong *et al.*, 2009). Strong upregulation of CD36 seems however to be linked to various pathologies. In fatty liver, the expression of CD36 is elevated compared to normal liver while in patients with hepatic steatosis, hepatocytes apoptosis was found to be positively correlated with high CD36 expression (Greco *et al.*, 2008;Bechmann *et al.*, 2010). In HepG2, CD36 was reported to be in intracellular compartments and played a minimal role in cholesterol efflux (Truong *et al.*, 2010). However, in NAFLD patients, CD36 is predominantly present on the plasma membrane of hepatocytes and its elevated expression is associated with insulin resistance (Miquilena-Colina *et al.*, 2011). In the second paper, we have been able to identify an important influence on cholesterol synthesis via the interaction of hexarelin with CD36 involving AMPK, PGC-1α and PPARγ.

1.1.2.1 Hexarelin and cholesterol

VLDL particles (containing ~50% TG and ~20% cholesterol) produced by the liver are eventually remodelled in the circulation into LDL particles (containing ~45% cholesterol and ~10% TG) following loss of TG due to LPL activity on peripheral tissues. LDL particles have a longer life span compared to other lipoprotein particles, and LDL-cholesterol was considered one of the major predictors of coronary heart disease (CHD) (Langer *et al.*, 1972). Today, other factors such as low HDL-cholesterol,

hypertriglyceridemia, hypertension, inflammation, elevated visceral adiposity are considered important predictors of coronary events. Cholesterol content in VLDL produced by the liver comes from the diet and *de novo* cholesterol synthesis. Statins, HMGR inhibitors, are first-line drugs to prevent CHD by lowering LDL-cholesterol (NCEP, 2002). The relationship between CD36, hexarelin and cholesterol is mainly associated with its impact on oxLDL uptake, HDL-cholesterol and the reverse cholesterol pathway. Indeed, oxLDL plasma level is elevated in CHD patients (Holvoet, 2004). While the effect of hexarelin against foam cell formation is well recognized, we still wondered whether a decrease in total cholesterol and in LDL-cholesterol levels observed in rats was solely due to an increase in cholesterol efflux associated with the reverse cholesterol pathway (De Gennaro-Colonna *et al.*, 2000;Pang *et al.*, 2010). More importantly, since hexarelin was shown to compete for the binding of oxLDL on CD36, unless the uptake of oxLDL is increased via other scavenger receptors, the decrease in LDL-cholesterol cannot be explained by an oxLDL-CD36 interaction (Demers *et al.*, 2004). In this second paper, we provided an alternative explanation to how hexarelin can possibly lower plasma LDL-cholesterol through a decrease in hepatic *de novo* cholesterol synthesis.

1.1.2.2 Activation of LKB1-AMPK by hexarelin

Upon activation of AMPK by an increase in AMP/ATP ratio following an increase in ATP spending, AMPK targets a plethora of pathways for the main purpose of reducing energy usage and produce readily available fuel for the cell. Fatty acid oxidation is increased to produce acetyl-CoA which will then be shuttle towards the TCA cycle and oxidative phosphorylation to produce ATP. Cell proliferation, lipid synthesis, protein synthesis and gluconeogenesis can be affected depending on the cell type and function. Hepatocytes have a higher rate of fatty acid oxidation and AMPK activity, and are a major site of cholesterol synthesis. Glycogen is the most readily available source of energy for muscles during exercise and liver during fasting. As another pathway to control energy usage, AMPK inhibits hepatic gluconeogenesis (Andreelli *et al.*, 2006). Treatment of adipocytes with hexarelin showed a marked increase in fatty acid oxidation and an

activation of PPARγ in a CD36-dependent manner (Rodrigue-Way *et al.*, 2007). Based on these results, we hypothesized that hexarelin would have a similar effect in hepatocytes and that this effect would also have a direct impact on cholesterol synthesis by means of the activation of AMPK.

While the activation of AMPK leads to CD36 translocation to the plasma membrane in muscle and adipose tissue, to date no reports showed that interacting with CD36 results in the activation of AMPK. Activation of LKB1-AMPK pathway by hexarelin binding to CD36, suggests a rapid shift in energy demand which explains in part the resulting increase in FAO. AMPK can be activated by different pathways; although not obligatory, the phosphorylation by LKB1 is often associated with a decrease in AMP/ATP ratio.

Dysregulation in the control of AMPK activity arises in different diseases or conditions. A decrease in AMPK activity is observed in obesity and it is mostly associated with development of insulin resistance and diabetes. AMPK is reduced in the heart and liver of genetic models of obesity while conflicting results on its activity in skeletal muscle have been reported (Steinberg *et al.*, 2004;Turdi *et al.*, 2011;Ha *et al.*, 2011). The activation of AMPK by AICAR increases glucose uptake and FAO in both animal models and in humans, and remains an interesting therapeutic potential to bypass skeletal muscle insulin resistance (Bergeron *et al.*, 2001;Koistinen *et al.*, 2003). Metformin, used to treat type 2 diabetes, reduces plasma glucose and lipids, and increases insulin sensitivity (Knowler *et al.*, 2002). The interest in AMPK was heightened following the discovery that metformin increased AMPK activity providing insight to some of its beneficial effects in diabetic patients (Zhou *et al.*, 2001). A recent study elucidated part of the mechanism of action of metformin (Xie *et al.*, 2008). Without affecting the AMP/ATP ratio, PKCζ was shown to be activated by metformin and translocated to the nucleus where it phosphorylated LKB1 at Ser428 resulting in its nuclear export and AMPK activation.

The phosphorylation site on HMGR (Ser872) by AMPK was identified by Grahame Hardie and colleagues in 1990 (Clarke & Hardie, 1990). Phosphorylation of HMGR was initially thought to be associated with its degradation until it was later disproven (Zammit

& Caldwell, 1992). Since then, HMGR phosphorylation and degradation were mainly handled as two distinct events, one regulated by AMPK activity while the other by sterol levels, respectively. Around 70% of HMGR is phosphorylated while 30% remains active and the interconversion is played between the activity of AMPK and the protein phosphatase 2A. The resulting ratio of unphosphorylated over total HMGR impacts the overall HMGR activity (Ching *et al.*, 1996;Gaussin *et al.*, 1997). AICAR drastically decreases cholesterol synthesis in rat liver causing a decrease in HMGR activity to almost 15% of its initial activity (Henin *et al.*, 1995). Metformin via AMPK activation also decreased cholesterol synthesis in HepG2 cells (Zang *et al.*, 2004). In my second paper, we have shown for the first time that an increase in AMPK phosphorylation resulted in both HMGR inhibition and degradation. Although no studies so far have shown a link between AMPK activity and HMGR degradation, studies on aging have shown an effect on both enzymes. Indeed, during aging, HMGR activity increased in *ad libitum* fed rats while AMPK activity is reportedly reduced (Marino *et al.*, 2002;Reznick *et al.*, 2007). In addition, the rate of degradation of HMGR was significantly lower in older rats resulting in hypercholesterolemia (Pallottini *et al.*, 2004;Trapani *et al.*, 2010).

1.1.2.3 Cholesterol fate

While the inhibition of HMGR by AMPK is based on energy demand, the expression and the degradation of HMGR is considered to be primarily affected by intracellular sterol levels. Our results demonstrate that hexarelin caused an inhibition of HMGR and a marked decrease in intracellular cholesterol. Other causes for a decrease in cholesterol content should be considered such as cholesterol efflux from cells triggered by LXR-ABC transporters pathway. LXRα and LXRβ regulate the expression of genes involved in cholesterol efflux but also those involved in cholesterol storage, catabolism and excretion (Lu *et al.*, 2001). In addition to ABCA1, the hepatic expression of ABCG5 and ABCG8 is increased during a high cholesterol diet by LXRα and LXRβ and are involved in the cholesterol catabolism into bile acids (Repa *et al.*, 2002). Even though PPARγ is

known to regulate the expression of LXRs, we have not determined the expression or activity of LXRs in hepatocytes in response to hexarelin. Based on these previous reports, a decrease in intracellular cholesterol through LXR-ABC transporters seemed unlikely since efflux occurs in excess of cholesterol while treatment of hepatocytes with hexarelin resulted in the inactivating phosphorylation of HMGR. A recent study reported that in response to energy depletion, dietary fuel absorption is activated mainly through the action of AMPK aimed at phosphorylating and activating steroid receptor coactivator-2, SRC-2 (Chopra *et al.*, 2011). In adipocytes, SRC-2 is associated with TG accumulation (Picard *et al.*, 2002). However, in a liver-specific manner, the AMPK-SRC-2 axis involving bile salt export pump (BSEP), ABCG5 and ABCG8 stimulate bile acid secretion into the gut. Bile acids are necessary for the excretion of cholesterol but also for dietary lipid uptake in the gut. We cannot therefore exclude the involvement of LXRs in the hepatocyte's response to hexarelin. And since bile acids are synthesized from cholesterol, we currently do not know the role that they might play in reducing intracellular cholesterol.

1.1.2.4 The impact of increased insig-2 expression

The decrease in HMGR protein combined with an increase in its ubiquitination coincides with the decrease in intracellular cholesterol content due to hexarelin. These results suggested that hexarelin in addition to inhibiting HMGR activity, unexpectedly caused its degradation. The multitude of reports demontrating that the interaction of insig with HMGR is the committed step in HMGR degradation and our results showing an increase in the expression of insig-2 due to hexarelin prompted us to further investigate its interaction with HMGR. Based on their role in sequestering SREBPs/SCAP in the ER, changes in the expression of insig-1 or insig-2 have been associated mostly with alterations in the expression of genes involved in TG or cholesterol synthesis; however, the impact on their interaction with HMGR in those instances was not always considered. Insigs have overlapping functions by interacting with HMGR and with SCAP. In hepatic insig-1/-2 double KO mice, TG and cholesterol accumulated in the liver owing to elevated levels of nuclear SREBPs and to decreased rate of HMGR degradation (Engelking *et al.*, 2005).One

study using siRNA inhibition of insigs showed that insig-1 had an important role in regulating SREBPs activity while insig-2 silencing had a less significant effect on SREBP target genes in human hepatoma cell lines, suggesting a different involvement of insig-2 in lipid metabolism (Krapivner *et al.*, 2008). An increase in the expression of insig-1 is consistent with an increase in the activity of SREBPs since it is a known target of SREBPs; however, insig-2 expression regulation is more elusive (Janowski, 2002). Both human insig-1 and insig-2 expression are increased in response to insulin. Insulin promotes the phosphorylation of SREBP-1c and its association with COPII vesicles therefore increasing the expression of insig-1 (Yellaturu *et al.*, 2009). Insulin also promotes the phosphorylation of SAP1a recently found to stimulate the expression of insig-2 (Fernandez-Alvarez *et al.*, 2010). However because of their differences in stability, they can play different roles in modulating cholesterol metabolism. As stated previously, insig-2 is more stable than insig-1 and is not degraded in presence of sterols. Therefore unlike insig-1, its expression level is paralleled by its protein level and upregulation of insig-2 can have a direct impact on the fate of SREBP and of HMGR (Lee & Ye, 2004;Gong *et al.*, 2006). In a hyperlipidemic setting, overexpression of insigs in obese Zucker diabetic rats and fasted/refed normal rats showed a marked decrease in liver steatosis and hyperlipidemia (Takaishi *et al.*, 2004). Interestingly, activation of either PPARγ or PPARα using respectively troglitazone or WY 14,643, resulted in an increase expression of insig-1 and insig-2 after 6 hours of treatment; with troglitazone having the strongest effect on insig-2 expression in rat hepatoma cells, Fao (Konig *et al.*, 2009). The increase in expression of insig-1 or insig-2 was followed by a reduction in nuclear SREBP-1 after 24 hours along with a decrease in intracellular TG and marked decrease in VLDL-secreted TG. Troglitazone was also shown to lower cholesterol synthesis by reducing nuclear SREBP-2 in both HepG2 and Caco-2 but its role in HMGR degradation was never assessed (Klopotek *et al.*, 2006). We detected a similar effect with hexarelin in which insig-2 expression was rapidly increased.

1.1.2.5 Role of PPARγ and PGC-1α in the recruitment of insig-2 to HMGR

Our previous studies indicated that hexarelin activated PPARγ in both macrophages and possibly adipocytes. In addition, troglitazone was reportedly shown to increase insig-2 expression, to influence cholesterol synthesis and to induce expression of HMGR. Hence, we also looked at the effect of troglitazone on insig-2 recruitment to HMGR. Indeed, treatment of hepatocytes with troglitazone also resulted in the recruitment of insig-2 to HMGR. However, troglitazone is known to also activate AMPK (LeBrasseur *et al.*, 2006). Therefore, to assure that the effect was dependent on PPARγ, cells were treated with hexarelin in combination with PPARγ antagonist, GW9662. We found that PPARγ in addition to regulating the expression of HMGR was also capable of possibly inducing its degradation. The involvement of PPARγ in promotiong insig-2 recruitment to HMGR due to hexarelin coincided with the activation of AMPK, the recruitment of AMPK to PGC-1α, and the recruitment of PGC-1α to PPARγ. Although we cannot fully describe the intricate steps that lead to HMGR degradation, it is clear that there is an important interplay among hexarelin, CD36, AMPK, PGC-1α and PPARγ involved in, and therefore capable of influencing cholesterol synthesis.

1.1.2.6 The dual effect of hexarelin/CD36 on PPARγ activity

We observed that PPARγ1 was activated in our luciferase assay experiments performed on hexarelin-treated hepatocytes. Assuming that activation of PPARγ was ligand-independent, we looked at 2 different kinases known to affect PPARγ activity: Akt for its activation and Erk for its inhibition (Adams *et al.*, 1997;Feige *et al.*, 2006;Demers *et al.*, 2009). Phosphorylation level of both kinases was affected by hexarelin. As stated in Section 8.3 (page 80), Erk inactivates PPARγ1via phosphorylation on Ser84 while Akt activates PPARγ through phosphorylation on a yet unknown site. Although we did not confirm Akt actions on PPARγ1, pAkt was found to be increased by hexarelin. Erk was rapidly dephosphorylated (inactivated) by hexarelin and was maintained in such a state

beyond 60 minutes. This inactivation of Erk resulted in the reduction of the inhibitory effect exerted on PPARγ1 as shown by a rapid and prolonged dephosphorylation of Ser84 and an increase in PPARγ1 activity. Surprisingly, a previous study using EP80317 (another GHRP analog) known to bind CD36, showed a slower increase in Erk phosphorylation in treated macrophages which express both CD36 and GHS-R1a (Bujold *et al.*, 2009). It was demonstrated that EP80317 did not displace ghrelin on GHS-R1a. However, a previous study demonstrated a competition between EP80317 and ghrelin on cells expressing GHS-R1a (Muccioli *et al.*, 2001). Assuming that EP80317 only binds to CD36, perhaps the differences observed between the effects of hexarelin and EP80317 on the phosphorylation level of Erk lie in the cell type used. Since hexarelin would bind to GHS-R1a and to CD36 on macrophages, experiments using EP80317 on hepatocytes would have to be performed to compare. Only one study so far looked at the effect of hexarelin on Erk in the brain, which expresses both GHS-R1a and CD36 (Brywe *et al.*, 2005). These investigators found no changes in phosphorylation of Erk in response to hexarelin. In macrophages expressing both GHS-R1a and CD36, binding to either receptor was shown to have opposite effects on phospho-Erk. We recently demonstrated that treating macrophages with ghrelin caused an increase in phospho-Erk (Demers *et al.*, 2009). While this activation could result in the inactivation of PPARγ, we found that Dok-1 associated to GHS-R1a restrains the inhibitory potential of Erk. Therefore, it would be interesting to determine the fate of phospho-Erk and phosphorylation of PPARγ on Ser84 in hexarelin-treated macrophages. In our second paper, we demonstrate that in the absence of GHS-R1a, the binding of hexarelin to CD36 in hepatocytes causes a decrease in phospho-Erk which can no longer phosphorylate PPARγ at Ser84. In addition, we indirectly demonstrated that following dephosphorylation of Erk, PPARγ is activated when compared to PPARγS84A mutant.

PPARγ2, mainly found in adipocytes, is more responsive to insulin, glucose and lipids and plays a major role in lipid accumulation (Werman *et al.*, 1997). While PPARγ1 is the predominant form in hepatocytes, oleic acid or insulin were found to increase the expression of PPARγ2 in cultured mouse hepatocytes (Edvardsson *et al.*, 2006). Normal

mice fed a HFD had an increased expression of PPARγ2 in the liver (Vidal-Puig *et al.*, 1996). Hepatocytes stably expressing PPARγ2, showed phenotype similar to hepatic steatosis with increase in SREBP-1 activity, expression of FAS and lipid accumulation (Schadinger *et al.*, 2005). However, treatment of NASH patients with pioglitazone showed marked improvement of liver fat content demonstrating the beneficial effect of activating PPARγ (Promrat *et al.*, 2004). While we showed an increase in the expression of PPARγ in hepatocytes treated with hexarelin, we did not determine whether the expression of PPARγ2 was induced.

In parallel with PPARγ activation through its dephosphorylation at Ser84, we found that AMPK was rapidly recruited to PGC-1α following treatment of hepatocytes with hexarelin, adding to PPARγ`s effect on insig-2 recruitment to HMGR. It might seem like a paradox that while AMPK inhibits gluconeogenesis it also activates PGC-1α, a key player in gluconeogenesis; however, AMPK inhibits gluconeogenesis primarily by targeting HNF-4, leaving the active PGC-1α to co-activate other targets involved in mitochondrial biogenesis and FAO (Hong *et al.*, 2003).

Finally, with the observation that hexarelin also increased phosphorylation of Akt, it seems unlikely that Akt would target and inactivate PGC-1α (Section 8.2.3.1, Figure 18A, page 80) since the inhibitory phosphorylation on Ser570 would result in its acetylation by GCN5 which in turn would prevent phosphorylation by AMPK and recruitment to PPARγ (Li *et al.*, 2007b;Xiong *et al.*, 2010).

1.1.2.7 Possible mechanism of action of hexarelin in its inhibition of cholesterol synthesis

1.1.2.7.1 Cholesterol trafficking

Taking into account the rigorous mechanism that regulates cholesterol synthesis which is governed mostly by cholesterol and sterol levels in the ER, we wondered whether other factors were able to disturb this elaborate equilibrium between cholesterol synthesis

and HMGR degradation. Newly synthesized cholesterol moves rapidly from the ER to the plasma membrane, to extracellular acceptors or to the Golgi with a half-time of 18 minutes (Baum *et al.*, 1997). Any event disturbing this trafficking would result in rapid accumulation of cholesterol in the ER surpassing its 5 mol% threshold and triggering cholesterol synthesis inhibition mechanisms (Sokolov & Radhakrishnan, 2010). Possible activation of other pathways involving cholesterol or intermediates such as isoprenoids, bile acids, steroid hormones and vitamin D would result in a decrease and not an accumulation of cholesterol. Due to the hydrophobic nature of cholesterol molecules, they require specific transporters in order to be move to other organelles following synthesis. The oxysterol-binding protein (OSBP)-related proteins (ORPs) became interesting candidates for hexarelin's effect on cholesterol synthesis. ORPs are members of a large family of lipid transfer proteins possessing a core lipid binding domain with a hydrophobic pocket that can accommodate a single lipid molecule shielded from the aqueous environment. ORPs are involved in sterol signaling and sterol transport functions between lipid rafts and various intracellular compartments such as ER and Golgi; possibly having distinct effects in cholesterol metabolism (Raychaudhuri & Prinz, 2010). The location, the regulation and the mode of action for many of these ORPs are still unknown.

As stated in the discussion of my second paper, recent studies demonstrated the impact that regulation of ORPs might have on cholesterol mobilization and on HMGR activity (Hynynen *et al.*, 2005;Wang *et al.*, 2005;Ngo & Ridgway, 2009;Nhek *et al.*, 2010). Probing into this field of research, we wondered if gene expression of some ORPs could be influenced by hexarelin or troglitazone. Although not all, the expression of some ORPs was rapidly upregulated by hexarelin and to some degree by troglitazone. Since changes in the rate of HMGR degradation occurs even earlier than 5 hours, hexarelin might also influence their activity; perhaps inactivation of Erk or activation of AMPK can have a repercussion on the export of cholesterol from the ER.

Conceivably, the key to hexarelin's effect on cholesterol synthesis may involve one of these ORPs and could even impact its activity. Nonetheless, we do know that hexarelin

and troglitazone are capable of modulating the expression of at least a few of these candidates. Future knowledge of their function and their influence on lipid trafficking could eventually single out specific candidates to be studied. Although we cannot be certain this is the mechanism by which hexarelin acts, it is certainly an interesting avenue to further explore.

1.1.2.7.2 Possible hypoxic state?

The combined observations that hexarelin triggers (1) a rapid AMPK phosphorylation, (2) a rapid increase in insig-2 expression, (3) a recruitment of insig-2 to HMGR, along with reports of similar effect of troglitazone on insig-2 expression and reduce cholesterol synthesis, suggest a complex interplay among these key players in lipid metabolism, which we attempted to elucidate. A potential effect that we have not addressed in our paper was the link between AMPK activation and hypoxia. Several reports have shown that hypoxia activates AMPK in response to increase in ROS but independently of AMP/ATP ratio (Laderoute *et al.*, 2006;Emerling *et al.*, 2009). However, while it can be damaging in certain pathologies or during exhaustive training, production of ROS during moderate exercise is necessary and beneficial for mitochondrial adaptation to increased energy demand. An interesting study depicted the effect of antioxidant in young men subjected to moderate exercise and on ROS, known to be increased during hypoxia (Gomez-Cabrera *et al.*, 2008). While antioxidants may be beneficial in aging and metabolic diseases, vitamin C in these young subjects caused a reduction in the exercise-induced expression of key regulators of mitochondrial biogenesis such as PGC-1α and mtTFA and reduced VO_2 max.

Given that the synthesis of cholesterol requires a large amount of oxygen, the shutdown of this pathway is obligatory to prevent accumulation of sterol intermediates such as lanosterol but this occurs without inhibiting SREBP activity (Nguyen *et al.*, 2007). ROS has been suggested to possibly prevent degradation of HIF-1α (Chandel *et al.*, 1998). As previously mentioned in section 9.2.2, HIF-1α escapes degradation and stimulates

degradation of HMGR while increasing HMGR expression via binding to HRE on its promoter region (Pallottini *et al.*, 2008). Lanosterol has an affinity for HMGR and therefore specifically targets HMGR degradation which explains the induction of HMGR degradation during hypoxia without affecting SREBP activity. During hypoxia, the expression of both insig-1 and insig-2 was dependent on HIF-1α and was observed in combination with HMGR degradation (Nguyen *et al.*, 2007;Kayashima *et al.*, 2011). We observed a time-dependent, multi-level effect of hexarelin involving (1) a rapid phosphorylation of AMPK, (2) an increase in the expression of insig-2, (3) a possible sequestration of SREBP (via SCAP), (4) but also a recruitment of insig-2 to HMGR which was shown to be dependent on CD36. With the exception of changes in SREBP activity and the involvement of CD36, all the other events are seen in hypoxia including increase in PGC-1α to induce mitochondrial biogenesis. However, unlike hypoxia, hexarelin did not influence the expression of insig-1. Interestingly, in a hypoxic state, the expression of CD36 in human retinal pigment epithelial cells is increased by HIF-1α as an adaptive response (Mwaikambo *et al.*, 2009). However, in hepatocytes (with the exception of 25-HC), we did not see an increase in the expression of CD36 due to hexarelin. Despite that, it is possible that the increased in FAO might trigger a hypoxic state similar to what is seen in moderate exercise and stimulating mitochondrial biogenesis. Looking at the protein level of HIF-1α for example in hepatocytes treated with hexarelin could determine whether hypoxia plays a role in the decrease in intracellular cholesterol.

2 Perspectives

The results generated during my graduate studies have led to several new and interesting mechanisms of action of hexarelin through its association with CD36. Nonetheless, while some questions are left unanswered, our results give rise to many other possible interesting projects.

2.1 Studies to complement our results

In adipocytes, we have shown that through CD36, hexarelin causes (1) an increase in the expression of genes involved in mitochondrial biogenesis, FAO and oxidative phosphorylation, (2) an induction of brown adipocyte phenotype, and (3) a decrease in lipid content. In adipocytes, secretion of some adipokines can have important impact on inflammation and lipid homeostasis. CD36 has been shown to play a role in the inflammatory response in adipocytes and macrophages in presence of oxLDL, LPS and serum amyloid A (Baranova *et al.*, 2010;Kennedy *et al.*, 2011). However, blocking ligand interaction with a CD36 peptide based on hexarelin's binding site reduced the inflammatory response mediated by JNK and to a lesser degree Erk. It would be interesting to look at the expression of TNF-α, resistin, leptin and various interleukins as well as anti-inflammatory adiponectin and SFRP5 in adipocytes treated with hexarelin. Since hexarelin blocks binding of pro-inflammatory ligands, we could expect a reduced inflammatory response. In addition to regulating FAO, AMPK also affects lipolysis; it would be interesting to measure the rate of lipolysis, and the levels of phosphorylation of HSL, perilipin and ATGL as well as their expression in hexarelin-treated adipocytes. A previous report indicated that hexarelin decreased lipolysis in rat adipocytes which was measured by glycerol release (Muccioli *et al.*, 2004). Due to the brown fat phenotype induced by hexarelin it is possible that glycerol kinase might be upregulated. Therefore, using [1-^{14}C]palmitate incorporated into TG then measuring labeled-FA released from cells and ^{14}CO2 produced from FAO might provide further knowledge on the capacity of hexarelin to influence lipolysis. A full metabolic profile on hexarelin-treated mice would provide further insight on its overall physiological impact. However, since hexarelin binds to both CD36 and GHS-R1a, results could be difficult to attribute solely to its binding to CD36. Generation of a specific CD36 hexarelin derivative such as EP 80317 would be a better study candidate. Since we have seen a reduction in adipocyte TG content, DEXA scanning could be used to measure total body composition and fat content following hexarelin treatment.

One important question that remains unanswered is whether hexarelin blocks fatty acid uptake in adipocytes. A possible blockage by hexarelin in FA uptake could have an important impact on lipid homeostasis. Therefore, simple approaches are accessible to determine hexarelin's influence on lipid uptake. Iodine 125 fatty acid analogs such as BMIPP are readily used to monitor FA uptake and have been extensively used for this purpose (Coburn *et al.*, 2000). Alternatively, the use of fluorescent FA analogs coupled with a quench technology such as QBT™ Fatty Acid Uptake Assay by Molecular Devices (Sunnyvale, California, United States) have been used on 3T3-L1 adipocytes and offer a rapid, one-step detection of the level of FA uptake in response to hexarelin.

In hepatocytes, we have observed (1) phosphorylation of AMPK, (2) a decrease in intracellular cholesterol, (3) a recruitment of insig-2 to HMGR dependent on AMPK and PPARγ, (4) a recruitment of AMPK to PGC1α and PGC-1α to PPARγ, (5) dephosphorylation of Erk resulting in activation of PPARγ and (6) increased expression of selected ORPs. It would be of great interest to determine the precise role of ORPs in regulating hexarelin's effect on HMGR activity, and to determine if AMPK targets members of this family. Cholesterol distribution within the cell would also provide insight as to whether cholesterol trafficking is affected by hexarelin mainly if it accumulates in the ER. Also as stated previously, whether hypoxia could play a role in the response of hepatocytes to hexarelin would be interesting to evaluate.

Since many studies on the role of CD36 in FA uptake and its translocation following activation of AMPK have been performed in skeletal muscles, it would certainly be appealing to look at the effect of hexarelin on muscle performance and skeletal muscle cell function.

2.2 Signaling cascade(s) involving the binding of hexarelin to CD36

Although we have identified AMPK and Erk as downstream kinases involved in CD36 response in hepatocytes, in macrophages other kinases such as JNK and p38 are also

involved in CD36 signaling in response to oxLDL and are known to target PPARγ. Therefore, it would be interesting to look at the response of these kinases following treatment with hexarelin. As presented earlier, Src family kinases are known to associate with CD36 and are activated in response to FA and oxLDL. The association of Src with CD36 is important in inflammation and foam cell formation as well as gustatory cell signaling in response to dietary fat. Interestingly, loss of Fyn resulted in the increase in FAO in adipose tissue and skeletal muscle (with less in liver) indicating Fyn as a negative regulator of FAO (Bastie *et al.*, 2007). Recently, studies done by the same group showed that Fyn targets LKB1 (tyrosine 261 and 365) trapping LKB1 in the nucleus where it is inactive. SU6656, a Src inhibitor, rapidly increases energy expenditure, fatty acid oxidation, and increases AMPK phosphorylation at Thr-172 in wild-type mice. Inhibition of Fyn causes LKB1 to relocate to the cytosol and target AMPK for activation (Yamada *et al.*, 2010). How hexarelin and CD36 would affect Fyn's activity is uncertain. Nonetheless, with our finding that hexarelin triggers FAO and that Fyn is known to bind to CD36, it would certainly be of great interest to determine how Fyn (or other Src kinases) would react in presence of hexarelin. A possible role of Src kinase in hexarelin's effect on lipid metabolism would provide us with a more concrete mechanism of action between CD36 and FAO in adipocytes.

2.3 Other pathways affected by CD36/hexarelin interaction

Binding to CD36 has the potential to impact several pathways. In the presence of oxLDL or TSP-1, in addition to increasing inflammation, CD36 impairs insulin signaling, induces apoptosis and influences cell proliferation (Kennedy *et al.*, 2011). Hexarelin suppressed cardiac fibroblast and VSMC proliferation while inhibiting cardiomyocyte apoptosis following heart failure in rats (Xu *et al.*, 2007;Pang *et al.*, 2010;Pang *et al.*, 2004;Xu *et al.*, 2005). Depending on the cell type, ghrelin can have a similar effect. In rat hippocampal progenitor cells, hexarelin and ghrelin showed increased cell proliferation; however only hexarelin had anti-apoptotic effect on these cells (Johansson *et al.*, 2008).

Since AMPK inhibits protein synthesis and cell proliferation it would be interesting to see if hexarelin via CD36 plays a role in these pathways.

2.4 Lipid rafts and CD36

Cholesterol is an important component in lipid rafts and caveola that helps to maintain caveolar structure intact and components together. Cholesterol depletion results in the disassembly of the rafts and their proteins such as CD36 and has been shown to inhibit FA uptake (Covey *et al.*, 2007;Ehehalt *et al.*, 2008). Therefore, since hexarelin reduces intracellular cholesterol, it would be important to determine if this could impact cholesterol distribution to plasma membrane and FA uptake. In absence of caveolin-1, CD36 is no longer present on the cell surface while in presence of CD36, caveolin-1 is not longer a target of Src kinases (Ring *et al.*, 2006). Thus, given that there is an inter-relationship among caveolin-1, Src kinases and CD36 it would be interesting to investigate the effect of hexarelin on the presence of CD36 in lipid rafts. Since AMPK induces translocation of CD36 to plasma membrane even in adipocytes, determining the distribution of CD36 in response to hexarelin would be important. However, one report showed that in stimulated muscle, inhibition of Erk using PD-98059 abolished the translocation of CD36 and increase in FA uptake following contractions (Turcotte *et al.*, 2005). Therefore, determining whether hexarelin affects FA uptake through activation of AMPK or inhibition of Erk would provide further insight on its impact in adipocyte lipid metabolism.

2.5 The impact of hexarelin on lipid metabolism mediated by other nuclear factors

We previously reported that hexarelin also activated PPARα and β/δ which are also involved in regulating lipid catabolism (Avallone *et al.*, 2006). Fatty acids and their derivatives activate PPARα which plays a major role in FAO, gluconeogenesis, lipid transport and ketogenesis in the liver. Recently, 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine (16:0/18:1-GPC) was identified as the physiologically relevant endogenous

PPARα ligand synthesized from DAG and CDP-choline by choline-ethanolaminephosphotransferase-1 (CEPT1) (Chakravarthy *et al.*, 2009). The PPARα-dependent gene expression, at least in the liver, is dependent on the expression of FAS which leads to production of DAG. Since in adipocytes, the absence of CD36 results in the accumulation of DAG perhaps hexarelin mimics a similar event by increasing FAS expression, by increasing DAG levels and indirectly increasing synthesis of 16:0/18:1-GPC which in turn activates PPARα.

Agonists of AMPK and of PPARβ/δ induced the expression of metabolic genes but also drastically increased exercise endurance even in sedentary mice (Narkar *et al.*, 2008). Moreover, in adipocytes, PPARβ/δ selectively activates genes involved in fatty acid oxidation and energy uncoupling (Wang *et al.*, 2003). Since PGC-1α can also be recruited to other nuclear receptors in response to activation by AMPK, it would be interesting to determine if hexarelin can also trigger those recruitments and influence the activity of PPARα and PPARβ/δ in adipocytes and hepatocytes.

3 Conclusions

The World Health Organization estimates that as of 2008, 1.5 billion adults worldwide were overweight with 500 million being clinically obese (body mass index equal to or greater than 30 kg/m2). In 2010, approximately 43 million children under the age of five were overweight. Obesity can lead to complications associated with type 2 diabetes, cardiovascular and fatty liver diseases. Healthy diet and exercise still remains the best approach to reducing weight. Despite that knowledge, lifestyles are difficult to modify. Research is aimed at providing further insight to factors leading to obesity and related complications and finding possible alternatives to reversing obesity. In addition to decreasing foam cell formation and atherosclerotic plaque area, the interaction of hexarelin with CD36 also reduces lipid content in adipocytes, induces fatty acid oxidation; and in hepatocytes, hexarelin decreases intracellular cholesterol by inhibiting HMGR activity and stimulating the recruitment of insig-2 to HMGR, the committed step in HMGR

degradation. Although the use of a CD36-specific synthetic peptide would be preferable, our results certainly have contributed to further the knowledge of the impact that this interaction has on lipid metabolism. To determine the beneficial role it might have on reducing obesity and improving circulating lipid profile and body composition, further in vivo investigation is required. In conclusion, the ligand-mediated manipulation of CD36 may provide a useful alternative approach to the modification of lipid metabolism.

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