

University of Montreal

Regulation of lectin-like
oxidized low-density lipoprotein receptor-1 (LOX-1).
Relevance to diabetic vasculopathy.

By

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University of Montreal
Faculty of graduate studies

This thesis entitled
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RÉSUMÉ

L'athérosclérose est la principale cause de décès et la complication majeure du diabète de type 2. Le récepteur-1 "lectin-like oxidized low-density lipoprotein" (LOX-1) est un récepteur vasculaire nouvellement identifié pour les LDL oxydées (LDLox) et les produits de glycation (AGEs). LOX-1 est surexprimé dans les lésions athérosclérotiques humaines et dans l'aorte de rats diabétiques, ce qui suggère un rôle de ce récepteur dans la pathogenèse de l'athérosclérose associée au diabète. Dans la présente étude, nous avons étudié la régulation vasculaire de LOX-1 par le glucose et la CRP et le rôle de LOX-1 dans l'adhésion des monocytes à l'endothélium et la formation de cellules spumeuses. Nos résultats démontrent que: 1) l'incubation de cellules endothéliales aortiques humaines (HAECs) et de macrophages dérivés de monocytes humains (hMDM) avec des concentrations de glucose élevées augmente l'expression génique et protéique de LOX-1 de manière dose- et temps-dépendant; 2) l'augmentation de l'expression protéique de LOX-1, induite par des concentrations élevées de glucose, est abolie par des agents antioxydants et des inhibiteurs de la protéine kinase C (PKC), des protéines kinases activées par les mitogènes (MAPK), du facteur nucléaire-kappaB (NF- κ B) et de la protéine activée-1 (AP-1). Des MDM humains incubés en présence de concentrations élevées de glucose démontrent une augmentation de l'expression de PKC β 2 et une phosphorylation des protéines kinases régulées par des signaux extracellulaires (ERK) 1/2. L'activation de ces kinases est inhibée par les antioxydants et par l'inhibiteur spécifique de PKC β 2, le LY379196. Des concentrations élevées de glucose augmentent également la liaison des protéines nucléaires extraites de MDM

humains aux séquences régulatrices NF- κ B et AP-1 présentes dans le promoteur du gène de LOX-1; 3) l'incubation de HAECs et de MDM avec des concentrations élevées de glucose augmente l'adhésion des monocytes à l'endothélium et la formation de cellules spumeuses, respectivement, par l'entremise de mécanismes de signalisation LOX-1-dépendant; 4) l'incubation de HAECs avec la CRP augmente l'expression génique et protéique de LOX-1 de façon dose- et temps-dépendant. Cet effet est atténué par une préincubation des cellules avec des anticorps anti-CD32 et anti-CD64. Enfin, le traitement des HAECs par la CRP induit une augmentation de l'adhésion des monocytes à l'endothélium. Cette augmentation est dépendante de LOX-1. Globalement, ces résultats démontrent que des concentrations élevées de glucose augmentent in vitro l'expression endothéliale et macrophagique de LOX-1 et que ces effets sont associés à une adhésion accrue des monocytes à l'endothélium et à la formation de cellules spumeuses. Nous avons également démontré que la CRP augmente, in vitro, l'expression endothéliale de LOX-1 et que cet effet est associé à une augmentation de l'adhésion des monocytes à l'endothélium. En résumé, ces données suggèrent un rôle clé de divers facteurs métaboliques et inflammatoires dans la régulation vasculaire de LOX-1 et suggèrent un rôle de LOX-1 comme médiateur de l'athérogenèse associée au diabète humain. Une meilleure compréhension du rôle des facteurs métaboliques et inflammatoires dans la régulation vasculaire de LOX-1 dans le diabète pourrait permettre le développement de nouvelles stratégies dans le traitement de l'athérosclérose associée au diabète.

Mots clés: Diabète, LOX-1, glucose, macrophages, cellules endothéliales, CRP.

SUMMARY

Atherosclerosis is the leading cause of death and the major complication of type 2 diabetes. Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) is a newly identified vascular receptor for oxidized LDL (oxLDL) and advanced glycation end products (AGEs). LOX-1 is highly expressed in human atherosclerotic lesions and is upregulated in the aorta of diabetic rats. In the present study, we sought to determine the regulation of vascular LOX-1 in diabetes. Our results demonstrated that: 1) incubation of human aortic endothelial cells (HAECs) and human monocyte-derived macrophages (hMDM) with elevated glucose concentration enhanced, in a dose- and time-dependent manner, LOX-1 gene and protein expression; 2) high glucose-induced macrophage LOX-1 protein expression was abolished by antioxidants, protein kinase C (PKC), mitogen-activated protein kinase (MAPK), nuclear factor-kappaB (NF- κ B) and activated protein-1 (AP-1) inhibitors. In human MDM cultured with high glucose, increased expression of PKC β 2 and enhanced phosphorylation of extracellular signal-regulated protein kinase (ERK) 1/2 were also observed. Activation of these kinases was inhibited by the antioxidant and by the PKC β 2 inhibitor, LY379196. High glucose also enhanced the binding of nuclear proteins extracted from human MDM to the NF- κ B and AP-1 regulatory elements of the LOX-1 gene promoter. High glucose-induced endothelial LOX-1 protein expression was abolished by antioxidants, PKC, MAPK, and NF- κ B inhibitors. High glucose enhanced the binding of nuclear protein extracted from HAECs to the NF- κ B regulatory element of the LOX-1 gene promoter; 3) incubation of HAECs and macrophages with high glucose increased monocyte adhesion to

endothelium and foam cell formation, respectively, through LOX-1-dependent signaling mechanism; 4) incubation of HAECs with CRP enhanced, in a dose- and time-dependent manner, LOX-1 mRNA and protein levels. This effect was reduced by anti-CD32 and -CD64 antibodies, whereas it was unaffected by hyperglycemia, tumor necrosis factor α (TNF α) and interleukin-6 (IL-6). In CRP-treated HAECs, a LOX-1-dependent induction of monocyte adhesion to endothelium was observed. Overall, these results demonstrate that high glucose enhances human endothelial and macrophage LOX-1 expression *in vitro* and that this effect is associated with enhanced monocyte adhesion to endothelium and foam cell formation. We also found that CRP increases human endothelial LOX-1 expression *in vitro* and that this effect is associated with increased monocyte adhesion to endothelium. Taken collectively, these data indicate a key role for metabolic and inflammatory factors in the regulation of vascular LOX-1 in diabetes. They identify LOX-1 as one potential important mediator in the pathogenesis of atherosclerosis in human diabetes. Better understanding of the role of metabolic and inflammatory factors in the regulation of vascular LOX-1 in diabetes might provide new strategies in the treatment of diabetic atherosclerosis.

Keywords: Diabetes, LOX-1, glucose, macrophages, endothelial cells, CRP.

CONTENTS

Summary.....	iii
List of figures.....	xvi
List of tables.....	xix
List of abbreviations.....	xx
Acknowledgements.....	xxiv
Dedication	xxv
I. Introduction.....	1
I.I. Atherosclerosis.....	1
I.I.1. General.....	1
I.I.2. Development and progression of the atherosclerotic lesion.....	1
I.I.2.1. Endothelial dysfunction in atherosclerosis.....	1
I.I.2.1.1. Endothelial function.....	1
I.I.2.1.2. Endothelial dysfunction.....	2
I.I.2.2. Fatty-streak.....	3
I.I.2.3. Advanced, complicated lesion of atherosclerosis.....	4
I.I.2.4. Unstable fibrous plaques in atherosclerosis.....	4
I.I.2.4.1. Local factors.....	4
I.I.2.4.2. Systemic factors.....	5
I.I.3. Risk factors.....	6
I.I.3.1. Traditional risk factors.....	6

I.I.3.1.1. Hypercholesterolemia and dyslipoproteinemia.....	6
I.I.3.1.2. Hypertension.....	8
I.I.3.1.2.1. Renin-angiotensin system (RAS).....	8
I.I.3.1.2.2. Endothelin.....	9
I.I.3.1.3. Diabetes.....	10
I.I.3.1.4. Family history, age and gender.....	10
I.I.3.1.5. Obesity and smoking habit.....	11
I.I.3.2. New risk factors.....	12
I.I.3.2.1. CRP.....	12
I.I.3.2.2. Lp(a).....	13
I.I.3.2.3. Fibrinogen.....	15
I.I.3.2.4. Homocysteine.....	16
I.I.3.2.5. Infection.....	12
I.I.4. Pathogenesis of atherosclerosis.....	17
I.I.4.1. Endothelial dysfunction hypothesis.....	17
I.I.4.1.1. Risk factors for endothelial dysfunction	18
I.I.4.1.2. Mechanisms of endothelial dysfunction in atherosclerosis.....	19
I.I.4.2. Lipid infiltration hypothesis.....	20

I.I.4.3. Oxidative stress hypothesis.....	21
I.I.4.4. Hypothesis of infection.....	24
I.I.4.5. Hypothesis of inflammation.....	24
I.II. Diabetes mellitus (DM).....	28
I.II.1. Definition.....	28
I.II.2. Classification.....	29
I.II.2.1. Type 1 diabetes.....	29
I.II.2.2. Type 2 diabetes.....	34
I.II.2.2.1. Prevalence.....	35
I.II.2.2.2. Etiology.....	35
I.II.2.2.3. Genetic factors.....	36
I.II.2.2.3.1 Gene candidates.....	38
I.II.2.2.3.2. Mutations in genes involved in insulin resistance.....	38
I.II.2.2.3.3. Mutation in genes encoding β -cell proteins involved in the quality and quantity of secreted insulin (figure 3).....	42
I.II.2.2.3.4. Mutations in genes involved in lipid metabolism and obesity.....	44
I.II.2.2.3.5. Mutations in genes relevant to insulin action.....	46
I.II.2.2.4. Environmental factors.....	47

I.II.2.2.4.1. Obesity.....	47
I.II.2.2.4.2. Diet.....	48
I.II.2.2.4.3. Physical activity.....	48
I.II.2.2.4.4. Stress.....	49
I.II.2.3. Pathophysiology of type 2 diabetes.....	49
I.II.2.3.1. Action of insulin.....	50
I.II.2.3.2. Insulin resistance.....	51
I.II.2.3.2.1. Genetics and environment.....	52
I.II.2.3.2.2. Hepatic insulin resistance.....	53
I.II.2.3.2.3. Insulin resistance in skeletal muscle.....	53
I.II.2.3.2.4. Insulin resistance in adipocyte.....	54
I.II.2.3.3. Hyperinsulinemia and glucose intolerance.....	57
I.II.2.4. Complications associated with type 2 diabetes.....	58
I.II.2.4.1. Macrovascular complications.....	61
I.II.2.4.1.1. Traditional risk factors.....	60
I.II.2.4.1.1.1. Dyslipidemia.....	60
I.II.2.4.1.1.2. Hypertension.....	61
I.II.2.4.1.1.3. Smoking.....	61
I.II.2.4.1.1.4. Obesity.....	62
I.II.2.4.1.1.5. Hyperglycemia.....	62

I.III.2.1.2. 5'-flanking region of the LOX-1 gene.....	94
I.III.2.1.3. Human LOX-1 mRNA structural organization.....	95
I.III.2.2. Structure of LOX-1 protein.....	96
I.III.2.2.1. The domain structures and functions of LOX-1.....	96
I.III.2.2.2. Post-translational modification of LOX-1.....	97
I.III.2.2.3. Soluble forms of LOX-1.....	98
I.III.2.3. LOX-1 tissue distributions.....	98
I.III.3. LOX-1 binding ligands.....	98
I.III.4. Regulation of LOX-1 expression.....	100
I.III.4.1. Inducing Factors.....	100
I.III.4.1.1. Pro-inflammatory cytokines.....	100
I.III.4.1.2. Pro-atherosclerotic stimuli.....	100
I.III.4.1.3. Oxidative stress.....	101
I.III.4.1.4. Pathological conditions.....	102
I.III.4.2. Inhibitory factors.....	103
I.III.5. Role of LOX-1 in atherosclerosis.....	104
I.III.5.1. Role of LOX-1 in endothelial dysfunction.....	104
I.III.5.2. Role of LOX-1 in the progression of the atherosclerotic lesion.....	105
I.III.5.3. Role of LOX-1 in atherosclerotic plaques stability.....	105

I.III.6. Role of LOX-1 in type 2 diabetes.....	106
I.IV. CRP.....	107
I.IV.1. CRP and atherosclerosis.....	108
I.IV.1.1. CRP as a predictive risk marker of cardiovascular events...	109
I.IV.1.2. Role of CRP in atherosclerosis.....	111
I.IV.1.2.1. Role of CRP on monocyte adhesion/recruitment...	112
I.IV.1.2.2. Role of CRP on foam cell formation.....	113
I.IV.1.3. Comparison of CRP to other risk factors.....	114
I.IV.1.4. Goal of screening and therapeutic options.....	115
I.IV.2. CRP and Diabetes.....	115
I.V. Hypothesis and objectives.....	117
II. Results.....	119
II.1. Glucose enhances endothelial LOX-1 expression.	
Role for LOX-1 in glucose-induced human	
monocyte adhesion to endothelium.....	120
II.2. Glucose enhances human macrophage LOX-1	
expression. Role for LOX-1 in glucose-induced	
macrophage foam cell formation.....	155
II.3. C-reactive protein (CRP) enhances lectin-like oxidized low-density	
lipoprotein receptor-1 (LOX-1) expression in human	
aortic endothelial cells. Relevance of LOX-1	

to CRP-induced endothelial dysfunction.....	192
III. Discussion.....	222
IV. Conclusions and perspectives.....	248
IV.1. Conclusion.....	249
IV.2. Perspectives.....	250
V. References.....	253

LIST OF FIGURES

Introduction :

Figure 1. Modulation of cellular function by ROS in cardiovascular diseases.....	22
Figure 2. Downstream effects resulting from insulin/insulin receptor interaction.....	40
Figure 3. Genetic defects in beta-cells in type 2 diabetes.....	43
Figure 4. Pathogenesis of type 2 diabetes.....	52
Figure 5. Model of development of diabetic vascular complications.....	58
Figure 6. Multiple cardiovascular risk factors in diabetes.....	59
Figure 7. The formation of AGEs.....	63
Figure 8. Hyperglycemia-induced activation of The diacylglycerol-PKC pathway.....	66
Figure 9. Exogenous and endogenous stimuli leading to reactive oxygen species generation.....	68
Figure 10. Inflammation : the link between obesity, insulin resistance, diabetes, and atherosclerosis.....	75
Figure 11. Hypothetical model of chronic inflammation and adipocyte insulin resistance.....	76
Figure 12. Diagrammatic representation of the LOX-1 gene and protein.....	95
Figure 13. Role of LOX-1 atherosclerosis.....	106
Figure 14. CRP level and cardiovascular risk.....	111
Figure 15. Direct comparison of CRP to several other lipid and non-lipid risk factors for cardiovascular disease.....	114

First article:

Figure.1. Time- and dose-dependent effect of high glucose on LOX-1 mRNA levels in HAECs.....	146
Figure.2. Time- and dose-dependent effect of high glucose on LOX-1 protein expression in HAECs.....	148
Figure.3. Effect of high glucose and TNF α on endothelial LOX-1 protein expression.....	150
Figure.4. Effect of PKC, MAPK, NF- κ B inhibitors and antioxidants on glucose-induced LOX-1 mRNA levels.....	151
Figure.5. Effect of high glucose on the binding of nuclear proteins extracted from HAECs to the NF- κ B sequence of the LOX-1 gene promoter.....	152
Figure.6. Effect of high glucose on human monocyte adhesion to endothelial cells.....	154

Second article:

Figure.1. Time- and dose-dependent effect of high glucose on LOX-1 mRNA levels in human MDM.....	180
Figure.2. Time- and dose-dependent effect of high glucose on LOX-1 protein expression in human MDM.....	181
Figure.3. Effect of high glucose on TNF- α -induced macrophage LOX-1 mRNA and protein expression.....	182
Figure.4. Effect of PKC, MAPK, NF- κ B, AP-1 inhibitors and antioxidants	

on glucose-induced LOX-1 mRNA levels.....	183
Figure.5. Effect of high glucose on PKC and MAPK activation in THP-1 MDM. Modulatory effect of NAC and PKC/MAPK inhibitors.....	185
Figure.6. Effect of high glucose on the binding of nuclear proteins extracted from THP-1 MDM to the NF- κ B sequence of the LOX-1 gene promoter.....	186
Figure.7. Effect of high glucose on the binding of nuclear proteins extracted from THP-1 MDM to the AP-1 sequence of the LOX-1 gene promoter.....	188
Figure.8. Effect of high glucose on oxLDL uptake by human MDM. Role for LOX-1.....	190

Third article:

Figure.1. Time- and dose-dependent effect of CRP on LOX-1 mRNA levels in HAECs.....	216
Figure.2. Time- and dose-dependent effect of CRP on endothelial LOX-1 protein expression.....	217
Figure.3. Dose-dependent effect of IL-6 on endothelial LOX-1 protein expression.....	219
Figure.4. Effect of CRP on human monocyte adhesion to endothelial cells.....	220
Figure.5. Effect of CRP on oxLDL uptake by HAECs.....	221

LIST OF TABLES

Introduction :

Table 1. Favorable and atheroprotective effects of the healthy endothelium.....	2
Table 2. Novel risk factors for atherosclerotic vascular disease.....	14
Table 3. The effects of oxidized LDL and/or its products-oxidized phospholipids and/or oxysterols.....	21
Table 4. Classification of the types of diabetes.....	31
Table 5. Etiologic classification of diabetes mellitus.....	32
Table 6. Diabetic risk factors.....	36
Table 7. Genetic Classification of type 2 diabetes.....	41
Table 8. Criteria for the diagnosis of diabetes mellitus.....	83

LIST OF ABBREVIATIONS

ACE	: angiotensin converting enzyme
AGEs	: advanced glycation end products
AP-1	: activator protein-1
APP	: acute phase protein
AT1	: angiotensin II type 1
BAECs	: bovine aortic endothelial cells
BMI	: body mass index
CD40L	: CD40 ligand
CHD	: coronary heart disease
CML	: carboxyl methyl lysine
<i>C. pneumoniae</i>	: <i>Chlamydia pneumoniae</i>
CRD	: carbohydrate-recognition domain
CRP	: C-reactive protein
CS	: cystathionine synthase
CVD	: cardiovascular disease
DAG	: diacylglycerol
DCCT	: diabetes control and complications trial
DCs	: dendritic cells
DM	: diabetes mellitus
DPP	: diabetes prevention program
EBT	: electron beam computed tomography
eNOS	: endothelial NO synthase
ET-1	: endothelin-1
ESRD	: end-stage renal disease

FDPS : finish diabetes prevention study

FFAs : free fatty acids

FGF-2 : fibroblast growth factor -2

FPG : fasting plasma glucose

GDM : gestational diabetes

HAECs : human aortic endothelial cells

HCAECs : human coronary artery endothelial cells

HDL : high-density lipoprotein

HF : high fat

HNF : hepatocyte nuclear factor

H. pylori : *Helicobacter pylori*

ICAM-1 : intercellular adhesion molecule-1

IDDM : insulin-dependent diabetes mellitus

IFG : impaired fasting glucose

IGT : impaired glucose tolerance

IFN- γ : interferon- γ

IL-1 : interleukin-1

IL-6 : interleukin-6

IL-8 : interleukin-8

IL-12 : interleukin-12

IRS-1 : insulin receptor substrate-1

IRAS : insulin resistance atherosclerosis study

8-iso-PGF-2 α : 8-iso-prostaglandin F2 α

LDL : low-density lipoproteins

LFA-1	: lymphocyte function-related antigen-1
LOX-1	: lectin-like oxidized low-density lipoprotein receptor-1
MAPK	: mitogen-activated protein kinase
MCP-1	: monocyte chemotactic protein-1
M-CSF	: macrophage-colony stimulating factor
MODY	: maturity-onset diabetes of the young
MR-FIT	: multiple risk factor intervention trial
MTHFR	: methylenetetrahydrofolate reductase
NAC	: N-acetylcysteine
NDDG	: national diabetes data group
NIDDM	: non-insulin-dependent diabetes mellitus
NF- κ B	: nuclear factor-kappa B
NO	: nitric oxide
NOS	: nitric oxide synthase
OGTT	: oral glucose tolerance test
oxLDL	: oxidized low-density lipoprotein
PAI-1	: plasminogen activator inhibitor-1
PC	: phosphocholine
PDGF	: platelet-derived growth factor
PKC	: protein kinase C
PI	: phosphatidylinositol
PLC	: phospholipase C
PPAR	: peroxisome proliferator-activated receptors
PS	: phosphatidylserine
RAGE	: AGE receptor

RAS	: renin-angiotensin system
ROS	: reactive oxygen species
SMCs	: smooth muscle cells
SSA	: serum amyloid A
SSRE	: shear-stress responsive element
TF	: tissue factor
TFPI	: TF pathway inhibitor
TGF- β	: transforming growth factor β
TNF- α	: tumor necrosis factor α
TRE	: tetradecanoylphorbol 13-acetate-responsive element
TRIPOD	: troglitazone in prevention of diabetes
tRNAs	: transfer RNA
UKPDS	: united kingdom prospective diabetes study
VCAM-1	: vascular cell adhesion molecule-1
VDCs	: vascular DCs
VLDL	: very low-density lipoproteins
WHHL	: watanabe hereditary hyperlipidemic
WHO	: world health organization



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DEDICATION

I dedicate my work to my loving and caring parents, who have always been there to encourage and support me. You both taught me the importance of friendship, respect, and love, for all aspects of life. I dedicate my work to my husband and lovely daughter, whose love and strength have sustained my energy to complete this work. In addition, I dedicate this work to my brother, as well as my best friends, who through their goodness of nature have given me, as the song goes, "the wind beneath my wings."



I. INTRODUCTION



I. Introduction

I.I. Atherosclerosis

I.I.1. General

Atherosclerosis is a chronic, multifactorial and progressive disease characterized by the accumulation of lipids and fibrous elements in the large and medium arteries. Epidemiological studies over the past 50 years have identified numerous risk factors for atherosclerosis. These can be grouped into factors with an important genetic component and those that are largely environmental. Among the primary events in atherosclerosis are the accumulation of low-density lipoproteins (LDL) in the subendothelial space [Guyton et al. 1996, Van De Graff et al. 1992] and the adhesion of inflammatory cells, such as monocytes and T-lymphocytes, to the endothelium. The notion that atherosclerosis is an immune-mediated inflammatory disease is now widely accepted and provides the basis for the development of new strategies in the diagnosis and management of this disease.

I.I.2. Development and progression of the atherosclerotic lesion

I.I.2.1. Endothelial dysfunction in atherosclerosis

I.I.2.1.1. Endothelial function

The vascular endothelium is considered to be a monolayer acting as a selectively permeable barrier between blood and tissues. It is a dynamic and heterogeneous organ. It has also become evident that the vascular endothelium

is an active paracrine, endocrine, and autocrine organ that is indispensable for the regulation of vascular tone and maintenance of vascular homeostasis as shown in Table 1 [Bonetti et al. 2003].

Table 1. Favorable and Atheroprotective Effects of the Healthy Endothelium.

- Promotion of vasodilation
- Antioxidant effects
- Antiinflammatory effects
- Inhibition of leukocyte adhesion and migration
- Inhibition of smooth muscle cell proliferation and migration
- Inhibition of platelet aggregation and adhesion
- Anticoagulant effects
- Profibrinolytic effects

Endothelium can generate effector molecules, such as nitric oxide (NO), prostacyclin, platelet-derived growth factor (PDGF), angiotensin II, and endothelin [Ross 1999]. These molecules control and regulate thrombosis, inflammation, vascular tone, and vascular remodeling. Furthermore, the endothelium has important anti-coagulant and fibrinolytic functions.

I.I.2.1.2. Endothelial dysfunction

Endothelial dysfunction is characterized by an imbalance between the production of relaxing and contracting factors, anticoagulant and procoagulant mediators, or growth-inhibiting and promoting factors. Endothelial dysfunction is closely linked to the occurrence of vascular diseases such as hypertension,

diabetes, inflammation, and aging and precedes the development of atherosclerosis. It is characterized by a reduction of the bioavailability of vasodilators such as NO and an increased production of endothelium-derived contracting factors [Bonetti et al. 2003]. This imbalance leads to an impairment of endothelium-dependent vasodilation, which represents the functional characteristic of endothelial dysfunction. Aside from denoting the impaired endothelium-dependent vasodilation, endothelial dysfunction also comprises a specific state of endothelial activation, which is characterized by a proinflammatory, proliferative, and procoagulatory milieu that favors all stages of atherogenesis [Anderson. 1999]. This results in increased permeability of endothelium to macromolecules such as LDL and enhanced migration of inflammatory cells to the subintimal space through the effect of oxidized LDL (oxLDL), monocyte chemoattractant protein 1 (MCP-1), interleukin-8 (IL-8), PDGF, macrophage-colony stimulating factor (M-CSF), and osteopontin, leading to the formation of early atherosclerotic lesions or fatty streaks [Kita et al. 2001].

I.I.2.2. Fatty-streak lesion

As mentioned above, early atherosclerotic lesions, or fatty streaks are characterized by the accumulation of LDL and the migration of monocytes and lymphocytes into the subendothelial space of the arterial wall. Fatty streaks initially consist of lipid-laden monocyte-derived macrophages (foam cells) mixed together with variable numbers of T lymphocytes [Ross 1999]. The formation of foam cells is mediated mainly by M-CSF, tumor necrosis factor α (TNF- α), and

interleukin-1 (IL-1) [Rosenfeld et al. 1990, Hamilton et al. 1999]. Later, these cells are joined by various numbers of smooth muscle cells (SMCs). SMC migration is stimulated by PDGF, fibroblast growth factor-2 (FGF-2) and transforming growth factor β (TGF- β) [Boring et al. 1997, Boisvert et al. 1998]. Platelet adherence and aggregation are also observed and are stimulated by integrins, P-selectin, fibrin, thromboxane A₂, and tissue factor (TF) [Ross 1999].

I.I.2.3. Advanced lesion of atherosclerosis

As fatty streaks progress to intermediate and advanced lesions, they tend to form a fibrous cap that walls off the lesion from the lumen. This represents a type of healing or fibrous response to the injury [Ross 1999]. Advanced atherosclerotic lesions result from a variety of pathogenetic processes, including macrophage foam cell formation and death, accumulation of extracellular lipid, reduction of structural intercellular matrix and SMCs, generation of mineral deposits, chronic inflammation, and neovascularization [Stary 2000]. Coronary artery calcification is rather a marker for atherosclerotic lesion. Ectopic vascular calcification occurs commonly in atherosclerosis. Its presence and location in coronary arteries have been documented by ultrafast computed tomography scanning [Fallavallita et al. 1994, Janowitz 2001]. Electron beam computed tomography (EBT) can also rapidly and noninvasively detect and quantify calcified atherosclerotic plaque in the coronary arteries [O'Rourke et al. 2000]. A higher quantity of coronary artery calcium is associated with likelihood of obstructive lesions [O'Rourke et al. 2000], and with an increased risk of future

cardiovascular disease (CVD) [Arad et al. 2000]. The recent finding that TNF- α promotes, through the cyclic adenosine monophosphate (cAMP) pathway, osteoblastic differentiation of vascular cells [Tintut et al. 2000] suggests a role of pro-inflammatory cytokines in the vascular calcification process.

I.I.2.4. Unstable fibrous plaques in atherosclerosis

I.I.2.4.1. Local factors

The influx of inflammatory cells, macrophages, and T lymphocytes in atherosclerotic plaques increases with plaque progression and these cells preferentially accumulate at sites of plaque rupture [Ross 1999, Hosono et al. 2003]. Inflammatory mediators and proteases released by these cells and expressed in human atherosclerotic lesions contribute to plaque rupture. For example, it is well known that macrophages release metalloproteinases and other proteolytic enzymes at sites of thinning of the fibrous cap. These enzymes cause degradation of the matrix, which leads to hemorrhage from the vasa vasorum or from the arterial lumen, resulting in thrombus formation and arterial occlusion [Ross 1999]. Apoptosis of vascular cells such as SMCs and macrophages are associated with plaque instability as well [Von der Thusen et al. 2002, Kockx et al. 2000, Geng et al. 1995, Libby et al. 1996]. Local modulators of plaque rupture also include factors involved in the coagulation cascade [Khrenov et al. 2002]. For example, both TF and TF pathway inhibitor (TFPI) are expressed in advanced human atherosclerotic lesions and are abundant at sites of plaque rupture [Kaikita et al. 1999]. Studies also

demonstrate that plasminogen activator inhibitor (PAI) and prothrombin are increased in advanced human plaques [Falkenberg et al. 1996, Smith et al. 1981]. Recent studies on gene profiling have identified a differential expression of various genes between stable and ruptured human atherosclerotic plaques including perilipin and cathepsin K. In addition to suggest an important role for these genes in plaque stability, this study also supports a role for many other unknown genes such as vasculin [Faber et al. 2001, Bijnens et al. 2003, Lutgens et al. 2003].

I.I.2.4.2. Systemic factors

It has been stated that plaque rupture does not occur as an isolated phenomenon but rather as a systemic disease [Lutgens et al. 2003]. Rupture of the fibrous cap leads to the exposure of the thrombogenic parts of the atherosclerotic plaque with subsequent activation of the coagulation cascade and platelet aggregation. This leads to thrombosis and acute ischemia resulting from abrupt luminal compromise. Histomorphological features of vulnerable plaques include a) positive remodeling producing less luminal stenosis; b) large lipid core ($\geq 40\%$ plaque volume); c) large number of inflammatory cells; d) a thin cap depleted of SMCs and collagen and finally increased neovascularity. Recently, the term "cardiovascular vulnerable patient" has been proposed to define subjects susceptible to an acute coronary syndrome or sudden cardiac death based on plaque, blood, or myocardial vulnerability [Naghavi et al. 2003]. Vulnerable patients often present with multiple ruptured plaques. Systemic

factors that are correlated with plaque rupture are altered blood rheology, increased coagulability, increased systemic inflammation, and recurrent infections. These unfavorable systemic changes often interact synergistically with risk factors of atherosclerosis, such as hyperlipidemia, smoking, and diabetes [Fuster et al. 1999, Corti et al. 2003, Sambola et al. 2003].

I.I.3. Risk factors

I.I.3.1. Traditional risk factors

I.I.3.1.1. Hypercholesterolemia and dyslipoproteinemia

High plasma levels of cholesterol and of pro-atherogenic lipoproteins represent major risk factors for cardiovascular diseases. In particular, increased levels of circulating LDL is considered as a major risk factor of coronary heart disease and one of the earliest events in atherogenesis is the accumulation of LDL in the vessel wall. Retention and modification of LDL are key factors in atherosclerotic lesion formation. LDL can be modified by several mechanisms, including oxidation, glycation, aggregation, association with proteoglycans, or incorporation into immune complexes [Steinberg et al. 1997, Khoo et al. 1998, Khoo et al. 1992, Navab et al. 1996]. Progressive oxidation of LDL takes place during the process of LDL particle trapping in arteries. OxLDL is internalized by macrophages through surface scavenger receptors [Steinberg et al. 1997, Khoo et al. 1992, Navab et al. 1996, Griendling et al. 1997, Han et al. 1997]. The uptake of oxLDL by macrophages results in intracellular cholesterol ester accumulation and foam cell formation. Macrophage-derived foam cells are

present in all stages of atherogenesis and play a key role in the development and progression of atherosclerosis. Lipoprotein (a) (Lp(a)) closely resembles LDL in its content of cholesterol and apolipoprotein B-100 but differs by the presence of an attached glycoprotein, known as apoprotein (a). The involvement of Lp(a) in the pathogenesis of atherosclerosis is strongly suggested by the presence of Lp(a) in the human atherosclerotic lesions [Rath et al. 1989, Jurgen et al. 1993]. Numerous cross-sectional and prospective studies have also revealed associations between high plasma levels of Lp(a) and atherosclerotic vascular diseases, such as coronary heart disease and stroke [Utermann 1995, Djurovic et al. 1997]. The effect of high plasma levels of Lp(a) in the development of atherosclerosis has also been well-studied in transgenic animal models [Lawn et al. 1992, Fan et al. 2001]. Although the importance of high LDL cholesterol as a risk factor for coronary artery disease is well established, the role of hypertriglyceridemia as an independent risk factor for the development of vascular diseases is still controversial. Various factors associated with hypertriglyceridemia, including dyslipoproteinemia, alterations of hemostatic processes, obesity and hypertension may account for the deleterious effect of high triglyceride levels on the atherosclerotic process [Malaguarnera et al. 2000]. Although, the meta-analysis of Austin and Hokanson, 1998 has recently suggested that hypertriglyceridemia is an independent risk factor for cardiovascular diseases, many investigators still believe that triglyceride-rich lipoproteins cannot migrate through the vascular endothelium [Faegerman, 1998]. Recently it has been suggested that postprandial hypertriglyceridemia

may cause endothelial dysfunction via enhanced oxidative stress [Bae et al. 2001]. In addition, a low level of HDL-cholesterol as an important risk factor for cardiovascular disease has been well established through epidemiological and clinical studies [Gordon et al. 1989]. A growing body of evidence suggests that HDL exerts part of its antiatherogenic effect by counteracting LDL oxidation [Assmann et al. 2004].

I.I.3.1.2. Hypertension

I.I.3.1.2.1. Renin-angiotensin system (RAS)

The RAS plays an important role in the pathogenesis of cardiovascular disease. Angiotensin II, the principal product of the RAS, increases blood pressure through increasing vascular resistance, stimulating aldosterone synthesis and release, increasing renal tubular sodium reabsorption, augmenting the release of antidiuretic hormone, and enhancing sympathetic outflow from the brain. Notably, angiotensin II induces cardiac and vascular cell hypertrophy and hyperplasia directly by activating the angiotensin II type 1 (AT1) receptor and indirectly by stimulating the release of several growth factors and cytokines [McConnaughey et al. 1999]. The mechanisms through which angiotensin II increases blood pressure include binding of angiotensin to specific receptors on SMC, eliciting the activation of phospholipase C (PLC) and leading to increased intracellular calcium concentrations and SMC hypertrophy [Gibbons et al. 1992]. Angiotensin II also decreases NO production, increases oxidative stress [Yanagitani et al. 1999] and induces the activation of nuclear factor- κ B (NF- κ B)

[Kranzhöfer et al. 1999], protein kinase C (PKC) [Henrion et al. 1996] and mitogen activated protein kinases (MAPK) [Haendeler and Berk, 2000].

I.I.3.1.2.2. Endothelin

Endothelin is a potent vasoactive peptide, produced by endothelial cells. Endothelin is secreted in an abluminal direction by endothelial cells and acts in a paracrine fashion on underlying SMCs to cause vasoconstriction and elevate blood pressure [Oparil et al. 2003]. Circulating endothelin levels are increased in some hypertensive patients, particularly African Americans and persons with transplant hypertension, endothelial tumors, and vasculitis [Ergul et al. 1996]. Da Silva et al. [2004] recently reported that endothelin-1 (ET-1) contributes to increased arterial pressure in a model of visceral obesity produced by feeding Sprague-Dawley rats with a high-fat (HF) diet.

I.I.3.1.3. Diabetes

Patients with diabetes are at high risk of cardiovascular diseases [Resnick et al. 2002]. Several risk factors associated with diabetes including dyslipidemia, obesity, hypertension, hyperglycemia and hyperinsulinemia contribute to the accelerated atherosclerosis in human diabetes. (See section I.II. for details)

I.I.3.1.4. Family history, age and gender

It is clear that positive family history, advanced age and male gender increase the risk of developing coronary artery disease [Saniko et al. 1990].

Numerous studies have shown that individuals with a family history of coronary heart disease (CHD) are at higher risk of atherosclerosis. For example, Pankow et al. [1997] and Li et al. [2000] investigated family CHD risk scores and found higher traditional CHD risk factors in individuals with a family history of heart disease.

Because of protection by female hormones, the onset of CHD tends to be delayed in women by about 10 years with some catch-up after menopause [Matthews et al. 1989].

I.I.3.1.5. Obesity and smoking habit

Obesity, especially visceral obesity, is associated with a cluster of metabolic complications increasing the risk of type 2 diabetes and CHD. For example, obese patients with visceral obesity show increased glycemic and insulinemic responses to an oral glucose load compared to normal weight individuals or compared to obese individuals with low visceral adiposity [Kothari et al. 1998]. Viscerally obese patients are also characterized by an unfavorable plasma lipid profile which includes elevated triglyceride and apolipoprotein B concentrations, reduced high density lipoproteins (HDL)-cholesterol levels as well as increased proportion of small, dense LDL particles [Despres et al. 2000].

Despite the epidemiological evidence linking cigarette smoking with cardiovascular disease, the precise components of cigarette smoke responsible for this relationship and the mechanisms by which they exert their deleterious effects have not yet been fully elucidated. Cigarette smoke is a complex mixture

and only a few components have been extensively studied. Nicotine and carbon monoxide are much less damaging than is whole smoke. There is considerable evidence that cigarette smoking can result in both morphological and biochemical endothelial disturbances both *in vivo* and *in vitro* [Michael Pittilo 2000].

I.I.3.2. New risk factors

In recent years, a number of new candidate risk factors or markers have been proposed as significant predictors of atherosclerosis and its complications. These risk factors and markers are summarized in Table 2 [Hackam et al. 2003]. Among these factors, four of them will be discussed emphatically due to their unique advantages. 1). There is substantial evidence on their predictive abilities. 2). A genetic basis for premature disease involves these factors. 3). Modifying treatments of these factors are available. 4). These factors are the subject of ongoing or completed clinical trials. These factors are C-reactive protein (CRP), Lp (a), fibrinogen, homocysteine, and infection.

I.I.3.2.1. CRP

CRP is a circulating acute-phase reactant that is increased many-fold during the inflammatory response to tissue injury or infection [Du Clos. 2000]. This protein has received substantial attention in recent years as a promising biological predictor of atherosclerotic disease [Pearson et al. 2003]. Studies have provided evidence that CRP is not only a risk marker for atherosclerosis but also promotes atherosclerosis. (See section I.VI. for details).

I.I.3.2.2. Lp(a)

Lp(a) is an LDL-like particle in which an apolipoprotein (a) moiety is linked via a disulfide bond to apoB-100 of LDL [Milionis et al. 2000]. Concentrations of Lp (a) are largely under genetic control and vary substantially between individuals depending on the size of the apo (a) isoform present. On the other hand, Lp(a) levels change little with diet or exercise, unlike other lipoproteins such as LDL and HDL [Kraft et al. 1996]. Cross-sectional and many prospective studies have revealed the association between high plasma levels of Lp(a) and atherosclerotic vascular diseases [Djurovic et al. 1997, Danesh et al. 2000]. These epidemiological evidence largely support the hypothesis that Lp (a) is a highly atherothrombotic lipoprotein. Lp(a) is an acute-phase reactant [Min et al. 1997], more than doubling in concentration in response to the proinflammatory cytokine interleukin 6 (IL-6) [Craig et al. 1992]. The mechanisms through which Lp(a) triggers atherosclerosis have been identified. Lp(a) binds avidly to endothelial cells, macrophages, fibroblasts, and platelets, as well as to the subendothelial matrix [Pillarsetti et al. 1997, Poon et al. 1997]. After binding, it promotes proliferation of vascular SMCs and chemotaxis of human monocytes [Pillarsetti et al. 1997, Grainger et al. 1994, Poon et al. 1997]. However, its most important putative role in atherothrombosis is to inhibit clot fibrinolysis at sites of tissue injury. As Lp(a) has unique structural homology to plasminogen, it is thought to compete with plasminogen for binding to plasminogen receptors, fibrinogen, and fibrin [Loscalzo 1990]. Lp(a) may also induce the production of PAI-1 and may inhibit the secretion of tissue-plasminogen activator by

endothelial cells [Li et al. 1997, Levin et al. 1994]. Thus, Lp(a) is considered as an emerging cardiovascular risk factor due to its properties as an atherogenic and prothrombotic molecule [Lippi et al [2003].

Table 2. Novel risk factors for atherosclerotic vascular disease.

Inflammatory Markers

- CRP
- Interleukins (e.g. IL-6)
- Serum amyloid A
- Vascular and cellular adhesion molecules
- Soluble CD40 ligand
- Leukocyte count

Homeostasis/Thrombosis Markers

- Fibrinogen
- Von Willebrand factor antigen
- PAI-1
- Tissue-plasminogen activator
- Factors V, VII
- D-dimer
- Fibrinopeptide A
- Prothrombin fragment 1+2

Platelet-Related Factors

- Platelet aggregation
- Platelet activity
- Platelet size and volume

Lipid-Related Factors

- Small dense LDL
- Lp (a)
- Remnant lipoproteins
- Apolipoprotein A1 and B
- HDL subtypes
- OxLDL

Other Factors

- Homocysteine
- Lipoprotein-associated phospholipase A (2)
- Microalbuminuria
- Insulin resistance
- PAI-1 genotype
- Angiotensin-converting enzyme genotype
- ApoE genotype
- Infectious agents: Cytomegalovirus, *Chlamydia pneumonia*, *Helicobacter Pylori*, Herpes simplex virus
- Psychosocial factors

I.I.3.2.3. Fibrinogen

Fibrinogen is a circulating glycoprotein, which acts at the final step in the coagulation pathway [Herrick et al.1999]. Epidemiological data support an independent association between elevated levels of fibrinogen and cardiovascular morbidity [Danesh et al. 1998, Maresca et al. 1999, Orem et al. 2003]. A recent study reports the effect of genetic variant of platelet fibrinogen receptor on the risk of cardiovascular event associated with elevated fibrinogen plasma levels [Boekholdt et al. 2004]. The potential pathophysiological mechanisms by which elevated fibrinogen levels mediate cardiovascular risk include the following aspects [Koenig et al. 2003]. 1). It forms the substrate for thrombin and represents the final step in the coagulation cascade. 2). It is essential for platelet aggregation and modulates endothelial function. 3). It promotes SMC proliferation and migration. 4). It interacts with the binding of plasmin with its receptor. 5). It represents a major acute phase protein.

I.I.3.2.4. Homocysteine

Homocysteine is a non-protein-forming sulfhydryl amino acid derived from the methionine metabolism [Mangoni et al. 2002]. Cells remethylate homocysteine by a number of possible pathways involving several different enzymes. These enzymes variously use B vitamins as substrates or cofactors, namely folate, cobalamin, and pyridoxine [De Bree et al. 2002]. Hyperhomocysteinemia is considered to be a novel risk factor for coronary, cerebral, and peripheral atherosclerotic disease. A common gene mutation

encoding one of the enzymes that metabolizes homocysteine, namely 5, 10-methylenetetrahydrofolate reductase [MTHFR] leads to moderate increase in homocysteine levels, particularly in the presence of low folate intake [Frosst et al. 1995]. Hyperhomocysteinemia seen in general adult populations may be associated with cystathionine synthase (CS) and MTHFR deficiencies [McCully 1996, Nehler et al. 1997, Nyard et al. 1997, Malinow 1995]. Mild homocysteine increase is observed in 20-30% of patients with atherosclerotic disease. Treatment with folic acid returns plasma homocysteine concentrations to normal [Yarnell 1991]. There is also considerable epidemiological evidence that the homocysteine concentration is a risk factor for venous thrombosis [den Heijer et al. 1998, Ray 1998].

Several mechanisms have been proposed to explain the atherogenic properties of homocysteine. They include endothelial dysfunction, oxidative damage, SMC proliferation, activation of the PKC/c-fos signaling pathway, platelet aggregation, and activation of the coagulation pathway [Dalton et al. 1997, Welch et al. 1997, Durand et al. 1997, Mangoni et al. 2002, De Bree et al. 2002, Werstuck et al. 2001].

I.I.3.2.5. Infection

There is increasing evidence that infectious pathogens, such as *Helicobacter pylori*, cytomegalovirus, and *Chlamydia pneumoniae*, can promote the atherosclerotic cascade. Proposed mechanisms include macrophage transformation, endothelial injury, chronic inflammation, and thrombosis.

Herpes viruses and *Chlamydia pneumoniae* are two main types of infectious microorganisms that have been shown to correlate with the incidence of atherosclerosis [Leinonen et al. 2002]. Both organisms have been found in coronary atheromatous lesions [Hendrix et al. 1990, Jackson et al. 1997]. A possible role of hepatitis A in the pathogenesis of atherosclerosis has also been proposed [Zhu et al. 2000]. The role of infectious agents in the pathogenesis of atherosclerosis will further be discussed in section I.I.4.4..

I.I.4. Pathogenesis of atherosclerosis

Atherosclerosis has been recognized for over a century, and the understanding of its pathogenesis has undergone many changes. We discuss some of the hypothesis associated with the development of atherosclerosis as follows.

I.I.4.1. Endothelial dysfunction hypothesis

The endothelium retains a reduced vasomotor tone, prevents leukocyte and platelet adhesion, and inhibits the proliferation of vascular SMCs under physiological conditions. Conversely, endothelial dysfunction refers to several pathological conditions, including altered anticoagulant and anti-inflammatory properties of the endothelium, impaired modulation of vascular growth, and dysregulation of vascular remodeling. Endothelial dysfunction plays a pathogenic role in the initial development of atherosclerosis [Ross 1999, Poredos 2002].

Abundant evidence has proven that endothelial dysfunction also precedes the development of atherosclerotic lesions [Celermajer 1997, Poredos 2001].

I.I.4.1.1. Risk factors for endothelial dysfunction

Endothelial dysfunction has been demonstrated in subjects with different risk factors of atherosclerosis, including hypercholesterolemia, diabetes, hypertension, smoking, obesity, and others [Celermajer et al. 1992, Libby et al. 2002]. Treatment of these risk factors results in improvement of endothelial dysfunction. Over the past few years, it has been documented that chronic inflammation is a key pathogenic factor in endothelial dysfunction [Verma et al. 2003]. Inflammatory activity and endothelial dysfunction are thought to be strongly interrelated [Ross 1999]. Inflammatory cytokines such as TNF α and IL-6 can cause endothelial dysfunction either directly or indirectly [Ross 1999, Stehouwer et al. 1997, Yudkin et al. 1999]. CRP is induced by cytokines such as IL-6 [Xing et al. 1998] under inflammatory conditions and has been shown to induce endothelial dysfunction [Verma et al. 2003]. For example, CRP potently downregulates eNOS transcription in endothelial cells and destabilized eNOS mRNA, with resultant decrease in both basal and stimulated NO release [Verma et al. 2002]. Induction of adhesion molecule expression has also been documented in CRP-treated endothelial cells *in vitro*. Importantly, investigators have shown a direct correlation between endothelial dysfunction and plasma levels of CRP has been demonstrated [Fichtlscherer et al. 2000a, Fichtlscherer et al. 2000b].

I.I.4.1.2. Mechanisms of endothelial dysfunction in atherosclerosis

Endothelial dysfunction implies diminished production or availability of NO. NO is generated by the conversion of the amino acid L-arginine to NO and L-citrulline by the enzyme NO synthase (NOS). It is the key endothelium-derived relaxing factor and plays a pivotal role in the regulation of vascular tone and vasomotor function [Albrecht et al. 2003]. Diminished NO production leads to decreased endothelium-dependent vasodilation, increased platelet aggregation and adhesion of monocytes to the endothelium, and enhanced vascular SMC proliferation. Reduction of NO production/bioactivity mediates oxLDL-induced endothelial dysfunction/activation [Cominacini et al. 2001]. NO released from the endothelium is decreased in patients with coronary atherosclerosis [Ludmer et al. 1986, Vita et al. 1990]. Endothelial dysfunction is elicited from imbalance between the release of endothelium-derived relaxing and contracting factors, such as ET-1 and angiotensin [Verma et al. 2002]. High plasma ET-1 concentrations have been reported in myocardial infarction, cardiogenic shock, unstable angina pectoris, coronary artery disease in general, cardiac failure, and essential hypertension [Poch et al. 1995, Agapitov et al. 2002]. Endothelium dysfunction/activation is associated with increased expression of endothelial cell adhesion molecules including selectins, vascular cell adhesion molecule (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1), which promote adherence of monocytes to endothelium. This process can be triggered by proinflammatory cytokines, CRP, oxLDL, and CD40/CD40 ligand (CD40L and CD154) interaction [Collins et al. 2001, Chen et al. 2002, Verma et al. 2002, Kaplanski et al. 1998,

Schonbeck et al. 2001]. Endothelial dysfunction is associated with major risk factors of atherosclerosis including hyperlipidemia, hypertension, smoking, diabetes, and aging.

I.I.4.2. Lipid infiltration hypothesis

In the early 19th century, von Rokitansky [1852] found that the main characteristic feature of atherosclerosis is cholesterol accumulation in the intima. Much later, the lipid infiltration hypothesis was supported by the studies of Deng et al. [1993] demonstrating the presence of atherosclerotic lesions in rabbits fed a cholesterol-enriched diet. The lipid infiltration hypothesis postulates that high plasma LDL levels associated with several risk factors including inappropriate diet, obesity, physical inactivity, cigarette smoking and low estrogen levels, results in the penetration of LDL into the arterial wall. This leads to subendothelial lipid accumulation, which attracts macrophages and leads to their conversion to foam cells. LDL also causes migration and multiplication of SMCs in the subendothelial region. This hypothesis has been reinforced over the past years by clinical studies showing a direct correlation between plasma cholesterol levels and CHD and also demonstrating a reduction of cardiovascular events by normalization of plasma lipid levels [Ito et al. 2001, Koizumi et al. 2002, Sasaki et al. 2003].

The lipid infiltration theory alone does not explain everything about the atherosclerotic process. Indeed, high cholesterol levels and inflammation appear to be two essential components in the pathogenesis of atherosclerosis and both

components seem to play a key role in the formation of the atherosclerotic lesion. In fact, both processes interact, with oxLDL and/or its products having proinflammatory activities [Witztum et al. 2001] (See Table 3).

Table 3. The effects of oxidized LDL and/or its products-oxidized phospholipids and/or oxysterols.

Mimic biological effects of platelet-activating factor.
Induce monocyte binding to endothelial cells.
Increase tissue factor activity in endothelial cells (minimally modified-LDL).
Increase expression of M-CSF, monocyte chemoattractant protein-1.
Increase expression of VCAM-1 (LPC and 13-HOO stearate).
Induce apoptosis.
Inhibit nitric oxide release or function.
Induce expression of interleukin-1 and interleukin-8.
Increase collagen synthesis in smooth muscle cells.
Increase intracellular calcium.
Inhibit lipopolysaccharide-induced expression of NF κ B.

I.I.4.3. Oxidative stress hypothesis

This hypothesis proposes that many pathogenic mechanisms in atherogenesis may result from increased oxidative stress. Oxidative stress is a state in which excess reactive oxygen species (ROS) overwhelm endogenous antioxidant system. ROS are reactive chemical entities that can be classified into two categories: free radicals and non-radical derivatives [Kukreja et al. 1994]. Free radicals are more reactive than the corresponding non-radicals. While ROS in low concentrations serve as signaling molecules, the excessive induction of ROS elicits harmful effects.

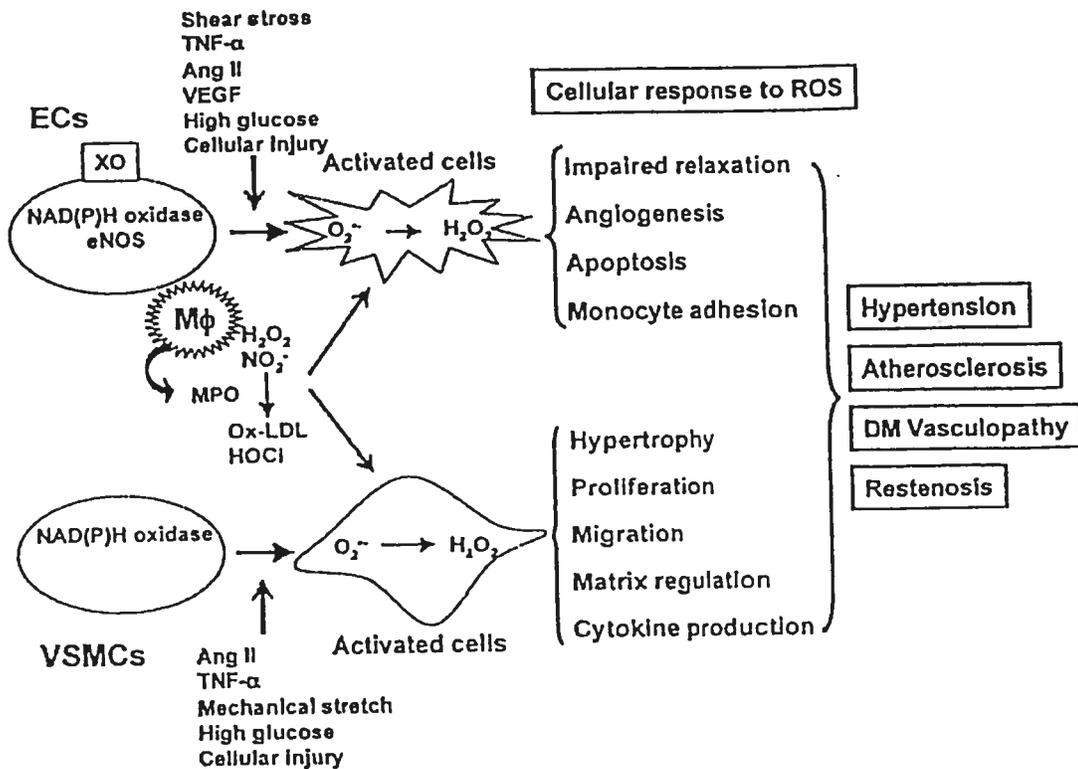


Figure 1. Modulation of cellular function by ROS in cardiovascular diseases. [Taniyama et al. 2003]

As shown in figure 1, ROS play major roles in the initiation and progression of cardiovascular dysfunction associated with diseases such as hyperlipidemia, diabetes mellitus (DM), hypertension, ischemic heart disease, and chronic heart failure. ROS affect many functions of the endothelium. The most well known is endothelium-dependent vasorelaxation, which is impaired by a loss of NO bioactivity in the vessel wall [Mugge et al. 1991]. ROS also cause endothelial apoptosis [Dimmeler et al. 2000, Li et al. 1999], increase monocyte adhesion [Marui et al. 1993, Khan et al. 1996, Chappell et al. 1998], and play a role in angiogenesis [Maulik et al. 2002, Kuroki et al. 1996, Arbiser et al. 2002]. ROS can cause vascular SMCs growth [Rao et al. 1992, Sundaresan et al. 1995, Patterson et al. 1999] and migration [Weber et al. 2002], as well as induce the

expression of matrix components [Rajagopalan et al. 1996] and inflammatory mediators such as NF κ B [Li et al. 2002].

Several studies have demonstrated the ability of various antioxidant compounds to prevent atherogenesis in animals. For example, it has been shown that probucol inhibited the formation of atherosclerotic lesions independently of its cholesterol-lowering properties in Watanabe hereditary hyperlipidemic (WHHL) rabbits [Carew et al. 1987]. This agent also reduces the formation of atherosclerotic lesions in cholesterol-fed monkeys [Sasahara et al. 1994]. Moreover, vitamin E has been shown to reduce atherosclerosis both in WHHL rabbits [Williams et al. 1992] and cholesterol-fed hamsters [Parker et al. 1995]. While these results suggest that antioxidants may protect against atherosclerosis, human studies are less convincing. Although in epidemiological studies a higher intake of antioxidant vitamins or a higher plasma concentrations of vitamin E seem to protect patients to some extent [Riemersma et al. 1989], clinical intervention studies in patients at high risk of cardiovascular events have been inconclusive [Steinberg et al. 2002]. This has led to a general consensus that antioxidant supplements are of no value in the prevention of CVD in subjects at high risk. For examples, several large-scale, double-blind, placebo-controlled trials have shown convincingly that neither β -carotene [Omenn et al. 1996, Hennekens et al. 1996, Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study Group 1994] nor vitamin E alone in the Heart Outcomes Prevention Evaluation (HOPE) study and the Heart Protection Study (HPS) [Yusuf et al. 2000, Heart Protection Study Collaborative Group 2002] or in combination with

other antioxidant vitamins [Brown et al. 2001], reduces the risk of fatal or nonfatal infarction in an unselected population of people with established CHD or at high risk of CHD.

I.I.4.4. Hypothesis of infection

There is reasonable evidence suggesting that infection may contribute to the pathogenesis of atherosclerosis. Several studies have addressed the possible role of infectious agents in the development of atherosclerosis. One interesting candidate is *Chlamydia pneumoniae* (*C.pneumoniae*), a human respiratory pathogen, which has been linked in both sero-epidemiological and immunohistochemical studies with CHD [Saikku et al, 1998, Elkind et al, 2000, Schmidt et al, 2000]. Results of animal experiments and preliminary intervention trials with antibiotics [Gurfinkel et al 1999] further suggest a role of this pathogen in atherogenesis. A second infectious agent is *Helicobacter pylori* (*H. pylori*), a pathogen involved in peptic ulcer [Danesh et al. 1997]. *H. pylori* DNA is found in atherosclerotic plaques, but is absent in healthy vascular walls [Farsak et al. 2000].

I.I.4.5. Hypothesis of inflammation

Several studies have firmly established that immune mechanisms play a key role in the pathogenesis of atherosclerosis. In fact, recent advances in basic science have demonstrated a fundamental role for inflammation in mediating all stages of atherosclerosis from initiation through progression, and ultimately

thrombotic complications. Whilst the inflammatory response and cell-mediated immunity may be initially protective in atherogenesis, persistent and excessive inflammation will favor the progression of the disease.

Signs of inflammation accompany the earliest accumulation of lipid within the arterial wall. Blood leukocytes adhere poorly to the normal endothelium. When the endothelial monolayer becomes inflamed, it expresses adhesion molecules that bind cognate ligands on circulating leukocytes. Once adherent to the endothelium, the leukocytes contribute to the local inflammatory response and simultaneously penetrate into the intima. MCP-1 is responsible for transmigration of monocytes into the intima and a family of T-cell chemoattractants is in charge for transmigration of lymphocytes. M-CSF is most essential for monocyte/macrophage differentiation in the atherosclerotic lesions. Monocyte-derived macrophages are scavenging and antigen-presenting cells. They secrete cytokines, chemokines, growth-regulatory molecules, metalloproteinases and other hydrolytic enzymes. These secretory products may play a critical role in converting fibrous plaque from stable to unstable ones. Proinflammatory cytokines such as IL-1, IL-6, IL-8, interleukin-10 (IL-10), interleukin-12 (IL-12), and TNF α produced by macrophages in the arterial wall provide chemotactic stimuli to adherent leukocytes directing their migration into the intima. Activated macrophages express class II histocompatibility antigens such as HLA-DR that allow them to present antigens to T lymphocytes [Raines et al. 1996]. Both CD4 and CD8 T cells are present in the lesions at all stages of the atherosclerotic process, particularly CD4 subtype [Zhou et al. 1996, Hansson et

al. 1989]. T cells are activated when they bind antigen processed and presented by antigen presenting cells including SMCs [Hansson et al. 1989], dendritic cells (DCs), and macrophages. Activated T cells elaborate inflammatory cytokines such as $TNF\alpha$ and interferon- γ (IFN- γ). IFN- γ is the predominant cytokine produced by intraplaque T cells. It activates macrophages and induces inflammatory responses. Several in vitro and in vivo data indicate that IFN- γ is proatherogenic. Among these, it has been shown that IFN- γ downregulates ABC1 a protein that regulates cholesterol efflux from macrophages [Wang et al. 2002], antagonizes the production of collagen [Billiau et al. 1998], which is widely believed to stabilize plaque structure and that blockage of IFN- γ results in an approximately 60% reduction in atherosclerosis [Gupta et al. 1997]. Thus, in human lesions, T cells may play a role in both lesion development and instability. OxLDL and heat-shock proteins are candidate antigens initiating adaptive immunity in atherosclerosis. A third proposed autoantigen is β 2-glycoprotein Ib. Some evidence also implicates microbial pathogens in atherogenesis and bacteria may induce innate immunity and autoimmunity.

DCs arise from a common CD34+ progenitor in the bone marrow and constitute a family of cells that are able to induce primary immune responses [Banchereau et al. 1998, Austyn 1998, Palucka et al. 1999]. DCs express high levels of both class I and class II MHC molecules and co-stimulatory molecules, and this thereby relates to their unique ability for activating naïve T cells [Banchereau et al. 1998, Austyn 1998, Palucka et al. 1999]. In the arterial wall, DCs are present in their immature forms and become activated during

atherogenesis [Bobryshev et al. 1995a, Bobryshev et al. 1995b, Bobryshev et al. 1998, Bobryshev et al. 1999]. For example, immunohistochemical and ultrastructural studies have shown that the number of DCs increased in atherosclerotic arteries [Bobryshev et al. 1995, Bobryshev et al. 1996a, Bobryshev et al. 1996b, Bobryshev et al. 1998]. Also, DCs have been identified in atherosclerotic lesions in rats with experimental hypercholesterolemia and in apo-E deficient mice [Ozmen et al. 2002, Bobryshev et al. 1999a, Bobryshev et al. 1999b]. Although vascular DCs become activated and differentiate at early stage of atherosclerosis, advanced atherosclerotic plaques are enriched either by adventitial vascular DCs invading directly [Bobryshev et al. 1998] or by blood DCs invading via inflamed neovessels [Bobryshev et al. 1998, Bobryshev et al. 1999]. Although the functional significance of DCs in atherosclerotic lesions requires clarification, it is reasonable to postulate that DCs in the arterial wall may be involved in antigen capture and antigen processing [Bobryshev et al. 1998]. Bobryshev et al [1998] observed in the atherosclerotic lesions. DCs frequently located in areas enriched with T cells, particularly within inflammatory infiltrates. DCs clustering with T cells display ICAM-1 and VCAM-1 [Bobryshev et al. 1998], which interact with LFA-1 and VLA-4, respectively and this co-localization is thought to be essential for T cell activation. It has also been found that inflammatory cytokines as well as heat-shock proteins [Springer 1994] are responsible for DC activation, while chemokines control DC migration [Banchereau et al. 1998, Lane et al. 1999, Palucka et al. 1999, Dieu et al. 1998,

Arnold-Schild et al. 1999, Todryk et al. 1999]. Taken together, DCs may play a crucial role in T cell activation in atherosclerosis.

Recent studies have provided evidence that mast cells are present in various stages of atherosclerotic lesions. Mast cells can assist in the recruitment of monocytes and lymphocytes into vascular tissues, thereby propagating the inflammatory response.

I.II. Diabetes mellitus (DM)

I.II.1. Definition

DM is a group of metabolic syndromes characterized by chronic hyperglycemia, the hallmark of the disease [Olefsky et al. 1992] and disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action or both. The effect of chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels. Symptoms of marked hyperglycemia include polyuria, polydipsia, weight loss, polyphagia, and blurred vision. In its most severe forms, ketoacidosis or a non-ketotic hyperosmolar state may develop and lead to stupor, coma and death in the absence of effective treatment.

I.II.2. Classification

The classification of DM was brought into order by the National Diabetes Data Group of the USA and the second World Health Organization (WHO) Expert

Committee on Diabetes Mellitus in 1979 and 1980. Apart from minor modifications by WHO in 1985, little has been changed since that time. This classification of DM is shown in Table 4. In light of the new knowledge regarding the etiology of different forms of diabetes, an etiologic classification of diabetes is also presented in Table 5.

I.II.2.1. Type 1 diabetes

Type 1 diabetes is a chronic disease that occurs when the pancreas produces too little insulin to regulate blood sugar levels appropriately. Type 1 diabetes have weak link with Class I antigens (B8 and B15), but have stronger link with Class II antigens (DR3 and DR4) and subtypes of DR4 antigens (Dw4, 10, and 14) [Braun 1992]. Approximately 95% of white patients with type 1 diabetes have either DR3 or DR4 antigens, and around 55 to 60% have both antigens. The HLA-DR3 and DR4 genes are believed to be the primary susceptibility genes for type I diabetes. The observation that heterozygosity for DR3/DR4 increases the risk for diabetes compared with homozygosity for other high-risk alleles suggests a polygenic mode of inheritance [Bertrams 1984]. Although the genetic contribution is important for developing type 1 diabetes, it is insufficient and requires an environmental factor to trigger its initiation.

Type 1 diabetes is classified as classic autoimmune (type 1A) and idiopathic (type 1B) diabetes [Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus 1997, Alberti et al. 1998]. Type 1A diabetes, results from a cellular-mediated autoimmune destruction of the beta

cells of the pancreas. It accounts for only 5-10% of this type of diabetes. It can become manifest with hyperglycemia presenting in the first days of life or in adults over the age of 60. Several evidences support that type 1 diabetes is an immune-mediated disease [Rewers et al. 1996, Wucherpfennig et al. 2001, Atkinson et al. 2001]: First, it is linked with class II HLA (D-region) antigens known to be associated with autoimmune disease. Second, it may occur with other forms of immune endocrinopathies and coupling of diabetes with immune endocrinopathy may cluster in families. Third, an early lesion in experimental and human type 1 diabetes consists of lymphocytic infiltration of the islets Langerhans (insulinitis or isletitis) resembling lymphocytic infiltrations in other autoimmune diseases. Fourth, antibodies directed against both cytoplasmic and cell-surface determinants on islet cells are present in many type 1 diabetic patients at diagnosis. Finally, immunotherapy prevents overt diabetes in experimental models of diabetes.

Markers of the immune destruction of the β -cell are detectable in type 1 diabetes. These antibodies include autoantibodies to islet cells, insulin, glutamic acid decarboxylase (GAD₆₅), and tyrosine phosphatases IA-2 and IA-2 β . Several of these autoantibodies are present in 85-90% of individuals with type 1 diabetes having impaired fasting hyperglycemia levels. In this form of diabetes, the rate of β -cell destruction is quite variable, being rapid in infants and children and slow in adults.

Table 4. Classification of the Types of Diabetes. [World Health Organization 1985]

Class name	Characteristics
Insulin-dependent diabetes mellitus (IDDM)	<p>Low or absent levels of circulating endogenous insulin and dependent on injected insulin to prevent ketosis and sustain life.</p> <p>Onset predominantly in youth but can occur at any age.</p> <p>Associated with certain HLA and GAD antigens.</p> <p>Abnormal immune response and islet cell antibodies are frequently present at diagnosis.</p> <p>Etiology probably only partially genetic, as only ~35% of monozygotic twins are concordant for IDDM.</p>
Non-insulin-dependent diabetes mellitus (NIDDM)	<p>Insulin levels may be normal, elevated, or depressed; hyperinsulinemia and insulin resistance characterize most patients; insulinopenia may develop as the disease progresses.</p> <p>Not insulin-dependent or ketosis-prone under normal circumstances, but may use insulin for treatment of hyperglycemia.</p> <p>Onset predominantly after the age of 40 years but can occur at any age.</p> <p>Approximately 50% of men and 70% of women are obese.</p> <p>Etiology probably strongly genetic as 60%~90% of monozygotic twins are concordant for NIDDM.</p>
Gestational diabetes (GDM)	<p>Glucose intolerance that has its onset or recognition during pregnancy.</p> <p>Associated with older age, obesity, family history of diabetes.</p> <p>Conveys increased risk for the woman for subsequent progression to NIDDM.</p> <p>Associated with increased risk of macrosomia.</p>
<p>Other types of diabetes, including diabetes secondary to or associated with:</p> <ul style="list-style-type: none"> Pancreatic disease Hormonal disease Drug or chemical exposure Insulin receptor abnormalities Certain genetic syndromes 	<p>In addition to the presence of the specific condition, hyperglycemia at a level diagnostic of diabetes is also present.</p> <p>Causes of hyperglycemia are known for some conditions, e.g., pancreatic disease; in other cases an etiologic relationship between diabetes and the other condition is suspected.</p>

Table 5. Etiologic classification of DM. [American Diabetes Association 2004]

I. Type 1 diabetes (β -cell destruction, usually leading to absolute insulin deficiency)

A. Immune-mediated

B. Idiopathic

II. Type 2 diabetes (may range from predominantly insulin resistance with relative insulin deficiency to a predominantly secretory defect with insulin resistance)

III. Other specific type

A. Genetic defects of (β -cell function)

1. Chromosome 12, HNF-1 α (MODY3)
2. Chromosome 7, glucokinase (MODY2)
3. Chromosome 20, HNF-4 α (MODY1)
4. Chromosome 13, insulin promoter factor-1 (IPF-1; MODY4)
5. Chromosome 17, HNF-1 β (MODY5)
6. Chromosome 2, *Neuro D1* (MODY6)
7. Mitochondrial DNA

8. Others

B. Genetic defects in insulin action

1. Type A insulin resistance
2. Leprechaunism
3. Rabson-Mendenhall syndrome
4. Lipotrophic diabetes
5. Others

C. Diseases of the exocrine pancreas

1. Pancreatitis
2. Trauma/pancreatectomy
3. Neoplasia
4. Cystic fibrosis
5. Hemochromatosis
6. Fibrocalculous pancreatopathy
7. Others

D. Endocrinopathies

1. Acromegaly
 2. Cushing's syndrome
 3. Glucagonoma
 4. Pheochromocytoma
 5. Hyperthyroidism
 6. Somatostatinoma
 7. Aldosteronoma
 8. Others
-

Table 5. Etiologic classification of DM (Continue).

E. Drug- or chemical-induced

1. Vacor
2. Pentamidine
3. Nicotinic acid
4. Glucocorticoids
5. Thyroid hormone
6. Diazoxide
7. β -adrenergic agonists
8. Thiazides
9. Dilantin
10. α -Interferon
11. Others

F. Infection

1. Congenital rubella
2. Cytomegalovirus
3. Others

G. Uncommon forms of immune-mediated diabetes

1. "Stiff-man" syndrome
2. Anti-insulin receptor antibodies
3. Others

H. Other genetic syndromes sometimes associated with diabetes

1. Down's syndrome
2. Klinefelter's syndrome
3. Turner's syndrome
4. Wolfram's syndrome
5. Friedreich's ataxia
6. Huntington's chorea
7. Laurence-Moon-Biedl syndrome
8. Myotonic dystrophy
9. Porphyria
10. Prader-Willi syndrome
11. Others

IV. GDM

Some patients, particularly children and adolescents, may present with ketoacidosis as the first manifestation of the disease. Others have modest fasting hyperglycemia that can rapidly change to severe hyperglycemia and/or

ketoacidosis in the presence of infection or other stress. Adults may retain residual β -cell function sufficient to prevent ketoacidosis for many years.

Unlike type 1A diabetes, type 1B is not triggered by an autoimmune response and is of unknown origin. These patients have permanent insulinopenia and are prone to ketoacidosis. This form of diabetes is strongly inherited, lacks immunological evidence for β -cell autoimmunity, and is not HLA associated. Individuals with this form of diabetes suffer from episodic ketoacidosis and exhibit varying degrees of insulin deficiency between episodes. Thus, some patients with type 1B require insulin replacement therapy [Banerji et al. 1989].

I.II.2.2. Type 2 diabetes

Type 2 diabetes is characterized by a state of resistance to insulin-mediated glucose disposal in muscle, liver, and adipose tissue associated with an absolute or relative deficiency in insulin secretion. It accounts for 90-95% of DM. Both genetic and environmental factors contribute to the development of type 2 diabetes. Obesity and insulin resistance are major risk factors for this form of diabetes [Haffner et al. 2003].

Type 2 diabetes is preceded by a long period of asymptomatic hyperglycemia and thus remains frequently undiagnosed for many years. Persistent metabolic derangement before the onset of frank diabetes is responsible for the high incidence of micro- and macro-vascular complications in patients with type 2 diabetes. Cardiovascular disease is responsible for 80% of

the mortality in patients with type 2 diabetes and importantly precedes overt diabetes in more than 50% of patients.

I.II.2.2.1. Prevalence

The number of diabetic patients is increasing worldwide. The WHO predicts that between 1997 and 2025, the number of diabetics will double from 150 to about 300 millions. The incidence has increased by about 33% over the last decade and is expected to increase further. However, the greater rise in the prevalence of type 2 diabetes is projected to occur in developing countries, particularly India and China.

I.II.2.2.2. Etiology

There is no single cause of type 2 diabetes. The development of this disease in high-risk subjects is caused by both polygenetic and environmental factors. Factors that predispose an individual to the development of type 2 diabetes are listed in Table 6 [American Diabetes Association. 2003]. Type 2 diabetes occurs primarily in individuals greater than 45 years of age [Harwell et al. 2001], however an increased prevalence of childhood obesity has promoted a marked increase of type 2 diabetes in adolescents and young adults [Pinhas-Hamiel et al. 1996]. Obesity is a major risk factor for type 2 diabetes [Chan et al. 1994, Colditz et al. 1995]. In fact, 80% of patients with type 2 diabetes are overweight.

Table 6. Diabetic risk factors.

Age > 45
Body mass index (BMI) $\geq 25\text{kg/m}^2$
A Family history of diabetes
Physical inactivity
Ethnicity
Impaired fasting glucose (IFG) or impaired glucose tolerance (IGT)
A History of gestational DM, or the delivery of a baby of >9 lb
Hypertension
Dyslipidemia
A History of vascular disease

Insulin resistance is also an essential determinant in the development of type 2 diabetes [Goldstein 2003]. Women with a history of GDM, and their children, have a relatively high risk of developing type 2 diabetes. Numerous epidemiological studies also indicate that a sedentary lifestyle is strongly and independently related to an increased incidence of type 2 diabetes. Several of these studies establish a dose-response relationship between physical activity and risk of diabetes [James et al. 1998, Astrup et al. 2001].

I.II.2.2.3. Genetic factors

There is considerable evidence that genes are important in the etiology of type 2 diabetes [McCarthy et al. 1994]. For example, it has been shown that 1) a history of type 2 diabetes in a first-degree relative doubles the risk of developing diabetes; b) identical twins of parents who have type 2 diabetes have 80-90% chance to develop diabetes [Kenny et al. 1995, Rewers et al. 1995]; c) parents and siblings of patients with type 2 diabetes, who share 50% of their genes, are 3-4 times more likely to have type 2 diabetes than subjects without diabetic

relatives [Kenny et al. 1995, Rewers et al. 1995]. Variation in prevalence between ethnic groups also provides evidence for the genetic component of type 2 diabetes. Indeed, migration studies show that genetic predisposition varies markedly between races that live in a similar environment [Kenny et al. 1995]. Detailed analysis of the challenges and progress towards defining genetic susceptibility to type 2 diabetes has been reviewed by Elbein et al [1994]. Current research suggests that only a small number of cases of type 2 diabetes are caused by a single-gene defect. These cases include maturity-onset DM of youth (mutated MODY gene), syndrome of insulin resistance (insulin receptor defect), and maternally inherited DM and deafness (mitochondrial gene defect) [So et al. 2000]. The genetic component of the more typical common form of type 2 DM is probably complex, involving the interactions of several genes and environmental factors [So et al. 2000]. It is now well accepted that the genetic component is subject to major influence from the environment, type 2 diabetes becoming manifest unless the environment permits for example excess dietary intake and a degree of obesity.

I.II.2.2.3.1 Gene candidates

Most of major advances in understanding the genetics of type 2 diabetes have been done in the rare subtypes caused by mutations of a single gene [Hattersley 1998]. Defining the genes that predispose patients to ordinary type 2 diabetes is considerably more difficult than defining causal genes in rare monogenic subgroups. Recent studies have shown that, unlike HLA genes in

type 1 diabetes, there is no single major predisposing type 2 diabetes gene. To date, more than 250 candidate genes have been investigated in type 2 diabetes but none has been found to play a major role in the common forms of type 2 diabetes. Genetic classification of type 2 diabetes is shown in Table 7 [Unger et al. 1998]. Recently, evidence has accumulated that a variant in the peroxisome proliferator-activated receptor (PPAR)- γ gene is protective for the development of type 2 diabetes [Altshuler et al. 2000]. Conversely, it has been reported that variations in the protease calpain 10 gene, is associated with susceptibility to type 2 diabetes in Mexican Americans [Horikawa et al. 2000].

I.II.2.2.3.2. Mutations in genes involved in insulin resistance

Insulin receptor gene: As shown in figure 2 [Kahn 1994], the insulin receptor consists of two α subunits and two β subunits that bind the insulin β - α - α - β heterotetramer. More than 40 mutations of the insulin receptor gene are known to cause insulin resistance. However, mutations in the structural sequence of the insulin receptor gene are not believed to cause insulin resistance in ordinary type 2 diabetes [Taylor et al. 1992].

Insulin receptor substrate-1 (IRS-1): IRS-1 is the primary substrate of the insulin receptor in most tissues (figure 2) and thus the IRS-1 gene is a candidate gene in type 2 diabetes. Although mutations in this gene have been identified in patients with type 2 diabetes, they are common in nondiabetic subjects as well [Stern 1995]. Genetic analysis of the IRS-1 gene has revealed several base-pair

changes that result in amino acid substitutions [Almind et al. 1993, Laakso et al. 1994, Imai et al. 1994]. The most common amino acid change is a glycine to arginine substitution at codon 972 (G972R), which has an overall frequency of ~6% in the general population [Hitman et al. 1995]. This mutation has been reported to significantly impair IRS-1 function in experimental models of diabetes [Almind et al. 1996]. Furthermore, clinical studies have shown that this genetic variant is associated with reduced insulin sensitivity. Although a variant of IRS-1 may reinforce the negative effect of obesity on insulin-sensitivity, it seems unlikely that mutations in the IRS-1 gene contribute to the pathogenesis of type 2 diabetes.

GLUT4: Activation of the insulin signal transduction system in insulin target tissues leads to the stimulation of glucose transport. GLUT4, the insulin-responsive facilitative glucose transporter, is expressed in insulin-sensitive tissues such as muscles and adipocytes. GLUT4 activity was considered a prime candidate for decreased glucose uptake in muscle and adipose tissue in obesity and diabetes. However, studies in large populations of type 2 diabetics have shown that GLUT4 polymorphisms are uncommon and occur with approximately equal frequency in controls and patients with diabetes [Kusari, et al. 1991, Choi et al. 1991].

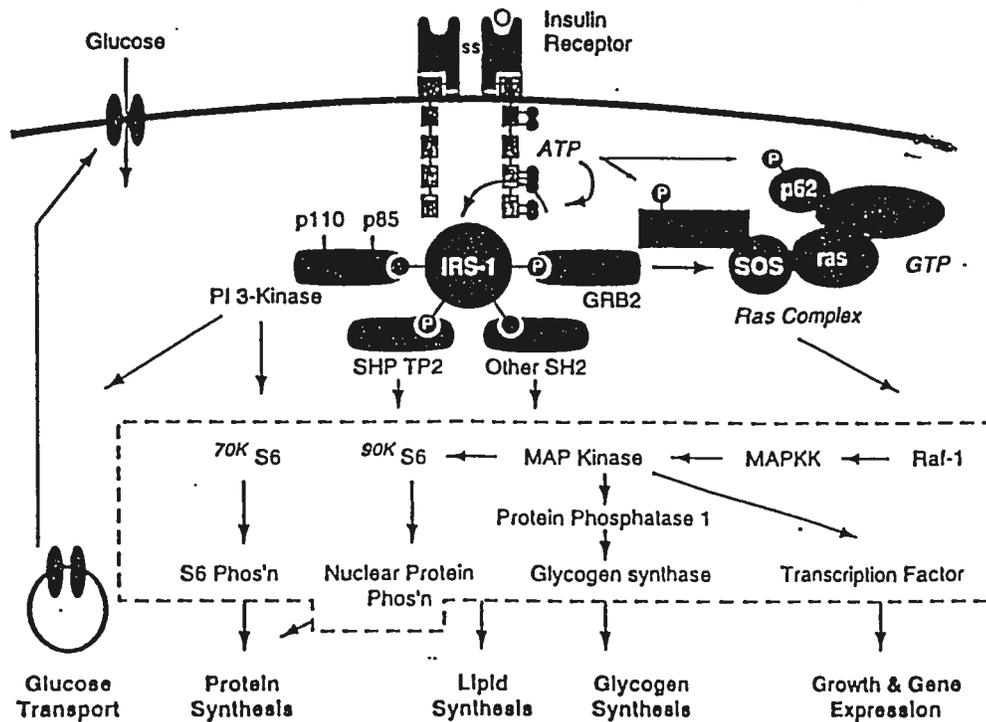


Figure 2. Downstream effects resulting from insulin/insulin receptor interaction. [Kahn 1994]

Hexokinase II: Hexokinase II is associated with GLUT4 and plays an important role in intracellular glucose metabolism by catalyzing the conversion of glucose to glucose-6-phosphate. Although it was considered to be a promising candidate gene for type 2 diabetes and insulin resistance, missense mutations in the hexokinase II gene that encodes the principal hexokinase isoenzyme occur with equal frequency in type 2 diabetic patients and in controls [Laakso et al. 1995]. Hexokinase II activity has been reported to be decreased in muscle of patients with type 2 diabetes [Lehto et al. 1995].

Table 7. Genetic Classification of type 2 diabetes. [Unger et al. 1998]

1. Genetically characterized forms of type 2 diabetes
 - A. Maturity-onset diabetes of the young (MODY)
 1. MODY1— linked to chromosome 20q
 2. MODY2— linked to glucokinase (7p13-15)
 3. MODY3— linked to chromosome 12q
 4. Others
 - B. Defects in the insulin gene (11p15)
 1. Familial hyperproinsulinemia
 2. Mutant insulin molecules
 - C. Defects in the insulin receptor (10p13)
 1. Leprechaunism
 2. Type A syndrome of insulin resistance
 3. Rabson-Mendenhall syndrome
 - D. Mutation in mitochondrial gene for tRNA
 1. Maternally inherited diabetes with neurosensory deafness
 2. MELAS syndrome
 - E. Mutation in GLUT2 glucose transporter (3q26) (one case only)
 2. Genes involved in ordinary type 2 diabetes
 - A. Genes with some evidence for involvement
 1. HLA locus-DR4 (6P21-23) – in elderly persons with type 2 only
 2. Glucagon receptor gene (17q25)
 3. Insulin receptor substrate-1 (2q76)
 4. Glycogen synthase (19q13)
 5. Intestinal fatty acid-binding proteins (4q)
 6. RAD (2q3637)
 - B. Genes for which significant involvement has been ruled out
 1. Insulin gene (11p15)
 2. MODY genes (20q, 7p, 12q)
 3. ATP-sensitive K⁺ channel (21q22)
 4. Glucagon-like peptide-1 (GLP-1) receptor (6p21)
 5. GLUT2 (3q36)
 6. GLUT4 (17p13)
 7. Insulin receptor (19p13)
-

Glycogen synthase: Glycogen synthesis and glycogen synthase are reduced in type 2 diabetes. A missense mutation of the muscle glycogen synthase gene (M416V) is associated with insulin resistance in the Japanese population and may play a role in the pathogenesis of type 2 diabetes [Shimomura et al. 1997].

I.II.2.2.3.3. Mutation in genes encoding β -cell proteins involved in the quality and quantity of secreted insulin (figure 3)

Pro-insulin and insulin: Mutations in pro-insulin gene have been reported. These mutations associated with hyperinsulinemia or hyperpro-insulinemia do not alter glucose homeostasis in the absence of other environmental factors such as age and obesity [kahn et al. 1996]. A polymorphism in the 5' region of insulin gene has been described in obese women [Weaver et al. 1992]. In addition, Olansky et al [1992] identified that an insertion in the promoter of insulin is responsible for decreasing promoter activation, and this alteration has been found to contribute to 5 to 6% in the development of type 2 diabetes in African-Americans. A mutation in insulin gene, responsible for hyperpro-insulinemia has also been reported by Collinet et al in 1998. Finally, a mutation of allele 2 in insulin gene has been shown to induce diabetes and severe dysfunction of pancreatic β -cells in mice [Wang et al. 1999]. Despite these results, it is important to note that insulin gene mutation is unlikely to play a major role in the development of type 2 diabetes.

GLUT2: GLUT2 is another glucose transporter and is also a candidate gene in type 2 diabetes [Davies et al. 1998]. GLUT2 mRNA and protein are decreased in diabetic animals, and this transporter is a target molecule for streptozotocin, an agent used to induce experimental diabetes. Nevertheless, GLUT2 mutations are not common in type 2 diabetes, although rarely they may contribute.

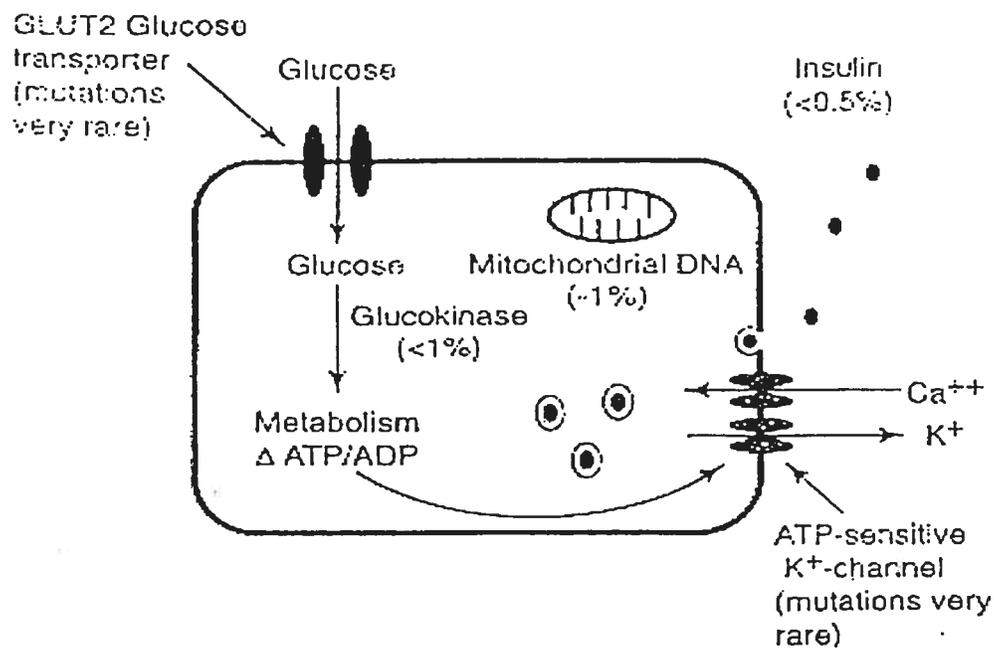


Figure 3. Genetic defects in Beta-Cells in type 2 diabetes. [Kahn et al. 1996].

Glucokinase: Glucokinase (hexokinase IV) is a rate-limiting enzyme in glucose metabolism and the predominant glucose phosphorylating enzyme in pancreatic beta cells. It induces the initial steps of glycolysis, that are critical for insulin secretion. Its importance has been confirmed by the finding that mutations in its gene cause a form of diabetes known as maturity-onset diabetes of the young

(MODY), which is characterized by mild hyperglycemia, decreased glucose-induced insulin secretion, and impaired hepatic glycogen storage [Hattersley 1998]. However, structural mutations on the glyckinase gene do not appear to play a role in the common forms of type 2 diabetes.

ATP-sensitive potassium (K⁺) channel subunit: ATP-sensitive K⁺ channels play important roles in many cellular functions by coupling cell metabolism to electrical activity. Studies in different diabetic populations do not support a role for these channels in type 2 diabetes.

Sulfonylurea receptor: One study has been reported that mutations in the sulfonylurea receptor gene on chromosome 11 cause familial persistent hyperinsulinemic hypoglycemia of infancy [Reis et al. 2002].

Mitochondrial Genes: Mitochondrial DNA is a circular molecule of 16,569 base pairs containing 37 genes encoding 22 transfer RNAs (tRNAs) and 13 enzymes of oxidative phosphorylation. Many studies have established an association between mitochondrial DNA mutation and maternally transmitted type 2 diabetes [Kameoka et al. 1998, Xiu et al. 1997, Gerbitz et al. 1996].

I.II.2.2.3.4. Mutations in genes involved in lipid metabolism and obesity

Lipid metabolism related gene defects: Lipoprotein lipase gene mutations may cause high levels of triglycerides and reduced levels of high-density lipoproteins,

which commonly occur in type 2 diabetes. Despite of this, polymorphic enzymes were not considered to explain the abnormalities of lipid metabolism in diabetic patients. An association between apolipoprotein D polymorphism and type 2 diabetes has been reported in Nauruan and South Indian patients [Baker et al. 1994]. Pima Indians have a polymorphism at codon 54 of the gene encoding the intestinal fatty acid-binding protein. An Ala → Thr substitution doubles the affinity of the protein for long-chain fatty acids and can increase the rate of uptake and oxidation of fatty acids, suppressing glucose utilization. Individuals who are heterozygous or homozygous for the Thr allele have greater insulin resistance and hyperinsulinemia.

Obesity-related gene defects: In human, the β -3-adrenoreceptor gene is expressed in visceral adipose tissue surrounding the gastrointestinal tract and is believed to influence both resting metabolic rate and lipolysis. A mutation in the beta 3-AR gene (Trp64Arg) has been associated with increased tendency to gain weight and early onset of type 2 diabetes [Sakane et al. 1998]. The product of the *ob* gene is a 167-amino-acid peptide called leptin that is expressed and secreted by adipocytes. Mutation or deletion of the leptin gene causes massive obesity and type 2 diabetes in *ob/ob* mice [Wang et al. 1998]. Reichart et al [2000] demonstrated that leptin-receptor-defective mice might develop type 2 diabetes. Adiponectin, also referred to as AdipoQ or ACRP30, is a plasma protein produced and secreted exclusively by adipose tissue. A recent study identified that missense mutation (I164T) of adiponectin is associated with type 2

diabetes [Kondo et al. 2002]. The hormone resistin has been suggested to link obesity to type 2 diabetes by modulating steps in the insulin-signaling pathway and inducing insulin resistance. However, a study of Sentinelli et al [2002] suggests that genetic defects of the resistin gene are unlikely to play a role in the etiology of metabolic disorders such as type 2 diabetes.

I.II.2.2.3.5. Mutations in genes relevant to insulin action

TNF α and IL-6: IL-6 and TNF α modulate tissue sensitivity to insulin. In 2003, Kubaszek et al [2003] investigated whether polymorphisms in the TNF α (G-308A) and IL-6 (C-174G) genes predict the conversion from IGT to type 2 diabetes. They reported that the -308A allele of the promoter polymorphism (G-308A) of the TNF α gene is a predictor for the conversion from IGT to type 2 diabetes.

Other candidate diabetogenes: MODY are monogenic forms of type 2 diabetes that are characterized by an early disease onset, autosomal-dominant inheritance, and defects in insulin secretion. Genetic studies have identified mutations in eight genes associated with different forms of MODY. The majority of the MODY subtypes are caused by mutations in transcription factors that include hepatocyte nuclear factor (HNF)-4 alpha [Pruhova et al. 2003], HNF-1 alpha [Prukova et al. 2003, Kim et al. 2003], PDX-1, HNF-1 beta, and NEURO-DI/BETA-2.

Islet amyloid deposition mainly composed of amylin, is a characteristic pathological finding in patients with type 2 diabetes. Amylin promoter gene mutation are known to predipose some populations such as Chinese, Japanese, and New Zealand Maori to type 2 diabetes [Poa et al. 2003]. Interestingly, a recent study found that S20G mutation of the amylin gene is associated with a lower body mass index in Korean type 2 diabetic patients [Cho et al. 2003].

I.II.2.2.4. Environmental factors

I.II.2.2.4.1. Obesity

The increased prevalence of obesity is accompanied by a parallel increase in the prevalence of type 2 diabetes. Body weight is determined by an interaction between genetic, environmental and psychosocial factors acting through the physiological mediators of energy intake and expenditure. Numerous cross-sectional studies on a range of Caucasian and non-Caucasian populations have reported an association between obesity and the risk of type 2 diabetes [Hodge et al. 2001]. In a 16-year study of nearly 85,000 female nurses, Hu et al [2001] found that the most important determinant of diabetes risk was BMI. It is important to note that the best predictor of diabetes is the quantity of abdominal or visceral fat [Ronnemaa et al. 1997, Gastaldelli et al. 2002]. Abdominal fat is also a predictor for metabolic complications of diabetes. Visceral fat can provide an increased flow of free fatty acids (FFA) to the liver with increased formation of VLDL triglycerides. As a consequence, hepatic steatosis and hepatic insulin resistance can develop. FFA can also be deposited as triglycerides in muscles,

resulting in peripheral insulin resistance. Thus, obesity is thought to predispose to type 2 diabetes primarily by causing insulin resistance.

I.II.2.2.4.2. Diet

Several dietary factors may affect the risk of developing type 2 diabetes. A low-fiber diet with a high glycemic index has been associated with an increased risk of diabetes [Salmeron et al. 1997, Liu et al. 2000]. A high-fat diet and specific dietary fatty acids have been shown to differentially affect insulin resistance and the risk of diabetes [Vessby et al. 2000, Hu et al. 2001]. A recent study demonstrated that an increased dietary polyunsaturated:saturated fat ratio is associated with a reduced risk of diabetes, independent of age, sex, family history of diabetes, and other lifestyle factors [Harding et al. 2004]. Montonen et al [2003] provided evidence that whole-grain consumption and cereal fiber intake are associated with a reduced risk of type 2 diabetes both in men and women.

I.II.2.2.4.3. Physical activity

Physical activity has acute and chronic effects on glucose, lipid and protein metabolism. It has been found, in cross sectional, as well as in prospective and longitudinal studies, to be independently associated with both prevalence and incidence of type 2 diabetes in men and women [De Courten et al. 1997, Tuomilehto et al. 1997, Kriska et al. 1992, Hill et al. 2004,]. Some prospective studies found a dose response relationship between exercise and diabetes incidence. In these studies, it was proposed that type 2 diabetes may be

reduced by 30-50% with regular exercise [Manson et al. 1994]. Physical activity may lower type 2 diabetes risk by reducing total body fat [Ballor et al. 1991] and abdominally distributed fat [Ross et al. 2000], and/or through improving insulin sensitivity by increasing GLUT4 expression in muscles [Mayer-Davis et al. 1998]. In obese diabetic patients, physical activity normalizes the levels of insulin secretion. [Giacca et al. 1998]. Average physical activity levels have decreased over recent years in many populations and this fact is considered as a major contributor to the current global rise of obesity [Prentice et al. 1995] and thus also of type 2 diabetes [Perry et al. 1995].

I.II.2.2.4.4. Stress

Some evidence suggest that depression may increase the risk of developing type 2 diabetes mellitus [Eaton et al .1996]. A recent longitudinal, biracial cohort study involving 11,615 nondiabetic adults aged 48-67 years, has reported that depressive symptoms predict incident type 2 diabetes [Golden et al. 2004]. Hormonal or oxidative stress could also be determinants of type 2 diabetes.

I.II.2.3. Pathophysiology of type 2 diabetes

There are marked differences in the phenotypic expression of type 2 diabetes with affected individuals exhibiting varying levels of insulin resistance due to the failure of peripheral tissues to respond to circulating insulin or impaired in insulin secretion of beta cells [saltiel 2001]. This range of abnormalities

includes metabolic derangements characterized by predominant defects in insulin sensitivity with relative β -cell dysfunction to metabolic derangements characterized by severe β -cell dysfunction accompanied by mild insulin resistance.

I.II.2.3.1. Action of insulin

Insulin inhibits lipolysis, stimulates lipogenesis, stimulates DNA and protein biosynthesis, inhibits protein breakdown, activates transport of glucose, amino acids, and ions, and promotes glycogen synthesis and glycolysis. Insulin inhibits catabolism by suppressing secretion of glucagon, the primary catabolic hormone, and by blocking the hepatic effects of glucagon through enhancement of hepatic phosphodiesterase activity, which inhibits cyclic AMP-dependent protein kinase and thereby inhibits glycogenolysis and gluconeogenesis. The anabolic/catabolic state of the organism therefore can be smoothly regulated by the relative concentration of these two antagonistic hormones.

Insulin action begins when it binds to the α subunit of the insulin receptor inducing the auto-phosphorylation of the β subunit of the insulin receptor. The β subunit of the insulin receptor, which has acquired tyrosine kinase activity phosphorylates the IRS-1, thereby initiating the signaling cascade. IRS-1 stimulates phosphatidylinositol (PI) 3-kinase, the enzyme that catalyzes the phosphorylation of PI, PI-4-P, and PI-4, and ultimately enhances protein synthesis and glucose transport. Phosphorylated IRS-1 acts as a docking protein that binds to and stimulates the P21 signaling pathway through its association

with GRB2, which interacts with SOS in the RAS complex. The RAS complex plays a role in cell growth and metabolism and modulates glycogen synthesis in a pathway that activates raf-1 kinase, which in turn phosphorylates and activates MAPK.

I.II.2.3.2. Insulin resistance

Insulin resistance can be defined as a condition of low insulin sensitivity, a blunted effect of insulin in promoting glucose uptake in peripheral tissues, mainly in skeletal muscles. As shown in figure 4 [Barbara et al. 2002], insulin resistance is a major feature of type 2 diabetes and obesity. Genetic and environmental risk factors such as obesity and physical inactivity are key determinants of insulin resistance. Insulin resistance in visceral fat leads to increased fatty acid production and increased cytokine secretion, which both exacerbate insulin resistance in the liver and muscles [Saltiel. 2001]. In the liver, increased glucose output and increased VLDL and TG production are observed, while in the skeletal muscle decreased uptake of glucose is well documented. The resulting combination of chronic free fatty lipotoxicity and hyperglycemia causes β -cell dysfunction. The consequent reductions in insulin secretion and β -cell mass, facilitate the progressive deterioration of glucose tolerance, and eventually provoke the onset of type 2 diabetes.

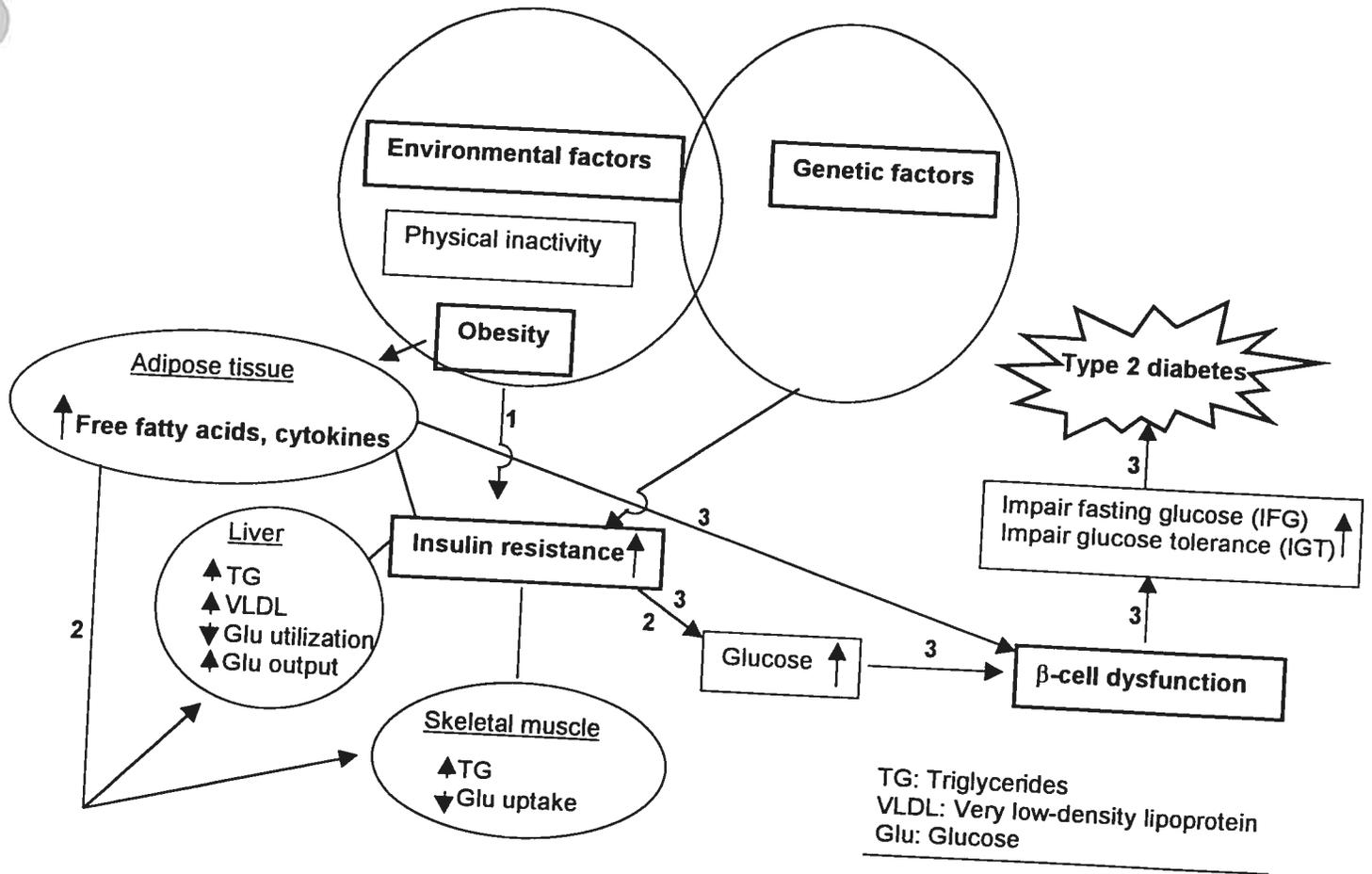


Figure 4. Pathogenesis of type 2 diabetes.

I.II.2.3.2.1. Genetics and environment

The sensitivity of target tissues to the glucoregulatory action of insulin is genetically determined and future risk for development of type 2 diabetes is inversely related to the level of insulin sensitivity [Hansen et al. 2003]. Evidence exists that 50% of first degree relatives of diabetic patients show insulin resistance [Kahn 1994]. Abdominal obesity is the major acquired pathogenic factor for insulin resistance [Bjorntorp 1994] and the effect of obesity on insulin

resistance is greater in members of diabetic families than in persons with no family history of diabetes [Gerich et al. 1998].

I.II.2.3.2.2. Hepatic insulin resistance

Type 2 diabetes with moderate fasting hyperglycemia show slight increased production of glucose by the liver [DeFronzo 1992]. Increased hepatic glucose production in type 2 diabetes is probably caused by a decreased inhibitory effect of insulin on glucagon secretion and glucose production. The importance of increased hepatic gluconeogenesis is underlined by the fact that when phosphoenolpyruvate carboxykinase, a regulatory enzyme of gluconeogenesis, is overexpressed in mice hyperglycemia results [Valera et al. 1994]. However, it is unlikely that increased production of hepatic glucose plays a primary role in the pathogenesis of human type 2 diabetes.

I.II.2.3.2.3. Insulin resistance in skeletal muscle

In normal individuals, 75% of the glucose in a carbohydrate meal is taken up by muscle, and it is stored as glycogen. In NIDD patients glucose uptake and glycogen synthesis in skeletal muscle is reduced [Kelly et al. 1992]. The fact that knockout of GLUT4 gene expression does not cause hyperglycemia in mice indicates that total loss of insulin-stimulated glucose transport is however not sufficient to cause type 2 diabetes [Tsao 1999].

I.II.2.3.2.4. Insulin resistance in adipocyte

Insulin resistance in type 2 diabetic patients is closely associated with increased visceral adiposity [Banerji et al. 1997]. It has been shown that visceral obesity rather than subcutaneous or total obesity is independently correlated with insulin resistance. The increased fat cell mass of obesity, in addition to being intrinsically insulin resistant due to a defect of GLUT4 translocation, may export insulin resistance to muscle, both by releasing FFAs into the general circulation and by infiltrating muscles.

1) FFAs

Concomitant increase in plasma levels of FFAs and insulin reflects the adipocyte resistance to the anti-lipolytic effect of insulin. Most obese individuals have elevated plasma levels of FFAs, which are known to cause peripheral muscle insulin resistance [Groop et al. 1991] by inhibiting insulin-stimulated glucose uptake and glycogen synthesis [Roden et al. 1996, Dresner et al. 1999]. FFAs also cause hepatic insulin resistance, through inhibiting insulin-mediated suppression of glycogenolysis [Roden et al. 2000]. On the other hand, FFAs support between 30 and 50 % of basal insulin secretion and potentiate glucose-stimulated insulin secretion. The insulin stimulatory action of FFAs is responsible for the fact that the vast majority of obese insulin resistant people do not develop type 2 diabetes. During the development of diabetes, excess in FFAs may alter the capacity of β cells to respond to glucose. The strong correlation documented between FFA concentrations and hepatic glucose production further supports

that insulin through its regulatory effect on adipocyte lipolysis may control hepatic glucose production.

2) Adipocytokines

IL-6 and TNF α : Recent data have revealed that the plasma concentration of inflammatory mediators, such as TNF α and IL-6, is increased in the insulin resistant states of obesity and type 2 diabetes, raising questions about the mechanisms underlying inflammation in these two conditions. These two cytokines are both overexpressed in the adipose and muscle tissue of patients with visceral obesity and patients with type 2 diabetes [Vozarova et al. 2001, Hotamisligil et al. 1995]. TNF α expression is also elevated in the adipose tissue of multiple experimental models of obesity. On a cellular level, TNF α is a potent inhibitor of the insulin-stimulated tyrosine phosphorylations on the beta-chain of the insulin receptor and insulin receptor substrate-1 [Hotamisligil et al. 1996]. Anti-TNF α antibody significantly improved insulin resistance in obese rodents [Ventre J, et al. 1997] but not in humans [Ofei et al. 1996].

Adiponectin: Adiponectin is an adipocyte-specific plasma protein. Evidence suggests that adiponectin is associated with obesity-like metabolic disease [Trayhurn et al. 2001]. For instance, increased serum adiponectin levels are coupled with increased insulin sensitivity and glucose tolerance [Yamauchi et al. 2002]. In a case-control study, Lindsay et al. [2002] found that subjects with low concentrations of adiponectin were more likely to develop type 2 diabetes than

subjects with high concentrations of this protein. In addition, it has been shown that first-degree relatives of type 2 diabetic patients have reduced adiponectin mRNA expression in adipose tissue compared to controls [Lihn et al. 2003]. These findings suggest a harmful effect of low levels of adiponectin in the development of type 2 diabetes.

Resistin: Resistin is a newly identified adipocyte hormone. Stepan et al [2001] demonstrated that plasma resistin concentrations are increased in obese mice, and that immunoneutralization of circulating resistin significantly improved insulin action. Consistent with these findings, it has been recently reported that serum resistin concentrations are elevated in both type 2 diabetic and obese nondiabetic patients [Jank et al. 2002, Savage et al. 2001]. Thus, resistin may provide a crucial link between obesity and insulin resistance.

Leptin: Leptin is a protein produced by the adipose tissue-specific *ob* gene, that appears to play an important role in the regulation of food intake and energy expenditure. Insulin resistance, obesity and type 2 diabetes are associated with hyperleptinemia. Notably, high serum leptin levels are accompanied by increased insulin levels [Cusin et al. 1995]. Recent studies have shown that leptin inhibits insulin secretion and exerts anti-insulin effects on liver and adipose tissue. If these effects are confirmed, leptin could participate in the insulin resistance of obesity and type 2 diabetes. It has been found that leptin is associated with impaired fibrinolysis, which is a common finding in obese humans [Skurk et al.

2002]. Recently, Kazumi et al [2003] found a positive association between CRP and leptin. Finally, plasma leptin levels have been shown to be associated with coronary atherosclerosis in type 2 diabetes [Wallace et al. 2001, Rahmouni et al. 2004]. Thus, leptin appears to be a novel risk factor for CVD [Wallace et al. 2001].

I.II.2.3.3. Hyperinsulinemia and glucose intolerance

Insulin resistance, hyperinsulinemia, and IGT are common abnormalities in individuals at high risk for type 2 diabetes [Rewers et al. 1995]. Current knowledge suggests that development of glucose intolerance or diabetes is initiated by insulin resistance. At the early stages, the pancreatic β -cell is able to compensate for insulin resistance by increasing insulin levels, thereby maintaining glucose levels in a normal range for a period of time (up to several years). IGT associated with mild postprandial hyperglycemia is a transitional period between prediabetes and mature diabetes. As insulin resistance worsens, the progression from IGT to early type 2 diabetes is marked by a decrease in β -cell function and thus a decline in insulin secretion. It is the failure over time of the β -cell to compensate for insulin resistance with hyperinsulinemia that marks the beginning of type 2 diabetes. As long as the pancreatic β -cell is able to compensate for insulin resistance by increasing insulin production and secretion, glucose levels remain normal or near normal. However, eventually the β -cell begins to fail, and insulin secretion falls, resulting in hyperglycemia and type 2 diabetes. Factors that contribute to decreased insulin secretion are leptin and

several agonists including norepinephrine, somatostatin, galanin, and prostaglandins [Sharp 1996, Kieffer et al. 1997].

I.II.2.4. Complications associated with type 2 diabetes

Chronic hyperglycemia in type 2 diabetes causes long-term damage to many tissues of the body, particularly to the vascular system. Vascular complications are the leading cause of morbidity and mortality in the type 2 diabetic population [Cooper et al. 2000]. These can be divided into microvascular and macrovascular complications. Figure 5 [Cooper et al. 2000] illustrates a pathophysiological model of the development of diabetes and diabetic vascular complications.

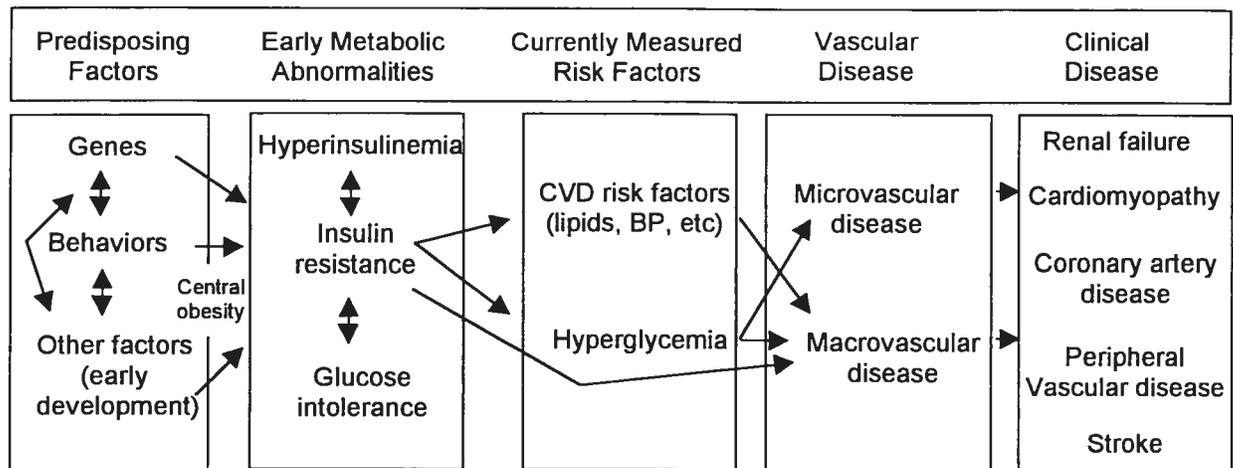


Figure 5. Model of development of diabetic vascular complications.

I.II.2.4.1. Macrovascular complications

Macrovascular complications of type 2 diabetes include cardiovascular, cerebrovascular, and peripheral vascular diseases. CVD is the most prevalent complication of DM and the leading cause of mortality in patients with type 2

diabetes. CVD is responsible for 80% of the mortality in diabetic patients, accounts for 75% of all hospitalization for diabetic complications [Pyorala et al. 1987, Haffner et al. 1998] and precedes overt diabetes in more than 50% of patients [Peters et al. 1998]. As shown in figure 6 [Libby et al. 2002], multiple factors contribute to the accelerated atherosclerosis in type 2 diabetes. These risk factors include excess prevalence of traditional risks such as obesity, hypertension, smoking, and dyslipidemia along with glycoxydation of lipoproteins and proteins, alteration of the coagulation system, inflammation, insulin resistance, and hyperinsulinemia. These factors have been clearly categorized into conventional and novel risk factors.

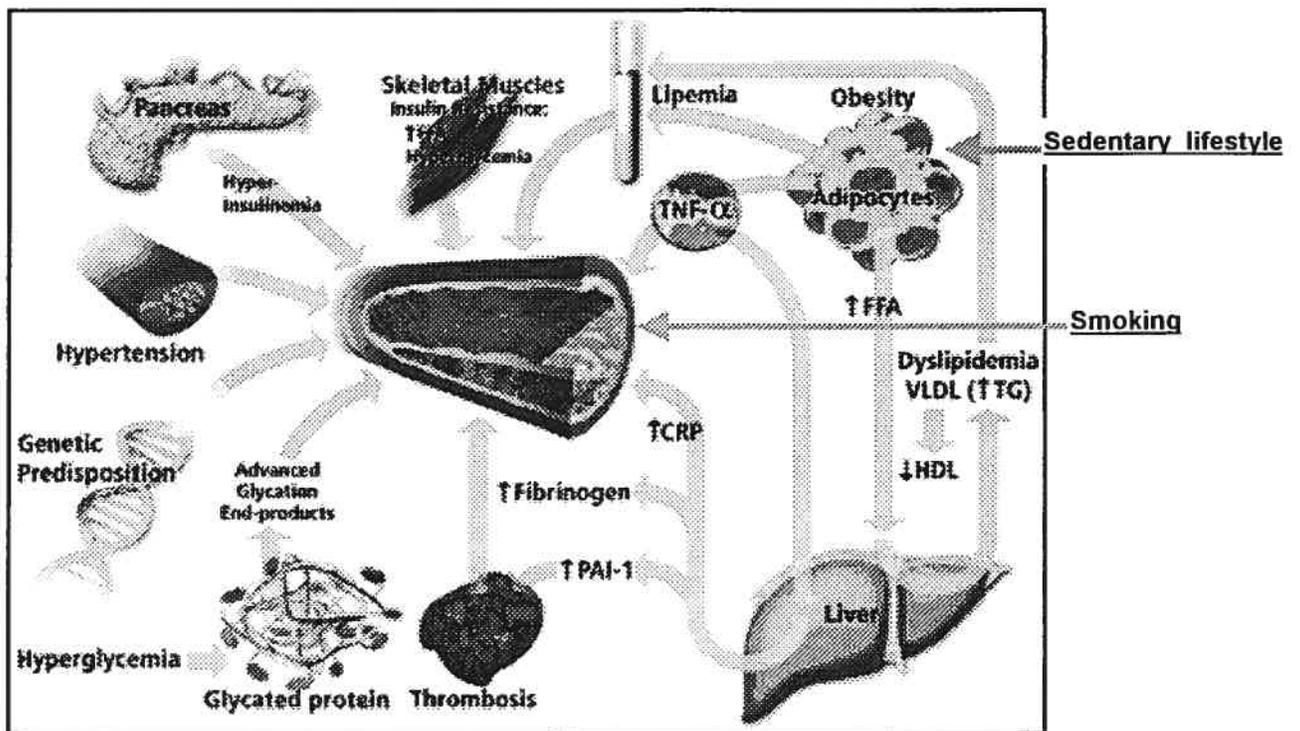


Figure 6. Multiple cardiovascular risk factors in diabetes.

I.II.2.4.1.1. Traditional risk factors

I.II.2.4.1.1.1. Dyslipidemia

Patients with type 2 diabetes have atherogenic dyslipidemia characterized by elevated levels of VLDL triglycerides and small dense LDL cholesterol particles and decreased levels of HDL cholesterol. Small dense LDL are more liable to oxidation and may more readily adhere to and subsequently invade the arterial wall [Laakso et al. 1995]. Although LDL are not usually increased in diabetes, two prevention trials clearly showed that lowering LDL cholesterol levels still reduces the risk of major cardiac events in diabetic patients [Pyorala et al. 1997, The Long-Term Intervention with Pravastatin in Ischaemic Disease Study Group 1998]. The prevalence of hypertriglyceridemia and low HDL cholesterol levels in diabetic subjects is approximately twice as high as in the nondiabetic groups [Garg et al. 1990]. There is increasing evidence supporting a role for VLDL triglycerides in macrovascular disease in patients with type 2 diabetes. The fact that type 2 diabetic patients with hypertriglyceridemia have increased small dense LDL [Yudkin 1993] and increased VLDL levels in type 2 diabetes are also associated with post-prandial hyperlipidemia that is compounded by impaired LPL activity [Krentz 2003]. Both small dense LDL and impaired LPL activity are associated with CVD. Low HDL levels have been found to be often associated with high levels of total VLDL triglycerides in type 2 diabetes [Laakso et al. 1995]. The Framingham study established low levels of HDL as a risk factor for CVD [Swan et al. 1999], because the reduction in cardioprotective HDL cholesterol means a reduction of cholesterol efflux from the

tissues, which is the first step in reverse cholesterol transport to the liver from peripheral tissues. In addition, the antioxidant and antiatherogenic activities of HDL cholesterol are reduced when circulating levels are low [Krentz 2003]. Overall, dyslipidemia is considered to be an independent risk factor for CHD in type 2 diabetes.

I.II.2.4.1.1.2. Hypertension

Hypertension is an independent risk factor for CVD in type 2 diabetes and is about 70% greater in type 2 diabetic patients than in non-diabetic subjects [Sowers et al. 2001]. The results from the UKPDS indicated that the risk of myocardial infarction correlate with blood pressure in patients with type 2 diabetes [Adler et al. 2000]. Hypertension is often associated with insulin resistance, hyperinsulinaemia [Laakso et al. 1997] and proteinuria [Stamler et al. 1993], which are also risk factors for CVD.

I.II.2.4.1.1.3. Smoking

The Multiple Risk Factor Intervention Trial (MR-FIT) [Stamler et al. 1993] has confirmed that cigarette smoking is an independent predictor and risk factor of CVD morbidity and mortality in persons with diabetes [Laakso et al. 1997]. Cigarette smoking appears to act synergistically with hypercholesterolemia to accelerate CVD in diabetes, possibly by enhancing the oxidation of LDL cholesterol [Heitzer et al. 1996].

I.II.2.4.1.1.4. Obesity

Obesity is an important risk factor of type 2 diabetes and is associated with insulin resistance, dyslipidemia, hypertension, and an increased risk of CVD [Bonadonna et al. 1990, Rexrode et al. 1996].

I.II.2.4.1.1.5. Hyperglycemia

Several studies have reported that hyperglycemia is not a particularly strong determinant for developing CHD in type 2 diabetic patients. While the UKPDS found that intensive blood-glucose control by either sulphonylureas or insulin substantially decreases the risk of microvascular complications, this approach did not reduce the risk of developing macrovascular disease in patients with type 2 diabetes [UK Prospective Diabetes Study (UKPDS) Group. 1998]. These results are in contrast with others showing that glycemetic control was important in decreasing cardiovascular risk in different populations including diabetic ones [Laakso. 1999, Gerstein et al. 1996, Fuller et al. 1983, Fuller et al. 1980, Singer et al. 1992]. Today, a debate still exists whether hyperglycemia per se is an important risk factor for diabetic macrovascular complications. Importantly, Bonora et al [Bonora et al. 2001, Bonora. 2002] recently documented that postprandial plasma glucose is an independent risk factor for cardiovascular disease in diabetic subjects. The mechanisms through which hyperglycemia causes vascular injury, include nonenzymatic glycosylation of proteins and lipids, oxidative stress, activation of PKC and of the polyol pathway.

1) Glycation of proteins

Definition and formation: Non enzymatic glycation is a biochemical process by which glucose is covalently bound to protein aminogroups through a series of chemical reactions described by Maillard [Maillard et al. 1912].

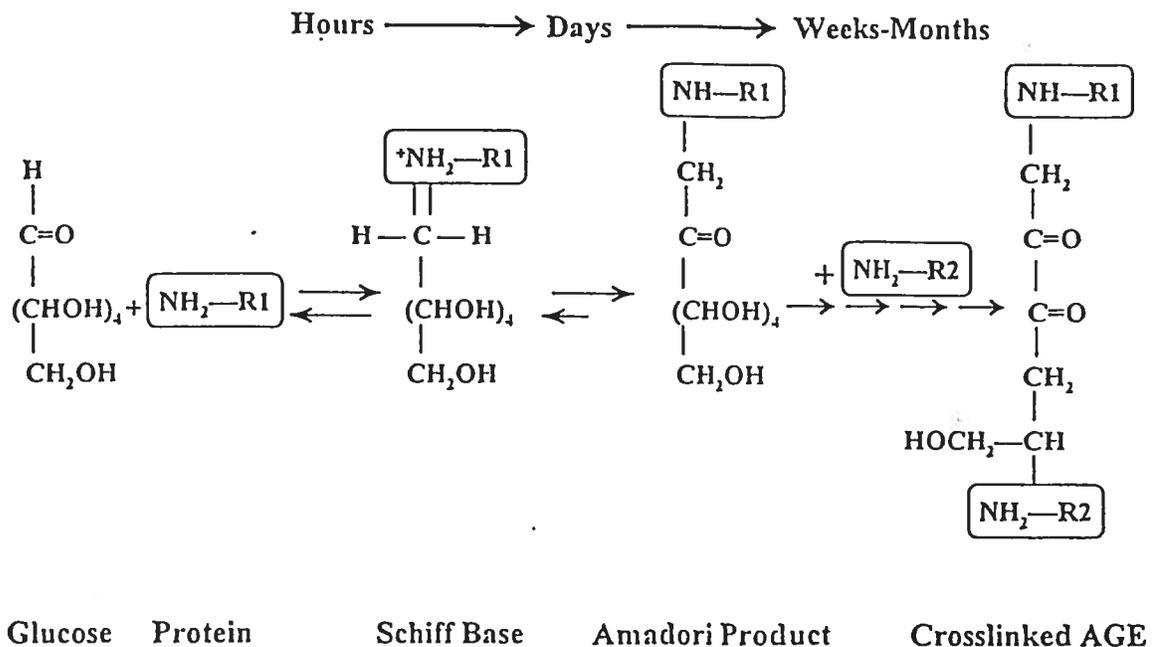


Figure 7. The formation of AGEs. [Aronson et al. 2002]

As shown in figure 7, glucose forms chemically reversible early glycosylation products with reactive amino groups of circulating or vessel wall proteins (Schiff bases), which subsequently rearrange to form the more stable Amadori-type early glycosylation products. Equilibrium levels of Schiff-base and Amadori products are reached in hours and weeks, respectively [Brownlee et al. 1988]. Some of the early glycosylation products on long-lived proteins such as

vessel wall collagen, continue to undergo complex series of chemical rearrangement to form AGEs [Brownlee et al. 1988]. Once formed, AGE-protein adducts are stable and virtually irreversible. The best chemically characterized AGEs compounds found in human are pentosidine [sell et al. 1989] and *N*-(carboxymethyl)-L-lysine (CML) [Ikeda et al. 1996].

Role of AGE in diabetic macrovascular complications: There is increasing evidence that AGE are involved in the pathophysiology of diabetic macroangiopathies. It has been shown that CML accumulates in atherosclerotic plaques and serum of diabetic patients [Schleicher et al. 1997]. Immunohistochemical analysis of coronary arteries from type 2 diabetic patients, has also documented high AGE-reactivity within the atherosclerotic plaque suggesting a role of AGEs in the accelerated development of arterial disease observed in diabetes [Nakamura et al. 1993].

AGEs can accelerate the atherosclerotic process by diverse mechanisms, which can be classified as non-receptor dependent and receptor-mediated. AGE binding to its specific receptors on endothelial cells quenches nitric oxide and increases vascular permeability, procoagulant activity, adhesion molecule expression and monocyte influx, and oxidative stress and thereby contributes to vascular injury [Aronson et al. 2002]. In addition, AGE also interact with macrophage receptors to induce cytokine and growth factor release, [Tanaka et al. 1988]. In the atherosclerotic lesion, linking of AGE to extracellular matrix enhances synthesis of extracellular matrix components and trapping of LDL in

the subendothelium [Brownlee et al 1986, Brownlee et al. 1985]. Evidence also suggests that AGE-modified LDL is less susceptible to LDL receptors, but more susceptible to macrophage scavenger receptors [Steinbrecher et al. 1984].

2) Polyol pathway

There are a number of tissues in which glucose uptake is not dependent on insulin, including the kidney, neural tissue, and the lens of the eye. In such tissues, hyperglycemia-induced tissue injury is associated with the activation of the polyol pathway, which is a two-step enzymatic process. First, intracellular D-glucose is reduced to sorbitol, coupled to oxidation of NADPH to NADP⁺ by aldose reductase. The second step is the subsequent oxidation of sorbitol to fructose, which is coupled to reduction of NAD⁺ to NADH by sorbitol dehydrogenase. Evidence indicates that an increased free cytosolic NADH/NAD⁺ ratio is observed in a variety of tissues of diabetic animals. This change was also found in cells and tissues exposed to elevated glucose levels in vitro [Obrosova et al. 2001, Tilton et al. 1992, Williamson et al. 1993]. The biochemical mechanisms responsible for a further reduced cytosolic state in diabetic tissues are not completely understood.

3) PKC activation

Activation of PKC, an intracellular second messenger, is reported in many diabetic tissues including the retina, kidney, heart, and aorta [Inoguchi et al. 1992]. In particular, the glucose-sensitive PKC β 2 isoform is activated in diabetic

vascular tissues [Inoguchi et al. 1992]. Evidence has been provided that LY333531, a specific PKC β 2 inhibitor, blocks various diabetes-related abnormalities in the retina and kidney [Ishii et al. 1996]. Moreover, ACE inhibitors and aminoguanidine have been shown to reduce renal PKC activity [Russo et al. 2000]. These findings suggest a role for PKC in mediating vascular injury in diabetes. Potential changes induced by hyperglycemia-induced activation of the DAG-PKC pathway are shown in figure 8.

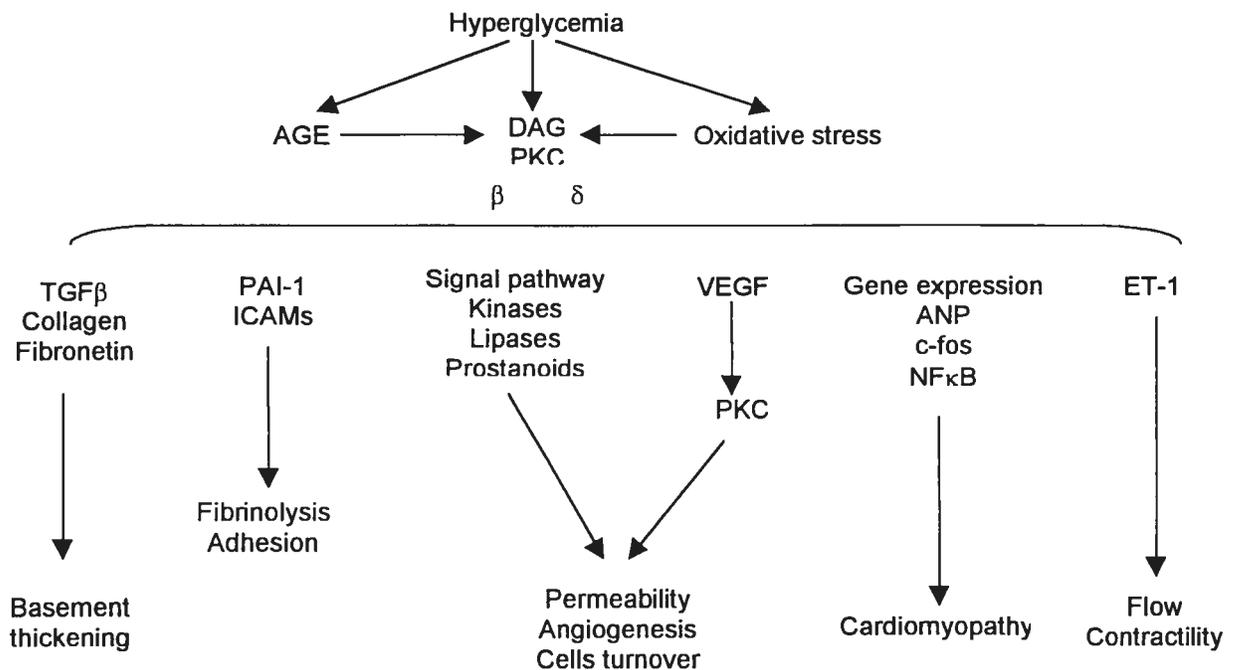


Figure 8. Hyperglycemia-induced activation of the DAG-PKC pathway. [Koya et al. 1998]

4) Oxidative stress

Oxidative stress may play a crucial role in the pathogenesis of diabetic complications [Evans et al. 2002]. Indeed, diabetes is characterized by a chronic, growing increase in oxidative stress-favoring stimuli as well as by a progressive decrease in the efficacy of the antioxidant system. This imbalance in the

oxidative stress equilibrium may underlie the much higher incidence of cardiopathy [Cai et al. 2001] and vasculopathy [Inoguchi et al. 2000] in diabetes. The theoretical importance of oxidative stress in diabetes is highlighted by its potential double impact on metabolic as well as vascular dysfunction in this disease [Wiernsperger 2003]. For example, oxidative stress can decrease insulin internalization by endothelial cells [Bertelsen et al. 2001] and therefore potentially interfere with the hormone transfer from plasma to tissues. In target cells, oxidative stress can reduce GLUT4 translocation, therefore limiting the capacity to transport glucose into muscle cells [Rudich et al. 1998].

Many studies have demonstrated the presence of oxidative stress in diabetic patients, as reflected by increased levels of indicators of oxidative stress and antioxidants [Vessby et al. 2002, Seghrouchni et al. 2002]. Oxidative stress in diabetes may originate from an exaggerated production of ROS, overcoming the antioxidant capacity, a breakdown of the antioxidants in front of a relatively normal ROS production, or a combination of both. As shown in figure 9, various sources of ROS exist. In particular, mitochondria are considered to be a privileged source of ROS due to the production and transport of electrons. Hyperglycemia, FFAs, and leptin have been shown to induce ROS generation from mitochondria [Evans et al. 2002, Wojtczak et al. 1993, Bakker et al. 2000, Yamagishi et al. 2001]. Hyperglycemia can also increase ROS via transition metal-catalyzed autoxidation [Wolff 1993, Evans et al. 2002]. In addition, since AGEs and lipids are constantly produced in overt diabetes, carbonyl stress has been proposed as a major source of oxidative stress in this disease [Baynes et

al. 2000, Taniguchi et al. 1996]. While high glucose level, FFA, insulin, and angiotensin II stimulate NAD(P)H oxidase to produce ROS [Griendling et al. 2000, Kashiwagi et al. 1999, Inoguchi et al. 2000, Griendling et al. 2000], high cholesterol, by activating xanthine oxidase, stimulates endothelial superoxide production [Ohara 1993]. Finally, oxidative stress-inducing carbonyl compounds are found in food [Cai et al. 2002].

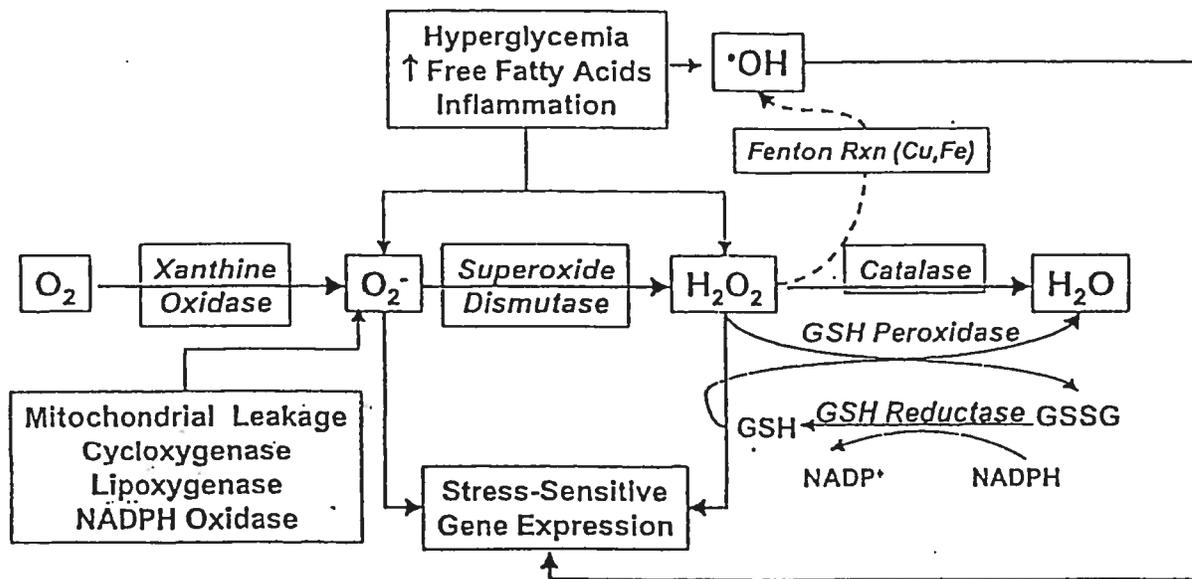


Figure 9. Exogenous and endogenous stimuli leading to ROS generation. [Evans et al. 2002]

The implication of oxidative stress in diabetes is a major concern for the development of therapeutics aimed at improving the metabolic and/or vascular dysfunctions of this burdening disease. However, there are currently no recommendations for antioxidant supplementation in diabetic patients. While an

endless number of in vitro and in vivo studies in both animals and humans have shown clearcut positive effects on individual metabolic or vascular parameters, there is still missing proof for a beneficial effect of antioxidant therapy in large-scale clinical studies. For example, antioxidants such as NAC remarkably protect retinal pericytes against AGE-induced apoptosis in vitro [Denis et al. 2002] and various antioxidants ameliorate diabetic neuropathy in animals. However human studies have failed to demonstrate a clear beneficial effect of antioxidant therapy. For example, whereas several reports exist showing improvements in surrogate endpoints such as endothelial reactivity, no effect has been found in all major clinical trials examining the long-term outcome of vitamin E on cardiovascular events [Yusuf et al. 2000, Lonn et al. 2001]. Very recently the vitamin E atherosclerosis prevention study (VEAPS) trial has reported that vitamin E reduces LDL oxidation but does not reduce atherosclerosis [Hodis et al. 2002]. The possible causes of apparent clinical failure of antioxidants may be due to the wrong conclusion about the relevance of vitamin E antioxidant effect and the effect of vitamin E as prooxidant. Let's hope that vitamin E is an unlucky choice and that the use of other antioxidants, alone or in combination, may contradict the actual pessimistic conclusion for antioxidants therapy. More promising approaches should be based on drugs preventing the ROS generation rather than their scavenging. This notion is supported by the result from a recent trial showing an improvement both in blood pressure and in glycemic control by administration of Coenzyme Q [Baynes et al. 1996].

I.II.2.4.1.2. Novel risk factors

Many studies have shown that assessment of conventional risk factors only partly account for the excessive risk of developing CVD in the diabetic population. More importantly, there is yet no definitive evidence that controlling hyperglycemia reduces macrovascular disease in type 2 diabetes. The recognition of these facts has led to the identification of novel risk factors that are commonly associated with insulin resistance, hyperinsulinemia and hyperglycemia. These factors include insulin resistance, inflammation, endothelial dysfunction, oxidative stress, and alteration of the coagulation.

I.II.2.4.1.2.1. Insulin resistance

Today, evidence exists that much of the increased risk of developing CVD in diabetes is related to insulin resistance. Insulin resistance is the hallmark of type 2 diabetes and precedes the disease for several years [Plutzky et al. 2002, Erkelens 2001]. Insulin resistance is related and is the preceding factor of both traditional and nontraditional CV risk factors, including hyperglycemia, hypertension, and obesity, linking all these abnormalities to the development of CVD [Haffner et al. 1999]. For such reasons, insulin resistance might be regarded as an accomplice in the pathogenesis of CV disease in type 2 diabetes. Importantly, it has been proposed that insulin resistance might also contribute to CVD in a direct and independent pathway [Bonora et al. 2002]. For example, it has been demonstrated that both insulin and proinsulin can augment the expression of PAI-1 *in vivo* [Nordt et al. 1998, Nordt et al. 1995].

I.II.2.4.1.2.2. Homocysteine

Elevated blood level of homocysteine is strongly related to an increased risk for atherosclerosis and cardiovascular disease [Bostom et al. 1999, Romm et al. 1998]. A high prevalence of hyperhomocysteinemia is found in patients with type 2 diabetes [Smulders et al. 1999, Hoogeveen et al. 1998, Munshi et al. 1996] and high serum levels of homocysteine are significantly associated with coronary events in type 2 diabetes, independently of traditional cardiovascular risk factors [Becker et al. 2003]. Plasma levels of homocysteine are also elevated in hypertensive diabetic patients [Qureshi et al. 2003].

I.II.2.4.1.2.3. Microalbuminuria

Microalbuminuria is defined as persistent elevation of albumin in the urine. Use of the albumin/creatinine ratio is recommended as the preferred screening strategy for all diabetic patients. Microalbuminuria is an independent risk factor for the development of CVD and a predictor of cardiovascular mortality in the diabetic population. It has been documented that microalbuminuria is associated with insulin resistance, dyslipidemia, central obesity, and hypertension [Bianchi et al. 1995, Sowers et al. 1999, Feldman et al. 1999]. Microalbuminuria is also related to endothelial dysfunction and increased oxidative stress, and thus is an important risk factor for CVD in diabetic persons [Kuusisto et al. 1995, Dinneen et al. 1997, Sowers et al. 1999].



I.II.2.4.1.2.4. Abnormal fibrinolysis and hypercoagulation

Recent studies have identified defects in the coagulation and fibrinolytic cascade as important pathological mechanisms leading to CAD. Type 2 diabetes is a state of increased plasma coagulability, increased platelet aggregability, and reduced fibrinolytic capacity [colwell 2001]. Diabetes is also associated with increased levels of von Willebrand factor, factor VII, factor VIII, platelet factor 4, and PAI-1 levels [Giuseppe et al. 2003]. PAI-1 predicts type 2 diabetes independently of insulin resistance and other known risk factors for diabetes [Festa et al. 2002]. Diabetic and hyperinsulinemic patients typically exhibit increased production of PAI-1 from visceral abdominal adipocytes. It is likely that elevated levels of TF and PAI-1 and reduced levels of tissue-type plasminogen activator are associated with increased production of proinflammatory cytokines such as IL-6 from adipocytes in type 2 diabetes [Giuseppe et al. 2003].

I.II.2.4.1.2.5. Inflammation

A decade ago, elevated systemic markers of inflammation were found to predict adverse CV events in patients with severe unstable angina [Liuzzo et al. 1994]. Since then, in view of the persuasive evidence indicating a role for inflammation in atherogenesis, several investigators have examined whether circulating markers of inflammation may predict CV risk in a variety of clinical settings. Results of their studies have supported a potential role for several biochemical markers of inflammation as predictors of the development of CHD as well as adverse CV-related outcomes in patients with known coronary

syndromes. Markers of inflammation appear to offer risk prediction information that is independent of, and possibly complementary to traditional risk factors for the development of CVD. Among these markers, elevated concentrations of CRP, IL-6, fibrinogen, adhesion molecules, serum amyloid A protein, leucocyte count have shown to be markers for increased risk of CVD [Altman et al. 2002, Liuzzo et al. 1994, Blake et al. 2001, Libby et al. 2002, Wu et al. 2003]. It should be noted that the notion that CRP may represent a predictor of CV risk has recently been challenged by Danesh et al [2004]. This point will be discussed further at section I.IV.1.1. Recently, chronic subclinical inflammation has emerged as a new risk factor for the development of type 2 diabetes and as a candidate mechanism underlying the common soil hypothesis linking type 2 diabetes and atherosclerosis. Indeed, it has been demonstrated that circulation markers of subclinical inflammation, in particular CRP and IL-6, are powerful independent predictors of both diabetes and CVD [Ridker et al. 2000, Pradhan et al.2001, Festa et al. 2002]. (see section I.IV. for details).

I.II.2.4.1.2.6. Endothelial dysfunction

Patients with type 2 diabetes exhibit endothelial dysfunction as determined by the assessment of vascular reactivity and/or the measurement of plasma markers of endothelial activation/dysfunction. Endothelial dysfunction can also be detected in obese patients, in relatives without diabetes as well as in the spectrum of insulin resistance before the detection of any distinct carbohydrate intolerance. Thus insulin resistance appears to be associated with endothelial

dysfunction. Some components of the metabolic syndrome, such as hypertension, dyslipidemia and increased uric acid, likely contribute to alterations in endothelial function. Evidence that markers of endothelial dysfunction are associated with CRP indicates the link existing between endothelial dysfunction and inflammation. Increasing evidence has revealed that endothelial cells play an important role in development and progression of atherosclerosis. Insulin resistance is clearly associated with increased cardiovascular risk. Therefore endothelial dysfunction builds a link between insulin resistance and atherosclerosis. Endothelial dysfunction is now considered as a new risk factor for type 2 diabetes.

I.II.2.4.1.3. Inflammation: the link between obesity, insulin resistance, type 2 diabetes and atherosclerosis.

The recent concept that type 2 diabetes is an inflammatory condition is an exciting and novel approach to better understanding of this condition. Inflammation is implicated both in terms of pathogenesis and complications of type 2 diabetes.

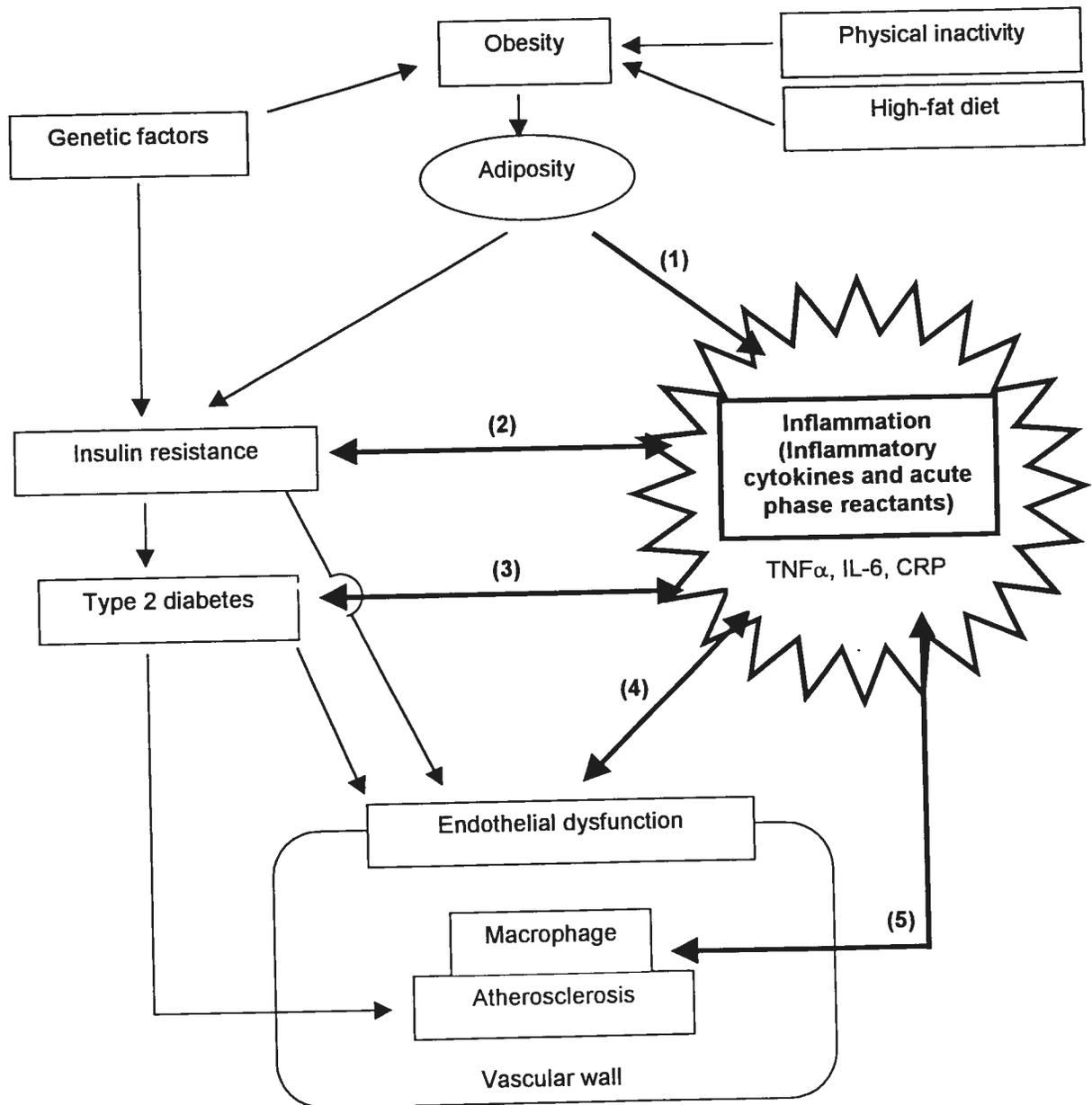


Figure 10. Inflammation : the link between obesity, insulin resistance, diabetes, and atherosclerosis.

I.II.2.4.1.3.1. Inflammation and obesity

It is now well established that obesity is a chronic inflammatory disorder. Consistent with this notion, elevated plasma concentrations of CRP [Yudkin et al. 1999], IL-6 [Mohamed-Ali et al. 1997], TNF α [Hotamisligil et al. 1993], and PAI-1

[Lundgren et al. 1996] have been found to be associated with obesity. Increasing evidence indicates that adipose tissue is an important source of cytokines including $TNF\alpha$, leptin, PAI-1, IL-6, IL-1, resistin and angiotensinogen [Ahima et al. 2000] and that adiposity contributes to a proinflammatory milieu [Yudkin et al. 1999]. High secretion rate of these proinflammatory cytokines is associated with decreased insulin release and action [Ahima et al. 2000]. As shown in figure 11, there is also some evidence that beside adipocytes, macrophage infiltration into adipose tissue can also take part in the inflammatory changes associated with obesity [Xu et al. 2003, Weisberg et al. 2003].

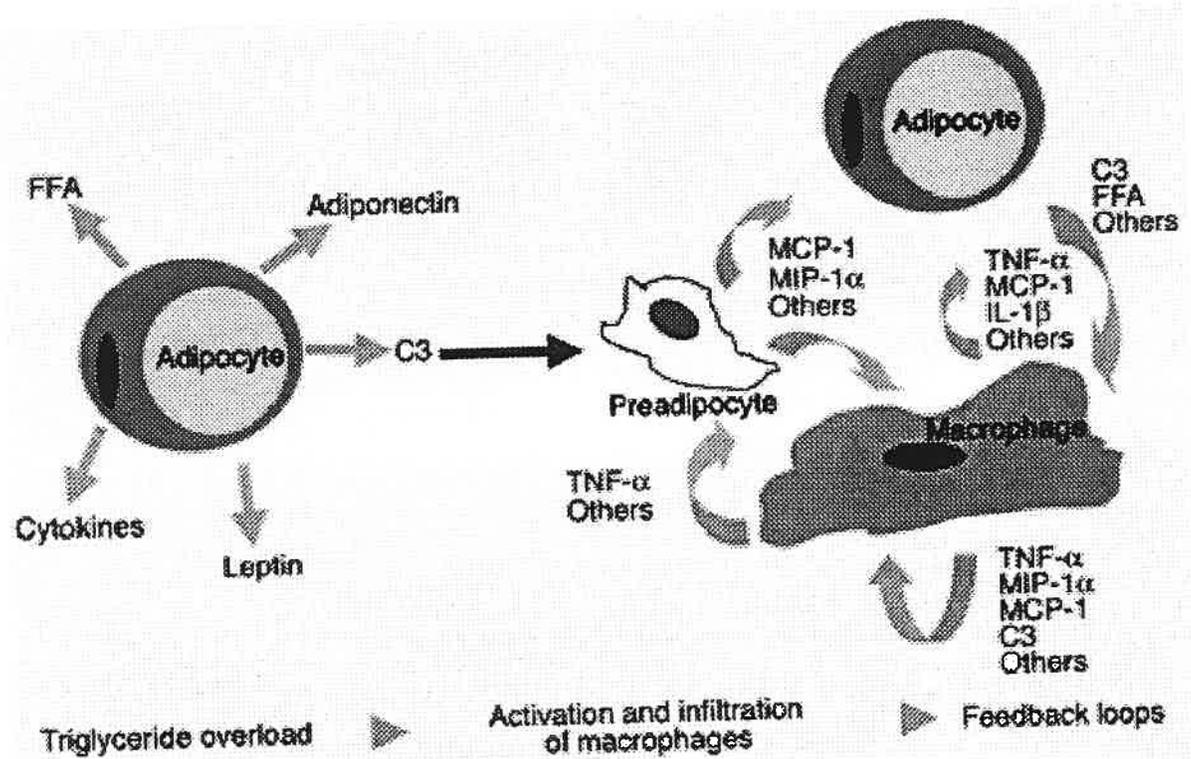


Figure 11. Hypothetical model of chronic inflammation and adipocyte insulin resistance. [Xu et al. 2003]

I.II.2.4.1.3.2. Inflammation and insulin resistance

As mentioned above, proinflammatory cytokines such as $\text{TNF}\alpha$ and IL-6 secreted from adipose tissue induce insulin resistance. Growing evidence has pointed to a correlative and causative relationship between inflammation and insulin resistance/type 2 diabetes. $\text{TNF}\alpha$ is overexpressed in white adipose tissue both in obese and insulin-resistant states and mediates insulin resistance in rodent obesity models [Hotamisligil et al. 1993, Hotamisligil et al. 1994]. Conversely, mice lacking the $\text{TNF}\alpha$ ligand or the p55 TNF receptor are partially protected from obesity-induced insulin resistance [Uysal et al. 1997, Uysal et al. 1998, Ventre et al. 1997, Moller 2000]. High secretion of IL-6 is associated with decreased insulin release and action. Plasma IL-6 levels are elevated in diabetic patients with features of the insulin resistance syndrome and serum IL-6 is associated with insulin resistance in men and women. Interestingly, low production capacity of IL-10, a cytokine with anti-inflammatory properties, is associated with the metabolic syndrome and type 2 diabetes [van Exel et al. 2002]. Elevated expression of SAA3, an acute phase reactant has also been recently documented in adipose tissue of mice, this fact being especially remarkable in the diabetic state [Lin et al. 2001]. The Insulin Resistance Atherosclerosis Study (IRAS) has clearly shown that CRP is independently related to insulin insensitivity [Festa et al. 2000]. In the liver, insulin seems to be one of the main regulators of the cytokine-associated acute-phase response. It attenuates cytokine stimulation of most acute phase protein genes in human hepatoma cells. Thus, lack of significant insulin action, as found in type 2

diabetes, or unsubstituted insulin deficiency, would result in loss of this anti-inflammatory effect.

Beside adipocytes, activated macrophages in the arterial wall also secrete numerous cytokines and chemokines such as $\text{TNF}\alpha$, IL-1, IL-6, and MCP-1, which all cause insulin resistance in adipocytes [Hotamisligil et al. 1994, Grimbble et al. 2002, Amrani et al. 1996, Fried et al. 1998]. As a consequence of sustained inflammation in the vascular wall, insulin signaling in adipocytes can become increasingly impaired.

I.II.2.4.1.3.3. Inflammation and type 2 diabetes

That type 2 diabetes may represent an inflammatory condition has been first suggested by Crook et al. [1993] then by Pickup et al in 1997. These investigators reported that type 2 diabetes is an inflammatory condition, characterized by elevated plasma concentrations of acute phase inflammatory reactants such as sialic acid and IL-6. Later on, an association between white blood cell count [Schmidt et al. 1999], CRP [Prahan et al. 2001], PAI-1 and type 2 diabetes [Festa et al. 2002] has also been documented. Interestingly, in 2002 elevated plasma levels of PAI-1 [Festa et al. 2002] and CRP [Prahan et al.2001] have been found to predict the development of type 2 diabetes independently of fat mass and BMI. These studies again add to the notion that low-grade inflammation is important in the pathogenesis of type 2 diabetes.

Although, type 2 diabetes is associated with chronic low-grade inflammation, the underlying mechanism(s) responsible for this inflammatory

response is not well understood. A recent study indicates that serum concentration of AGEs is an independent determinant of plasma CRP levels suggesting that subclinical inflammation in these patients may be partly due to activation of the inflammatory response by AGEs [Tan et al. 2004]. Considering the strong correlation between insulin resistance, inflammation, and type 2 diabetes, it is also possible that the association of type 2 diabetes with inflammation may be due to the state of insulin resistance. Alternatively, elevated levels of inflammatory markers in diabetes, may be the result of preexisting atherosclerosis.

I.II.2.4.1.3.4. Inflammation in endothelial dysfunction and atherosclerosis

Endothelial dysfunction predicts CV events and is documented in type 2 diabetes. It is also detected in obese and insulin resistant subjects. Thus, insulin resistance itself appears to be related to endothelial dysfunction. Insulin resistance may contribute to endothelial dysfunction through systemic defect of the PI3 kinase pathway and decreased insulin-mediated endothelial NO production. Endothelial dysfunction may also be induced by proinflammatory cytokines such as IL-1 β and TNF α , and by the acute-phase protein CRP. Indeed, recent studies indicate that human recombinant CRP, at concentrations known to predict vascular disease, elicits a multitude of effects on endothelial biology favoring a proinflammatory and proatherosclerotic phenotype.

The activation of proinflammatory mechanisms and the accumulation of monocytes and macrophages in the intima (in addition to lipid infiltration) are

characteristic of atherosclerosis. The cytokine, IL-6, has proinflammatory activity by itself and through increasing the levels of IL-1 and TNF α . All of these cytokines have been implicated in atherogenesis [Yudkin et al. 2000]. Importantly, IL-6 stimulates liver production of CRP. Serum CRP is an important marker of vascular inflammation and predictor of atherosclerosis [Ridker et al. 2003, Ridker et al. 2001, Ridker et al. 2000, Van Der Meer et al. 2002, Hashimoto et al. 2001]. Recent data suggest that CRP also promotes atherosclerosis. These findings together with the fact that CRP is present in the intima and media layers of human atherosclerotic arteries [Torzewski et al. 2000, Reynolds et al. 1987] and on the surface of foam cells [Torzewski et al. 1998], further suggest the involvement of CRP in atherosclerosis. Besides the production of CRP by the liver, macrophages and smooth muscle-like cells present in the atherosclerotic lesion also secrete CRP [Yasojima et al. 2001].

I.II.2.4.2. Microvascular complications

The major diabetic microvascular complications include retinopathy, nephropathy, and neuropathy [Greene. 1986]. As shown in figure 4, the Diabetes Control and Complications Trial (DCCT) [The Diabetes Control and Complications Trial Research Group. 1993], the UKPDS [UK Prospective Diabetes Study (UKPDS) Group. 1998], and the study by Ohkubo et al. [Ohkubo et al. 1995] studies point to a definite association between the severity and duration of hyperglycemia and microvascular complications in patients with type

2 diabetes. Specially evidence exists that patients with hemoglobin A1c levels greater than 8 %, are at high risk for these complications [Peters et al. 1998].

I.II.2.4.2.1. Retinopathy

Diabetic retinopathy is one of the earliest signs of the effects of sustained hyperglycemia on the microvasculature and the leading cause of adult blindness. While 20% of patients had retinopathy at the time of diagnosis [Peters et al. 1998], more than 60% of type 2 diabetic patients will develop some degree of diabetic retinopathy during the course of the disease [American Diabetes Association. 1998]. The retinopathy is usually categorized as background or proliferative. Notably, the UKPDS demonstrated that a significant decrease in the progression of retinopathy could be achieved in patients, treated with intensive insulin therapy compared to conventional therapy [The Diabetes Control and Complications Trial Research Group. 1993].

I.II.2.4.2.2. Nephropathy

Diabetes mellitus is the most common single cause of end-stage renal disease (ESRD) and accounts for about half of the patients receiving long term renal dialysis in the USA. When type 2 diabetes is diagnosed, the presence of nephropathy is already present in 8% of patients [Peters et al. 1998]. The earliest clinical evidence of nephropathy in type 2 diabetes is microalbuminuria. Progression to ESRD is associated with persistent proteinuria and hypertension.

In type 2 diabetes, high levels of glucose and glycohemoglobin are strongly associated with microalbuminuria [Alzaid et al. 1996].

I.II.2.4.2.3. Neuropathy

Diabetic neuropathy is classified as either peripheral or autonomic. Peripheral neuropathy is the most common manifestation of diabetic neuropathy [Singh et al. 1995], with symptomatic disease occurring in approximately 25% of patients with diabetes mellitus [Koda-Kimble et al. 1995]. The clinical presentation of peripheral neuropathy can range from mild-to-severe pain to decreased sensation primarily in the lower extremities [Koda-Kimble et al. 1995]. Autonomic neuropathy can manifest as impotence, gastroparesis, urinary retention, and diarrhea [Koda-Kimble et al. 1995]. Neuropathies can develop anytime during the progression of type 2 diabetes.

I.II.2.5. Diagnosis

The diagnostic criteria for diabetes mellitus have been modified from those formerly recommended by the NDDG [National Diabetes Data Group 1979] or WHO [World Health Organization 1985]. The revised criteria for the diagnosis of diabetes are shown in Table 8 [American Diabetes Association 2004]. Three ways to diagnose diabetes are possible, and each must be confirmed, on a subsequent day, by any one of the three methods given in Table 8. For example, one instance of symptoms with casual plasma glucose ≥ 200 mg/dl (11.1 mmol/l), confirmed *on a subsequent day* by 1) fasting plasma glucose (FPG) ≥ 126 mg/dl

(7.0 mmol/l), 2) an oral glucose tolerance test (OGTT) with the 2-h postload value ≥ 200 mg/dl (11.1 mmol/l), or 3) symptoms with a casual plasma glucose ≥ 200 mg/dl (11.1 mmol/l), warrants the diagnosis of diabetes.

Table 8. Criteria for the diagnosis of DM.

1. Symptoms of diabetes plus casual plasma glucose concentration ≥ 200 mg/dl (11.1 mmol/L). Casual is defined as any time of day without regard to time since last meal. The classic symptoms of diabetes include polyuria, polydipsia, and unexplained weight loss.

or

2. FPG ≥ 126 mg/dl (7.0 mmol/L). Fasting is defined as no caloric intake for at least 8 h.

or

3. 2-h postload glucose ≥ 200 mg/dl (11.1 mmol/L) during an OGTT. The test should be performed as described by WHO, using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water.

In the absence of unequivocal hyperglycemia, these criteria should be confirmed by repeat testing on a different day. The third measure (OGTT) is not recommended for routine clinical use.

I.II.2.6. Treatment

I.II.2.6.1. Diet and physical activity

I.II.2.6.1.1. Weight loss

The study by Long et al. [1994] indicated that weight loss in patients with severe clinical obesity prevented more than 30-fold, the progression of IGT to diabetes.

I.II.2.6.1.2. Life style changes

Changing lifestyle is the primary target in the prevention of type II diabetes. In a Swedish uncontrolled study, Eriksson et al. [Eriksson et al. 1991] initially reported that an increase in physical activity and a moderate loss of body weight, reduces the incidence of type 2 diabetes by 50% in middle-aged men with IGT. A Chinese study in patients with IGT, confirmed that a 6-year intervention with diet and physical activity leads, both in overweight and normal weight individuals, to an approximate 30-40% reduction risk of developing diabetes [Pan et al. 1997]. In the Finnish Diabetes Prevention Study (FDPS) [Tuomilehto et al. 2001], reduction in the incidence of diabetes in patients with IGT, was directly associated with their changes of lifestyle. Data from this study showed that lifestyle changes resulted in a 58% reduction of diabetic risk. Recently, the Diabetes Prevention Program (DPP) group performed the first study comparing the effects of lifestyle modification vs pharmacological therapy on the reduction of diabetic risk. This study found that lifestyle modification reduced diabetic risk by 58% and that compared to placebo, metformin treatment reduced diabetic risk by only 31% [DPP Group 2002]. This study also found that losing even 5-7 kg of body weight, reduced diabetic risk. This beneficial effect was accompanied by lower cholesterol levels as well as by a reduction in other cardiovascular risk factors [DPP Group 2002]. Liao et al [2002] recently reported that modification of lifestyle could influence BMI and fat distribution in Japanese American with IGT.

Although it is clear from these data that lifestyle changes may reduce the development of type 2 diabetes, substantial lifestyle modification still cannot be achieved in many developed countries. Additionally, in these countries, healthy individuals do not maintain long-term lifestyle changes and are subject to the danger of dietary carelessness and sedentary lifestyle. Due to the limitations of lifestyle modification in the prevention of type 2 diabetes, use of pharmacological agents is often required in type 2 diabetes.

I.II.2.6.2. Oral hypoglycemiants

Since type 2 diabetes is defined as a syndrome characterized by insulin deficiency, insulin resistance, and increased hepatic glucose output, medications used to treat type 2 diabetes are designed to correct one or more of these metabolic abnormalities. Currently, there are five distinct classes of hypoglycemic agents, including sulfonylureas, meglitinides, biguanides, thiazolidinediones, and alpha-glucosidase inhibitors. Each class of these agents displays unique pharmacological properties.

I.II.2.6.2.1. Sulfonylureas

Sulfonylureas derived from sulfonic acid and urea have remained the mainstay of antidiabetic therapy about five decades. Glyburide, glipizide, glimepiride, and gliclazide are the second-generation sulfonylureas, which are more potent than the first-generation sulfonylureas in the therapy of type 2

diabetes and in the prevention of diabetic vascular complications [Luna et al. 1999].

The sulphonylureas act by triggering insulin release from the pancreatic beta cell and may slightly improve insulin resistance in peripheral target tissues such as muscle and fat. A specific site on the adenosine triphosphate-sensitive potassium channels is occupied by sulphonylureas leading to closure of the potassium channels and subsequent opening of calcium channels resulting in insulin secretion [Zimmerman et al. 1997]. The overall clinical efficacy of sulphonylureas in patients with type 2 diabetes is related to the pre-treatment levels of fasting plasma glucose and HbA_{1c}. The higher the fasting glucose level, the greater the effect will be [DeFronzo et al. 1999, Feinglos et al. 1999, Luna et al. 1999]. Hypoglycemia is the most worrisome side effect of the sulphonylureas. It is of particular concern with agents that are metabolized to an active metabolite with significant renal excretion. These agents are long-acting compounds including chlorpropamide and glyburide, both of which should be avoided in the setting of impaired renal function and used with caution in elderly patients. Thus, shorter-acting compounds like tolbutamide and gliclazide have been relatively well tolerated and appear to be the best choice to treat elderly patients [Graal et al. 1999]. Glimepiride is associated with a lower incidence of hypoglycemia [Langtry et al. 1998].

I.II.2.6.2.2. Meglitinide

Meglitinides are rapid-acting insulin secretagogues targeting postprandial hyperglycaemia. This class of drugs is useful for patients who are at risk of hypoglycaemia with longer-acting sulfonylurea drugs [Loh et al. 2002]. Repaglinide is the first meglitinide analogue to become available for use in patients with type 2 diabetes. Neteglinide, the newest member of the class, has recently become available. Repaglinide achieves similar overall glycemic control to that provided with glyburide [Plosker et al. 2004]. Meglitinides rapid onset and relatively short duration of action allow for flexible meal schedules.

I.II.2.6.2.3. Metformin

Metformin is an insulin-sensitizing biguanide, widely used in the treatment of patients with type 2 diabetes. Metformin has a wide volume of distribution to the targeted organs because it is not protein bound [Davidson et al. 1997]. Moreover, metformin undergoes no modifications in the body, and is in its unchanged form rapidly excreted by the kidneys [Davidson et al. 1997]. Metformin improves hyperglycemia by reaching high concentrations in the small intestine [Wilcock et al. 1994, Wiernsperger et al. 1999] and decreasing intestinal absorption of glucose [Ikeda et al. 2000, Wilcock et al. 1991]. This action may contribute to decreased postprandial blood glucose levels [Wu et al. 1990]. Several reports indicate an antihypertensive effect of metformin in animals [Petersen et al. 1996, Katakam et al. 2000, Bhalla et al. 1996, Verma et al. 2000] and humans [Landin et al. 1991, Giugliano et al. 1993]. Potential mechanisms of

antihypertensive action of metformin are complex and include both insulin-dependent and insulin-independent vasodilatory actions [Kirpichnikov et al. 2002].

Metformin was recommended in a single drug therapy especially for obese subjects [Hermann et al. 1994, Johansen 1999]. In conjunction with diet, metformin reduces fasting glucose concentrations, and hemoglobin A1c values [Davidson et al. 1997, Hermann et al. 1994, DeFronzo et al. 1995]. The effectiveness of metformin monotherapy on lowering glucose has been shown to be independent of age, body weight, ethnicity, insulin and C-peptide levels [Hermann et al. 1994, Johansen 1999]. Treatment with metformin also significantly reduces both total and visceral fat [Pasquali et al. 2000]. In the liver, metformin only partially suppresses gluconeogenesis. It did not however, stimulate insulin production [DeFronzo et al. 1999, Wiernsperger et al. 1999].

Studies demonstrated that metformin achieved 10% to 30% reduction of free fatty acid oxidation [Wiernsperger et al. 1999, Perriello et al. 1994]. Based on the decreased levels of free fatty acids by metformin treatment, this drug could not only improve insulin secretion but might also help to correct impaired insulin secretion by β -cell [Patane et al. 2000]. Several pieces of evidence showed that metformin reduced both hepatic glucose and VLDL production through an activation of the hepatic AMP protein kinase pathway [Zhou et al. 2001]. Besides increasing insulin sensitivity in diabetic and nondiabetic subjects, metformin also lowers plasma insulin levels [Cusi et al. 1996, Bailey et al. 1996].

Importantly, the DPP study showed that metformin may directly delay or prevent the onset of type 2 diabetes by 31% [DPP Group 2002]. This study involved more than 3000 participants from 26 centers, using patients with IGT tested at 6-month intervals. These findings suggest that metformin may decrease the risk of development of type 2 diabetes. This beneficial effect may be related to the ability of metformin to decrease CRP [Arbar et al. 2003] and PAI-1 [Seli et al, 2002], two predictors of the development of diabetes.

Compared to other antidiabetic agents, metformin has several advantages such as lowering body weight, causing reduction levels of triglycerides and LDL-cholesterol, avoiding hypoglycemia, and improving fibrinolysis. These advantages of metformin accompanied by its good tolerability profile, place metformin as a first-line agent in the prevention and treatment of type 2 diabetes.

I.II.2.6.2.4. Thiazolidinediones

Thiazolidinediones act as insulin sensitizers and decrease peripheral insulin resistance by enhancing insulin-mediated glucose uptake by muscles. They have a lesser effect on insulin action in the liver. Thiazolidinediones play a beneficial role in the prevention of type 2 diabetes in individuals with IGT and other risk factors for diabetes [Nolan et al.1994]. Studies demonstrated that troglitazone improved β -cell function in subjects with IGT [Ehrmann et al. 1997]. In the Troglitazone in Prevention of Diabetes (TRIPOD) study [Azen et al. 1998], a 56% reduction in diabetes risk was observed following troglitazone treatment. Thiazolidinediones have rapid anti-inflammatory effects [Garg et al. 2000, Aljada

et al. 2001, Ghanim et al. 2001, Mohanty et al. 2001] and these effects may contribute to their beneficial action on insulin sensitivity. Troglitazone reduces ROS generation, NF κ B binding activity, and plasma CRP concentrations [Ghanim et al. 2001]. Troglitazone can cause fatal hepatic toxicity and has been withdrawn from the market, therefore alternatives such as pioglitazone and rosiglitazone are now being used in the clinical intervention trials.

I.II.2.6.2.5. Acarbose

Acarbose is a α -glucosidase inhibitor, which can improve insulin sensitivity, as well as decrease postprandial hyperglycemia through reducing the digestion of oligosaccharides. Thus, it may help reduce the hyperglycemic stress on the β -cells [Chiasson et al. 1996]. In the STOP-NIDDM trial [Chiasson et al. 2002], obese adults with IGT were treated with acarbose. Results of this study demonstrated that this drug induces in a 25-36% reduction of type 2 diabetic risk. The same study found that acarbose has anti-hypertensive effect, reduces postprandial glucose levels, and lowers insulin concentrations [Chiasson et al. 2002]. The specific advantages of acarbose can be summarized as follows [Levovitz et al. 2002]. It specifically lowers postprandial plasma glucose levels; Its action is independent of, and additive to, all other forms of pharmacologic therapy; It does not cause weight loss or gain, and it is relatively nontoxic [Lebovitz et al. 1998, Raptis et al. 2001].

I.II.2.6.2.4. Other medications that may reduce the risk of type 2 diabetes

Data obtained in a recent multi-centered international trial, have shown that the use of angiotensin converting enzyme (ACE) inhibitors such as Enalapril or Ramipril may prevent the onset of diabetes in people at risk of coronary heart disease [Simpson et al. 2003]. Other drugs such as angiotensin receptor blockers (ARBs) have been shown to reduce macrovascular complications in diabetes [Dahlof et al. 2002]. Because angiotensin II is a proinflammatory peptide, these drugs may exert their beneficial effects by reducing inflammation [Brasier et al. 2002, Phillips et al. 2002].

I.II.2.6.3. Insulin therapy

As β -cell function deteriorates during the course of type 2 diabetes, insulin secretagogues eventually become ineffective. At that stage, the patient needs insulin administration. Many studies have shown that insulin therapy greatly improves insulin secretion in patients with type 2 diabetes, presumably by reducing hyperglycemia [Della Casa et al. 1991, Ilkova et al. 1997]. Some studies have also demonstrated an improvement in peripheral insulin sensitivity after insulin therapy in type 2 diabetes [Garvey et al. 1985, Groop et al. 1989, Yki-Järvinen et al. 199, Foley et al. 1983]. Even short-term insulin therapy appears to result in long-term improvement in blood glucose control, especially when administered in the earliest stages of diabetes [Skyler et al. 1997, Ilkova et al. 1997]. Based on these observations, some diabetes experts have advocated initiating intensive insulin therapy early in the course of type 2 diabetes, or

immediately after a diet on exercise regimen fails, in an effort to preserve the remaining β -cell function and improve long-term glycemic control [Skyler et al. 1997, Glaser et al. 1999].

I.II.3. Gestational diabetes (GDM)

GDM is defined as any degree of glucose intolerance developing with the onset or discovered during pregnancy. Approximately 7% of all pregnancies are complicated by GDM, resulting in more than 200,000 cases annually. The prevalence of this disease may range from 1 to 14% of pregnancies, depending on the population studied [American Diabetes Association 2004]. Women with marked obesity, personal history of GDM, glycosuria, or a strong family history of diabetes are at high risk to develop GDM. GDM is associated with insulin resistance and endothelial dysfunction, and 60% of patients with GDM will develop diabetes within 15 years.

I.II.4. Other type of diabetes

This is a large inclusive group of less common and diverse disorders causing or associated with hyperglycemia. These probably account for 1 to 2 % of the cases of DM. They are listed in Table 4.

I.III. Lectin-like oxidized low density lipoprotein receptor-1 (LOX-1)

I.III.1. General

LOX-1 is a type II membrane protein belonging to the C-type lectin family of molecules. It is a newly identified endothelial receptor for oxLDL, which is also expressed by other vascular cell types, such as macrophages and SMCs [Sawamura et al. 1997]. LOX-1 expression is induced by various pathophysiological stimuli relevant to atherosclerosis and inflammation, including cytokines such as TNF α [Kume et al. 1998] and TGF β [Minami et al. 2000], fluid shear stress, oxidative stress [Murase et al. 1998] and homocysteine [Nagase et al. 2001], oxLDL [Aoyama et al. 1999, Dayan et al. 2000], angiotensin II [Li et al. 1999, Morawietz et al. 1999], endothelin [Morawietz et al. 2001], and AGE [Chen et al. 2001]. More importantly, studies demonstrate that LOX-1 is expressed in vivo in the vessels of hypertensive [Nagase et al. 1997], diabetic [Chen et al. 2001] and hyperlipidemic [Chen et al. 2000, Kakutani et al. 2001] animals and is upregulated in early human atherosclerotic lesions [Kataoka et al. 1999], suggesting that LOX-1 may be involved in the initiation of atherosclerosis.

I.III.2. LOX-1 properties

I.III.2.1. Structure of LOX-1 gene

I.III.2.1.1. Human LOX-1 gene

The length of the LOX-1 gene is more than 7 kb with 6 exons and 5 introns. The organization of the human LOX-1 gene is shown in figure 12.

I.III.2.1.2. 5'-flanking region of the LOX-1 gene

As shown in figure 12, a number of potential transcription-factor-binding sites are located in the 5'-flanking region of the gene. The *GATA-2*-binding sites are present at -180 and -1676 bp and are known to mediate endothelium-specific expression of endothelin-1 [Lee et al. 1999]. *c-ets-1* is present at -2274 bp and is expressed in endothelial cells during angiogenesis and tumour vascularization. It is also involved in the regulation of vascular endothelial growth-factor receptor [Macleod et al. 1992, Morishita et al. 1995]. 12-O-Tetradecanoylphorbol 13-acetate-Responsive Elements (*TREs*) are present at -60, -984 and -1714 bp. These elements are the binding sites for *AP-1* and are responsible for the induction of transcription of related genes by protein kinase C or phorbol ester [Angel et al. 1987]. A shear-stress responsive element (*SSRE*, GAGACC) is present at -1447 and -1011 bp. *SSRE* may be involved in the NF- κ B-mediated induction of LOX-1 gene expression [Collins et al. 1995, Khachigian et al. 1995]. The TATA box is located at -29 bp while the CAAT box is located at -99 bp. Sequence analysis of Rat LOX-1 5'-flanking region performed by computer analysis finds that there are six AP-1 sites, four AP-2 motifs, one NF κ B site, and one *SSRE* site [Nagase et al. 1998]. The consensus of NF κ B sequence presented in the promoter of rat LOX-1 gene is encoding as "gggattttac". The presence of these elements in the LOX-1 promoter suggests that LOX-1 is an inducible regulated gene.

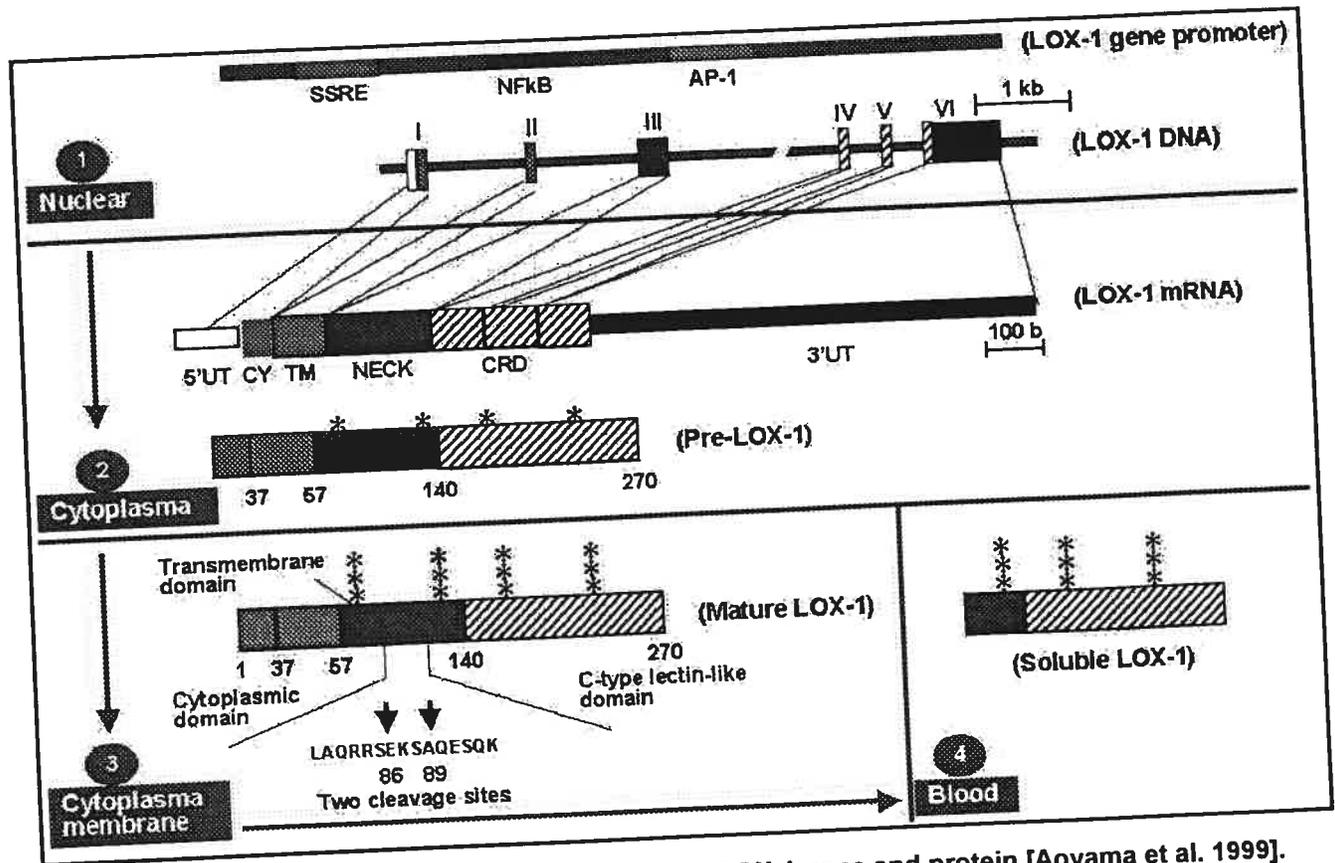


Figure 12. Diagrammatic representation of the LOX-1 gene and protein [Aoyama et al. 1999].

I.III.2.1.3. Human LOX-1 mRNA structural organization

The structural organization of the human LOX-1 mRNA is shown in figure 12. Exon 1 of the LOX-1 gene encoded the 5'-untranslated region and the cytoplasmic domain; the remainder of the cytoplasmic domain and the transmembrane domain of the protein are encoded by exon 2; the neck domain is encoded by exon 3, and finally the lectin domain and 3'-untranslated region are encoded by exons 4 to 6. Poly(A)⁺ is attached to the LOX-1 mRNA at 23 bp downstream of the AATAAA motif.

I.III.2.2. Structure of LOX-1 protein

As shown in figure 12, a Ca^{2+} -dependent carbohydrate-recognition domain (CRD) is present in C-type animal lectins. Based on the overall architecture of proteins, the position of the CRD domain, and the degree of homology of their CRD domains, C-type animal lectins have been classified into seven groups [Aoyama et al. 1999]. Group I includes proteoglycans, group II type-II membrane proteins including hepatic lectins, the Kupper cell receptor and the CD23 lymphocyte-activation antigen. Group III includes collectins with mannose-binding proteins, the pulmonary surfactant apoprotein and conglutinin. Group IV includes L-, P-, and E-selectins. Group V is a second group of type-II membrane proteins including NKR-P1, Ly49 and CD69. Group VI is the mannose receptor family. Group VII is the free CRD. LOX-1 belongs to the group V of the C-type lectin family [Dirckamer. 1988]. It has a type II membrane protein structure with a short N-terminal and a long carboxy-terminal hydrophilic domains separated by a hydrophobic domain of 26 amino acids [Sawamura et al. 1997].

I.III.2.2.1. The domain structures and functions of LOX-1

As shown in figure 12, the LOX-1 protein consists of four domains including the short N-terminal cytoplasmic domain, the transmembrane domain, the neck domain, and the C-type lectin-like domain [Sawamura et al. 1997]. Several potential phosphorylation sites including Thr 21 and Ser 28 (for PKC) and Thr 2 (for casein kinase II) have been found in the N-terminal cytoplasmic domain. Phosphorylation of these sites is involved in signal transduction and

LOX-1 regulation. Six repeats of cysteines in the lectin-like domain are totally conserved among all species. Truncation or deletion of the lectin domain of LOX-1 results in loss of oxLDL binding [Chen et al. 2001], as well as in loss of aged/apototic cell and negatively charged phospholipid binding ability [Chen et al. 2001] indicating its function as a ligand-binding domain, and initiator of the processes of internalization and phagocytosis [Chen et al. 2001].

I.III.2.2.2. Post-translational modification of LOX-1

As shown in figure 12, several potential N-linked glycosylation sites locate in the extracellular C-terminal domain of the LOX-1 protein [Sawamura et al. 1997]. Evidence indicates that LOX-1 is first synthesized as a 40-kDa precursor (pre-LOX-1) with N-linked high mannose carbohydrates, and then is subsequently glycosylated and processed into a 50-kDa mature LOX-1 protein, which is then transported to the cell surface [Kataoka et al. 2000]. Treatment of LOX-1 with N-glycanase reduces the molecular mass to 32 kDa, which is the predicted molecular size of LOX-1 lacking all the N-linked oligosaccharide moieties [Kataoka et al. 2000, Shi et al. 2001]. It has been proposed that N-linked glycosylation of LOX-1 regulates, in part, its intracellular transport and ligand binding activity. Altered glycosylation of LOX-1 may thus affect its biological functions *in vivo*.

I.III.2.2.3. Soluble forms of LOX-1

Many membrane proteins can be converted into soluble molecules by proteolytic cleavage [Ehlers et al. 1991, Rose-John et al. 1994, Hooper et al. 1997]. LOX-1 is one of these trans-membrane proteins which, can also be cleaved at the juxtamembrane region of the extracellular domain. The secreted soluble form of LOX-1 [Kume et al. 2001], has a molecular weight of 35 kDa. Because phenylmethylsulfonyl fluoride inhibits the release of soluble LOX-1 *in vitro*, a phenylmethylsulfonyl fluoride-sensitive serine protease(s) may be involved in the process of soluble LOX-1 secretion. Examination of the serum levels of LOX-1 *in vivo* may be a novel diagnostic strategy for the evaluation and prediction of atherosclerosis and vascular disease [Kume et al. 2001].

I.III.2.3. LOX-1 tissue distributions

LOX-1 is expressed not only in cultured vascular cells such as endothelial cells, macrophages, SMCs, fibroblasts, and platelets, but also in various tissues *in vivo* [Sawamura et al. 1997, Draude et al. 1999, Moriwaki et al. 1998, Yoshida et al. 1998]. For example, LOX-1 is expressed *in vivo* in aortic, carotid, thoracic, and coronary arteries and veins. It is mainly expressed in aortic intima and vascular-rich organs, such as placenta, lungs, brain and liver.

I.III.3. LOX-1 binding ligands

LOX-1 demonstrates binding activity for multiple ligands, which can be classified into five groups. Group 1 contains modified lipoproteins including ox-

LDL, acetylated LDL, and hypochlorite-modified HDL [Marsche et al. 2001, Moriwaki et al. 1998, Sawamura et al. 1997]. Group 2 contains polyanionic chemicals including polyinosinic acid and carrageenan. Group 3 contains anionic phospholipids including phosphatidylserine (PS) and phosphatidylinositol [Moriwaki et al. 1998, Oka et al. 1998]. Group 4 contains cellular ligands including apoptotic/aged cells, activated platelets, and bacteria [Kakutani et al. 2000, Oka et al. 1998, Shimaoka et al. 2001]. Group 5 includes AGEs [Jono et al. 2002].

Study of Kakutani's research team found that a novel sandwich enzyme immunoassay system utilizing anti-apolipoprotein B antibody and recombinant soluble LOX-1 can detect the lipoprotein LOX-1 ligand *in vivo* [Kakutani et al. 2001]. The same research team also evaluated the level of the lipoprotein LOX-1 ligand in plasma under some pro-atherogenic conditions. Their data demonstrated that plasma of WHHL rabbits shows increased levels of lipoprotein of LOX-1 ligand and that supplementation with antioxidants can attenuate the rapid increase of the lipoprotein LOX-1 ligand [Chen et al. 2002]. Taken together, this method may suggest a convenient approach for quantitative measurement of the lipoprotein LOX-1 ligand in routine clinical tests. This assay determining the level of modified LDL in the plasma *in vivo* may be useful for predicting ischemic heart diseases.

I.III.4. Regulation of LOX-1 expression

I.III.4.1. Inducing Factors

LOX-1 expression can be upregulated by pro-inflammatory factors stimuli, markers of oxidative stress, and under various pathological conditions.

I.III.4.1.1. Pro-inflammatory cytokines

LOX-1 expression is induced by the pro-inflammatory cytokine TNF- α in both endothelial cells and macrophages [Moriwaki et al. 1998, Kume et al. 1998]. It can also be upregulated by other proinflammatory cytokines including interleukin-1 β [Nakagawa et al. 2002] and endothelin-1 [Morawietz et al. 2001, Morawietz et al. 2002] in endothelial cells.

I.III.4.1.2. Pro-atherosclerotic stimuli

Besides pro-inflammatory cytokines, multiple pro-atherosclerotic stimuli regulate LOX-1 expression in vascular cells *in vitro*. For example, TGF- β 1 has been reported to upregulate LOX-1 expression in endothelial cells, macrophages, and SMCs [Draude et al. 2000, Chen et al. 2001, Minami et al. 2000]. Fluid shear stress has been shown to modulate various endothelial functions, including the expression of several genes such as endothelin-1 [Malek et al. 1993, Kuchan et al. 1993], TGF- β 1 [Ohno et al. 1995], and MCP-1 [Shyy et al. 1994]. Murase et al [1998] provided evidence that fluid shear stress transcriptionally induces LOX-

1 expression in vascular endothelial cells thus suggesting a role for LOX-1 in the localized formation of atherosclerotic lesions *in vivo*.

LOX-1 expression is also upregulated by phorbol 12-myristate 13-acetate (PMA) [Yoshida et al. 1998, Kume et al. 1998], lipopolysaccharide [Kume et al. 1998], and angiotensin II [Morawietz et al. 1999, Li et al. 1999]. PPAR α ligands, such as fenofibrate and WY14643, also enhance LOX-1 expression in cultured bovine aortic endothelial cells (BAECs) [Hayashida et al. 2001].

I.III.4.1.3. Oxidative stress

As mentioned before, oxidative stress is firmly implicated in the pathogenesis of atherosclerosis. Evidence exists that LOX-1 expression is not only upregulated by oxLDL, but also by the atherogenic lipid constituent of oxLDL, lysophosphatidylcholine [Aoyama et al. 2000, Mehta et al. 1998]. In addition, LOX-1 expression is induced by many stimuli associated with oxidative stress, such as 8-iso-prostaglandin F₂ α (8-iso-PGF₂ α), a marker of oxidative stress *in vivo* [Morrow et al. 1992]. Elevated formation of 8-iso-PGF₂ α is associated with cardiovascular risk factors such as hypercholesterolemia and diabetes mellitus [Patrino et al. 1997]. Halvorsen et al [2001] demonstrated that 8-iso-PGF₂ α increases LOX-1 expression both at the gene and protein levels in human JAR cells. Importantly, it has also been shown that AGEs, which are well known inducers of oxidative stress, bind to LOX-1 [Jono et al. 2002] and increase LOX-1 expression in human aortic endothelial cells (HAECs) [Chen et al. 2001]. Superoxide anions, as well as hydrogen peroxide, also increase LOX-1 mRNA

expression in cultured aortic endothelial cells [Nagase et al. 2001]. Finally, homocysteine, an atherogenic substance which exerts its effects through oxidative stress, enhances endothelial LOX-1 gene expression, an effect inhibited by *N*-acetylcysteine (NAC) [Nagase et al. 2001, Holven et al. 2003]. Overall, these results suggest a redox-sensitive regulation of LOX-1 expression.

I.III.4.1.4. Pathological conditions

LOX-1 expression is increased in experimental hypertension. Indeed, Nagase et al [1997] reported that Dahl salt-sensitive hypertensive rats spontaneously expressed high levels of LOX-1 mRNA in the aorta. Importantly, treatment of rats with the AT1 receptor blocker, losartan, not only decreases atherosclerosis, but also reduces endothelial LOX-1 expression. Morawietz et al [1999] demonstrated that endothelial LOX-1 is upregulated by angiotensin II *in vivo* and *in vitro*. The induction of LOX-1 expression is mediated by angiotensin II AT1 receptor. High LOX-1 expression is also documented in hyperlipidemia, as reflected by the upregulation of LOX-1 expression in the aorta of Watanabe-heritable hyperlipidemic rabbits and in high cholesterol-fed New Zealand white rabbits [Chen et al. 2000]. This augmented expression is primarily localized within the intima of early atherosclerotic lesions. Finally, increased LOX-1 expression has been observed in experimental diabetes, with increased LOX-1 expression in the endothelium and aorta of the diabetic rat [Chen et al. 2001].

I.III.4.2. Inhibitory factors

Li et al [2002] demonstrated that the 3-Hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors, simvastatin and atorvastatin, significantly reduce oxLDL-induced LOX-1 expression with consequent reduction of oxLDL-mediated activation of the redox-sensitive NF- κ B. Similar results were obtained by Mehta et al [2001] who reported that these drugs significantly reduced oxLDL-induced LOX-1 expression as well as eNOS expression. These results also provided evidence that both statins attenuated oxLDL-mediated activation of MAPK, and uptake of oxLDL by human coronary artery endothelial cells (HCAECs). Li et al [2001] reported that the effect of statins on LOX-1 expression is associated with an increase in PKB activity in HCAECs. Among other inhibitory factors of LOX-1 expression, Tempo, a superoxide dismutase and losartan, an ACE inhibitor, have been shown to reduce LOX-1 expression induced by angiotensin II [Nagase et al. 2001]. PPAR γ ligands including the natural ligand 15-deoxy-Delta (12,14)-prostaglandin J (2) (15d-PGJ(2)), and the thiazolidinediones, pioglitazone and troglitazone, have also been reported to decrease TNF α -induced LOX-1 mRNA expression in cultured BAECs [Chiba et al. 2001]. Finally, a recent study provided evidence that L-arginine was effective in decreasing LOX-1 expression in the donor organ after organ transplantation [Kosaka et al. 2003].

I.III.5. Role of LOX-1 in atherosclerosis

Uptake of oxLDL by vascular cells is considered a crucial step in the initiation and progression of atherosclerosis. As mentioned above, LOX-1 is expressed by vascular cells and is expressed *in vivo* in the vessels of hypertensive [Nagase et al. 1997], diabetic [Jono et al. 2002, Chen et al. 2001], and hyperlipidemic animals [Chen et al. 2000, Kakutani et al. 2001]. Importantly it is also upregulated in human atherosclerotic lesions [Kataoka et al. 1999] suggesting a role of LOX-1 in atherogenesis. The potential role of LOX-1 in atherosclerosis is shown in figure 13.

I.III.5.1. Role of LOX-1 in endothelial dysfunction

Numerous studies support a role of LOX-1 as a potential mediator of endothelial dysfunction. First, oxLDL binding to LOX-1 in endothelial cells generates superoxide anions, decreases NO, activates NF- κ B pathway [Cominacini et al. 2000, Cominacini et al. 2001], and induces the production of endothelin-1 and MCP-1 [Li et al. 2000]. Second, binding of oxLDL to LOX-1 induces apoptosis, a process involved in endothelial dysfunction [Li et al. 2000]. Third, LOX-1 is an adhesion molecule, favoring monocyte adhesion to endothelium [Honjo et al. 2003, Li et al. 2002]. Finally, it has been documented in a hypercholesterolemic rabbit model, that endothelial LOX-1 expression is upregulated during the early stage of atherogenesis i.e. before intimal accumulation of macrophage foam cells, thus suggesting a role for LOX-1 in endothelial activation during early atherogenesis [Chen et al. 2000].

I.III.5.2. Role of LOX-1 in the progression of the atherosclerotic lesion

Even though LOX-1 is a major receptor for oxLDL by endothelial cells, foam cell formation still has not been identified in endothelial cells either *in vitro* or *in vivo*. While uptake of oxLDL via LOX-1 in vascular endothelial cells may not result in massive lipid accumulation, this process may lead to endothelial activation and/or dysfunction, monocyte adhesion, and cell injury in the early stage of atherosclerosis. LOX-1 is not only expressed in endothelial cells but also in macrophages and SMCs [Yoshida et al. 1998, Draude et al. 1999]. Uptake of oxLDL by macrophages and subsequent foam cell formation play key roles in atherogenesis. It has been shown that LOX-1 expression is more prominent in macrophages and SMCs that have accumulated in the intima [Kataoka et al. 1999], thus suggesting a role for LOX-1 in foam cell transformation of macrophages and SMCs. Furthermore, Mehta et al [2002] have documented LOX-1 expression in the intima of advanced human atherosclerotic lesions, where LOX-1 co-localizes with apoptotic cells.

I.III.5.3. Role of LOX-1 in atherosclerotic plaques stability

It has been suggested that the composition of the atherosclerotic plaque is the most important determinant of plaque disruption and development of acute coronary syndromes [Libby. 1995]. MMPs are an endogenous family of enzymes that are responsible for vascular remodeling [Newby et al. 1994, Li et al. 2000]. Release of MMPs and enhanced collagenase activity may degrade various components of the fibrous cap and contribute to the vulnerability of plaques to

rupture. Recent studies have shown that increased activity and expression of MMPs play a crucial role in the composition of atherosclerotic plaques [Silence et al. 2001, Lemaitre et al. 2001]. The finding that oxLDL upregulates the expression of MMP-1 and -3 via LOX-1 activation strongly supports a role for LOX-1 in vasculature remodeling and plaque rupture [Li et al. 2003].

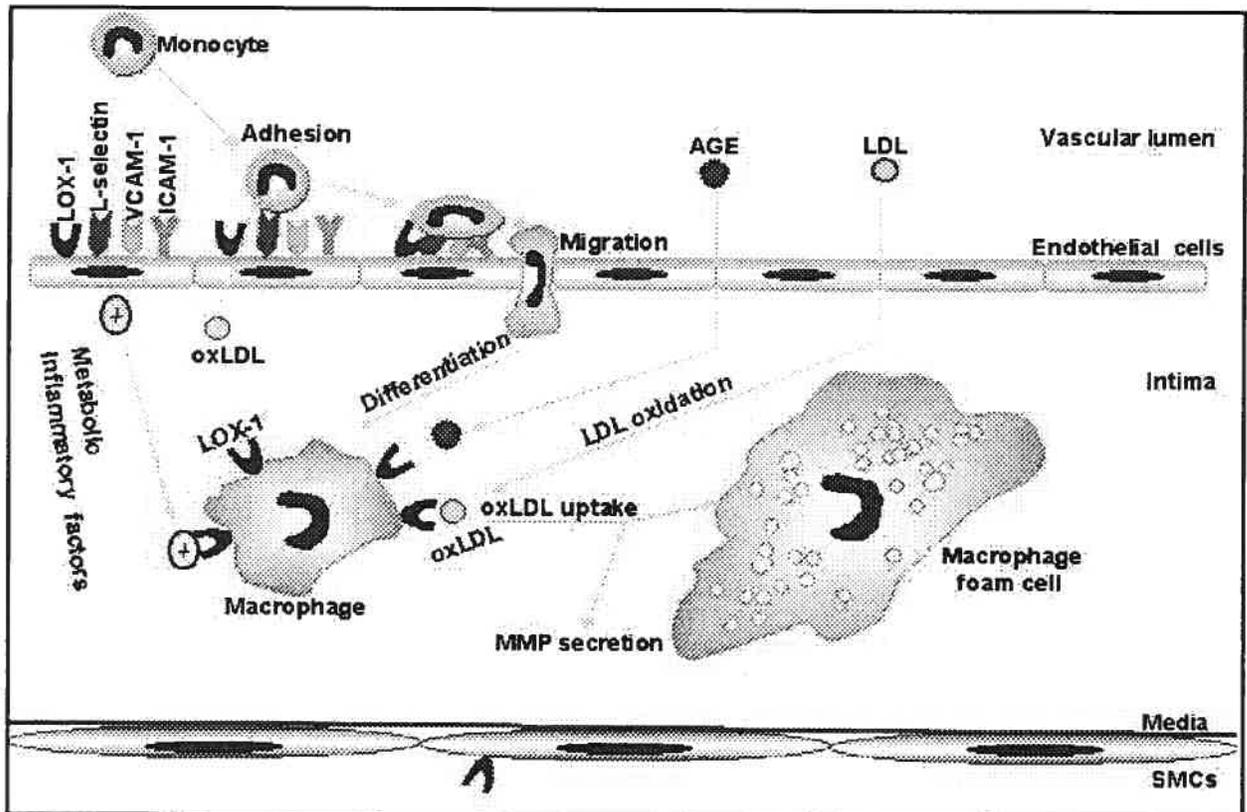


Figure 13. Role of LOX-1 in atherosclerosis. LOX-1 is expressed in endothelial cells, macrophages, and SMCs. LOX-1 is an adhesion molecule, involved in endothelial dysfunction and initiation and progression of atherosclerosis. LOX-1 is upregulated by multiple inflammatory cytokines and pathological stimuli. AGE and oxLDL are ligands for LOX-1.

I.III.6. Role of LOX-1 in type 2 diabetes

Elevated blood glucose levels can adversely affect the vessel wall through various mechanisms. Diabetes is characterized by a state of increased oxidative

stress, endothelial dysfunction, up-regulated expression of endothelial cell adhesion molecules, and glycosylation of virtually every protein in the body. It leads to enhanced oxidative modification of lipoproteins and AGEs formation. It has been shown that LOX-1 expression is increased in the aorta of STZ-induced diabetic rat [Chen et al. 2001]. In the same study, it was further demonstrated that diabetic rat serum and AGEs, which bound to LOX-1 [Jono et al. 2002] induce endothelial LOX-1 expression and that ligand activity is increased in the serum of diabetic rat. Our results showing that macrophage LOX-1 expression is increased in human type 2 diabetes, further support a role for LOX-1 in human diabetic vascular complications [Li et al. 2004].

I.IV. CRP

CRP was discovered in the plasma of patients during the acute phase of pneumococcal pneumonia. Its name was derived from a protein that binds to the C-polysaccharide of the pneumococcal cell wall [Tillett et al. 1930]. CRP is considered as a prototypical APP, although it accompanies both acute and chronic inflammatory disorders.

CRP is secreted by the liver in response to a variety of inflammatory cytokines including IL-6, IL-1 β , TNF α , interferon- γ , TGF- β , and possibly IL-8 [Wigmore et al. 1997]. A variety of other cell types including adipocytes produce these cytokines, but its most important sources at the inflammatory site are macrophages and monocytes [Wigmore et al. 1997].

Evidence suggests that CRP is a part of the innate immune system where it acts as a pattern recognition molecule to activate the adaptive immune response [Du Clos. 2000]. CRP is a member of the pentraxin family is composed of five identical 23-kDa subunits arranged in a calcium-dependent cyclic pentamer shape [Shrive et al. 1996]. Each subunit binds to phosphocholine (PC) with high affinity [Anderson et al. 1978]. The five ligand-binding sites of CRP are all on the same face of the pentamer [Du Clos. 2000]. On the opposite side of each subunit is an effector molecule-binding site that mediates the protein's interaction with C1q, Fc γ RI and Fc γ RII [Agrawal et al. 1994, Marnell et al. 1995, Bharadwaj et al. 1999]. Ligand recognition and binding by CRP may thus contribute to a range of metabolic, scavenging and host-defense functions.

Besides CRP, there are many other plasma proteins named APPs, which have been defined as those whose plasma concentration increases (positive APPs) or decreases (negative APPs) by at least 25 percent during inflammatory disorders. Among these are serum amyloid A (SSA), fibrinogen, albumin, transferrin, and many others [Du Clos. 2000]. After a moderate inflammatory stimulus, CRP and SSA levels increase rapidly up to 1000-fold within 1-2 days, whereas other APPs increase only by 50- to 200-fold in a slow response lasting several days [Du Clos. 2000].

Conditions that lead to substantial changes in plasma concentrations of APPs, include infection, trauma, surgery, burns, tissue infarction, and various immunological-mediated and crystal-induced inflammations [Gabay et al. 1999]. Because CRP decreases just as rapidly with the resolution of these conditions,

shows long-term stability during storage, has a very long half-life about 18-20 hours, does not show diurnal, age, and sex variations [Edward et al. 2003], its measurement appears a convenient biomarker to monitor various inflammatory states.

I.IV.1. CRP and Atherosclerosis

As discussed earlier, inflammation is considered as a major contributor to the pathogenesis of atherosclerosis. In this regard, the predictive association between markers of inflammation and CV events has been extensively confirmed. The interplay between the inflammatory process, cardiovascular risk factors, and atherothrombosis is complex [Liuzzo et al. 1994]. Since each step in atherogenesis involves cytokines, bioactive molecules, and inflammatory cells, the relationship between CRP and each parameter must be examined.

I.IV.1.1. CRP as a predictive risk marker of CV events

Numerous epidemiological studies have provided evidence that CRP is a predictive risk marker of CVD. Initially, in a large dyslipidemic population, Blackburn et al [1962] found that elevated CRP is an independent predictor of advanced carotid plaques in male subjects. Later on, Liuzzo et al [1994] and Haverkate et al [1995] established the prognostic usefulness of CRP in the setting of angina. Then, Tracy R et al [1997] identified CRP as an independent prospective CVD risk factor in healthy elderly men and women. Paul et al [1997] showed that men in the quartile with highest CRP values had three times the risk

of myocardial infarction and two times the risk of ischemic stroke compared to men in the lowest quartile. In 1999, Koenig et al reported that CRP predicts CHD in initially healthy middle-aged men in the Monitoring Trend and Determinants in CVD (MONICA) Augsburg cohort study. A recent prospective study provided evidence that CRP levels correlate with sudden cardiac death [Albert et al. 2002]. Taken together, these findings indicate that CRP is a powerful predictive marker of CV events. This notion has recently been challenged by results from a large study showing that the predictive value of CRP measurement in the serum adds relatively little to that provided by assessments of traditional risk factors [Danesh et al. 2004], suggesting that the clinical value of measuring CRP as a major predictor for the risk of CVD needs to be further evaluated.

As mentioned above, CRP has now emerged as one of the most powerful predictors of cardiovascular risk. CRP levels should be below $1\mu\text{g/mL}$ in an individual without inflammation. Patients with bacterial infections, autoimmune diseases, and cancer may have CRP levels higher than $100\mu\text{g/mL}$. As shown in figure 14, CRP levels in healthy individuals of less than 1, 1 to 3, and greater than $3\mu\text{g/mL}$ correspond to low-, moderate-, and high-risk groups respectively, for future CV events [Edward et al. 2003, Ridker. 2003]. If CRP levels show above $10\mu\text{g/mL}$, CRP testing should be repeated to exclude other pathological processes [Edward et al. 2003, Ridker. 2003]. Even though CRP levels correlate minimally with lipid levels, CRP levels should be interpreted in conjunction with the lipid profile. In 2000, Ridker et al reported that CRP is a better predictor of the

risk of CV events than LDL cholesterol in a study comprising 28,000 women and the same authors reported that CRP adds prognostic information at all levels of the Framingham Risk Score [Ridker. 2003]. For example, the Adult Treatment Panel III (ATP III) cut-points for LDL levels on coronary risk prediction were less than 130, 130 to 160, and greater than 160 mg/dL. Studies with LDL cholesterol below 130 mg/dL who have CRP levels greater than 3 $\mu\text{g/mL}$ represent a high-risk group often missed in clinical practice.

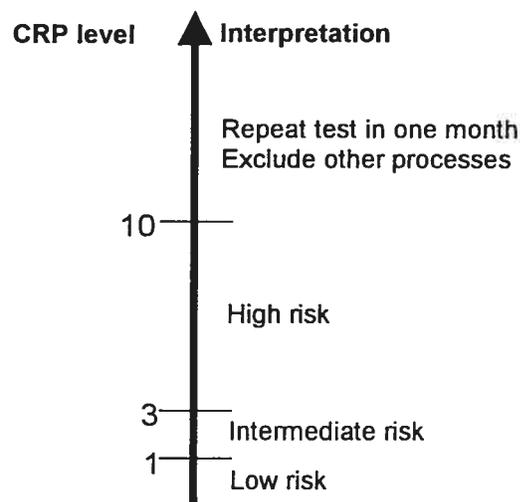


Figure 14. CRP level and CV risk. CRP levels are listed on the left and interpretations are on the right. [Yeh et al. 2003]

I.IV.1.2. Role of CRP in atherosclerosis

As mentioned above, inflammation plays a pivotal role in all stages of atherogenesis, and low-grade chronic inflammation as measured by high sensitivity CRP is thought to predict future risk of acute coronary syndrome independent of traditional cardiovascular risk factors. Increasing evidence

suggests that CRP may be directly involved in atherothrombogenesis that extends beyond its previously accepted role as an inflammatory marker. CRP is present in the intima and media layers of human atherosclerotic arteries. It is produced by macrophages and SMCs [Yasojima et al. 2001] and expressed on surface of foam cells [Torzewski et al. 1998], and serum CRP correlates with macrophage accumulation in coronary arteries of hypercholesterolemic pigs [Turk et al. 2003].

I.IV.1.2.1. Role of CRP on monocyte adhesion/recruitment

Monocyte adhesion to the endothelium and subsequent migration into the subendothelium, where they differentiate into monocyte-derived macrophage foam cells is believed to be the very early step in the inflammatory process of atherosclerosis. Evidence has been provided that CRP promotes chemotaxis of monocytes and increases expression of cell adhesion molecules and chemokines in human endothelial cells [Woollard et al 2002], thereby favoring monocyte-endothelial cell adhesion and monocyte recruitment into the vessel wall. Importantly, CRP deposition precedes the appearance of monocytes in early atherosclerotic lesions. CRP binds to monocytes through Fc γ RI/cd64 with low affinity [Marnell et al. 1995], as well as Fc γ RIIa/CD32 with high affinity [Bharadwaj et al. 1999]. Interaction of CRP with monocytes leads to enhanced release of proinflammatory cytokines including TNF α , IL-1 β and IL-6.

I.IV.1.2.2. Role of CRP on foam cell formation

Macrophages are key proinflammatory cells, which secrete abundant proatherosclerotic cytokines. After uptake of oxLDL through specific scavenger receptors, macrophages become foam cells, the hallmark of early atherosclerosis. It has been shown that CRP binds to apolipoprotein B – containing LDL and VLDL at their Ca²⁺-dependent PC binding sites and mediates LDL uptake by macrophages. These data suggest that foam cell formation in atherogenesis may result in part from this CRP effect. Further evidence for a role of CRP in early atherogenesis is the finding that CRP decreases eNOS expression and increases ET-1 expression. Importantly, Paul et al [2004] recently demonstrated that human CRP transgenic expression causes accelerated aortic atherosclerosis in apo E^{-/-} mice. These results provide final proof of the proatherogenic effects of CRP *in vivo*.

CRP may contribute to the development of atherosclerosis through several mechanisms. First, it may do so, by activating macrophage and SMC function leading to increased release of proinflammatory mediators and growth factors. Second, it may promote atherosclerotic lesion by activating the complement system. Through its ability to increase TF and PAI-1, it may also favor the progression of thrombosis and have a negative impact on vascular remodeling. Recently, Danenberg et al [2003] have provided the first *in vivo* evidence that arterial injury in CRP-transgenic mice results in an expedited and higher rate of thrombotic occlusion. These data suggest that CRP is a risk factor

and possible causal agent rather than merely a risk marker for increased rate of arterial thrombosis.

I.IV.1.3. Comparison of CRP to other risk factors

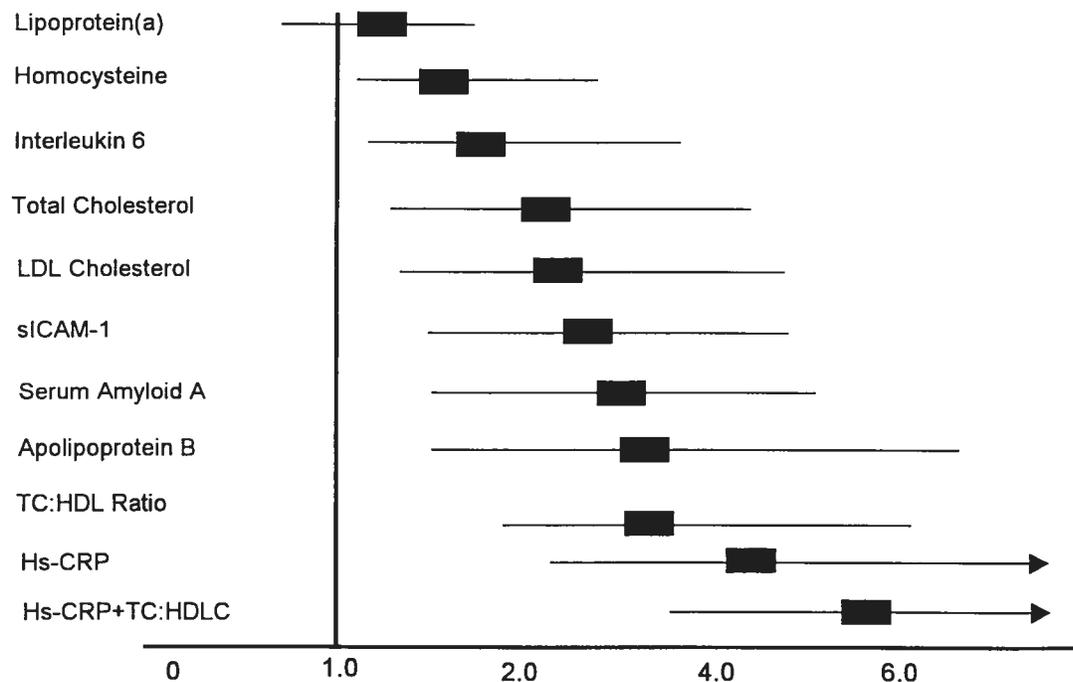


Figure 15. Direct comparison of CRP to several other lipid and non-lipid risk factors for CVD.

CRP is not the only inflammatory biomarker that has been shown to predict CV events. Many novel risk factors are also elevated among individuals at increased vascular risk [Blake et al. 2001]. Unfortunately, the assays required for their assessment are either inappropriate for routine clinical use or the protein of interest has too short half-life for clinical evaluation. For example, measurements of fibrinogen, a biomarker involved in both inflammation and

thrombosis remain poorly standardized. Comparison of CRP to other risk factors for CVD demonstrated that CRP is a stronger predictor of risk for CVD than all other markers as shown in figure 15 [Paul et al. 1997].

I.IV.1.4. Goal of screening and therapeutic options

The first goal of CV screening programs should be the identification of high-risk individuals. This group of individuals can be targeted for smoking control programs, improved diet and exercise regimes, and blood pressure control techniques. Even though there is currently no definitive evidence that lowering CRP will reduce CV event rates, many interventions known to reduce CV risk have been linked to lower CRP levels.

The benefits of statin drugs to decrease CRP levels to less than 3.6 μ g/mL and to decrease the number of CV events have been extensively demonstrated [Paul et al. 1997]. The peroxisome proliferator-activated receptor γ ligand, rosiglitazone has been found to reduce CRP levels in MI patients [Haffner et al. 2002]. Aspirin was also shown to reduce CRP levels in the primary prevention of CVD. Finally, the PPAR α agonists, fibrates, have also been found to decrease CRP levels [Kleemann et al. 2003].

I.IV.2. CRP and Diabetes

Many studies have demonstrated elevated serum levels of CRP among individuals with both features of the metabolic syndrome and overt type 2 diabetes. McMillan [1989] first reported that CRP increases markedly in both

diabetic and glucose intolerant subjects. Later, Rodriguez-Moran et al [1999] and Inukai et al [1999] reported that hyperglycemia is related to increased serum CRP levels in non-controlled type 2 diabetes. Importantly, Pradhan et al [2001] and Freeman et al [2002] recently demonstrated that CRP is a strong independent predictor of incident diabetes in large nationwide cohort studies. CRP has been shown to be associated with body mass index, fasting glucose levels, hyperinsulinemia and insulin resistance. In contrast to IL-6 whom effect was considerably attenuated after adjustment for BMI, the relation between CRP and the incidence of diabetes was still observed after adjustment of all risk factors. Ouchi et al [2003] recently demonstrated that elevated CRP levels are negatively correlated with the level of plasma adiponectin, an adipocyte-specific plasma protein associated with obesity-related metabolic and vascular diseases [Trayhurn et al. 2001]. It is important to note that CRP concentrations are significantly reduced by loss of body weight [Heilbronn et al. 2001, Tchernof et al. 2002].

I.V. Hypothesis and objectives

Endothelial dysfunction predicts CV events and is documented in patients with type 2 diabetes. Endothelial dysfunction can also be detected in obese patients, in relatives without diabetes as well as in the spectrum of insulin resistance. Thus, insulin resistance itself appears to be associated with endothelial dysfunction. Multiple interrelated mechanisms may contribute to endothelial dysfunction in insulin resistance. First insulin resistance may induce endothelial dysfunction through elements of the insulin syndrome, such as hypertension, dyslipidemia, and hyperglycemia. Insulin may also exert this effect independently of classic risk factors. One candidate mechanism underlying the effect of insulin resistance on endothelial dysfunction is low-grade inflammation. Indeed insulin resistance is closely associated with chronic subclinical inflammation and each component of the insulin resistance syndrome has been linked to the inflammatory cascade. In this regard, increased CRP levels have been shown to correlate significantly with features of the insulin resistance syndrome. Recently, CRP has been found to promote atherosclerosis and to predict the development of type 2 diabetes.

Endothelial LOX-1 is a novel identified receptor for oxLDL and a potential key mediator of oxLDL-induced endothelial dysfunction. LOX-1 is overexpressed in endothelium and aortas of diabetic rats, thus supporting a role of LOX-1 in human diabetic vasculopathy. In addition to endothelial cells, macrophages also express LOX-1. Expression of LOX-1 at the macrophage cell surface may favor foam cell formation. The nature of the metabolic and inflammatory factors

responsible for vascular LOX-1 upregulation in diabetes is unknown. Thus, the working hypothesis of our proposal was that human diabetes is associated with increased vascular LOX-1 expression and that this alteration contributes to the accelerated atherosclerosis associated with this disease.

The specific objectives of this study were:

- 1). To determine the direct *in vitro* effect of high glucose on LOX-1 expression in human endothelial cells and macrophages.
- 2). To determine the direct *in vitro* effect of CRP on LOX-1 expression in human endothelial cells.
- 3). To demonstrate the role of LOX-1 in glucose- or CRP-induced monocyte adhesion to endothelium as well as in oxLDL uptake by macrophages or endothelial cells.
- 4). To characterize the molecular mechanisms involved in the upregulation of endothelial and macrophage LOX-1 expression by glucose.

II. RESULTS

II.1. The first article

Glucose enhances endothelial LOX-1 expression. Role for LOX-1 in glucose-induced human monocyte adhesion to endothelium.

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ABSTRACT

Endothelial dysfunction is an early and key determinant of diabetic vascular complications that is elicited at least in part by oxidized low-density lipoprotein (oxLDL). The recent observation that lectin-like oxLDL receptor-1 (LOX-1) expression is increased in the vascular endothelium of diabetic rats suggests a role for LOX-1 in the pathogenesis of diabetic vascular dysfunction. Because postprandial plasma glucose has been recently proposed as an independent risk factor for cardiovascular diseases in patients with diabetes, we evaluated, in the current study, the in vitro effect of high glucose on LOX-1 expression by human aortic endothelial cells (HAECs) and the role of this receptor in glucose-induced human monocyte adhesion to endothelium. Exposure of HAECs to high D-glucose concentrations (5.6-30mmol/L) enhanced, in a dose- and time-dependent manner, LOX-1 expression, both at the gene and protein levels. The stimulatory effect of glucose on LOX-1 gene expression in HAECs was abolished by antioxidants as well as by inhibitors of nuclear factor- κ B (NF- κ B), protein kinase C (PKC) and mitogen-activated protein kinases (MAPK). Electrophoretic mobility shift assay data demonstrated that high glucose enhanced, in HAECs, the nuclear protein binding to the NF κ B regulatory element of the LOX-1 gene. Finally, our results showed that incubation of HAECs with high glucose increased human monocyte adhesion to endothelium through a LOX-1-dependent signaling mechanism. Overall, these results demonstrate that high glucose induces endothelial LOX-1 expression. This effect appears to be exerted at the transcriptional level through increased oxidant stress and NF- κ B, PKC and MAPK activation. The study also suggests a role for LOX-1 as mediator of the stimulatory effect of high glucose on monocyte adhesion.





INTRODUCTION

Atherosclerotic cardiovascular disease is the leading cause of death and the major complication of diabetes (1-5). Endothelial dysfunction is a key, early and potentially reversible event in atherogenesis that is commonly present in human diabetes (6-8) and plays a key role in the pathogenesis of diabetic vasculopathies (9). Several mechanisms may cause or contribute to endothelial dysfunction in diabetes mellitus. These include hyperlipidemia, oxidative stress, oxidized low-density lipoprotein (oxLDL), insulin resistance, formation of advanced glycated end products (AGE), activation of protein kinase C (PKC) and hyperglycemia (10-16). Recently, a role for lectin-like oxLDL receptor-1 (LOX-1), a novel endothelial cell receptor for oxLDL and AGE (17-18), in vascular cell dysfunction and monocyte adhesion has been proposed (19-20). The finding that LOX-1 expression is increased in the vascular endothelium of diabetic rats (21) suggests a role for this receptor in endothelial dysfunction associated with diabetes. The mechanisms responsible for the upregulation of vascular LOX-1 in diabetes are unknown. Pathophysiological stimuli relevant to atherosclerosis in diabetes that may contribute to this alteration include oxLDL, tumor necrosis factor- α (TNF- α) and advanced glycation end products (AGEs) (21-23). Since postprandial plasma glucose has recently been proposed as an independent risk factor for cardiovascular disease in patients with diabetes (24), we sought to investigate in the present study the regulation of endothelial LOX-1 expression by high glucose and the molecular mechanisms involved in this effect. On the basis of previous observations showing that hyperglycemia increases leukocyte-endothelial interaction (25-28) and that LOX-1



supports adhesion of leukocytes to endothelium (19-20), we further examined the role for LOX-1 in glucose-induced monocyte adhesion.

MATERIALS AND METHODS

Reagents

Fetal calf serum (FCS) was purchased from Wisent (St Bruno, Quebec). RPMI 1640 medium, phenylmethylsulfonyl fluoride (PMSF), Nonidet-P-40 (NP-40), Hank's balanced salt solution (HBSS), lymphoprep, penicillin-streptomycin, glycine, sodium dodecyl sulfate (SDS) and Trizol reagent were obtained from Gibco BRL (Burlington, Ontario, Canada). Human aortic endothelial cells (HAECs), endothelial growth culture medium (EGM) and EGM bullet kit were obtained from Clonetics (San Diego, CA, USA). D-glucose, L-glucose, mannitol, bovine serum albumin (BSA) fraction V, dianisidine dihydrochloride, hexadecyltrimethylamine ammonium bromide (HTAB), dithiothreitol (DTT), dimethylsulphoxide (DMSO), Vitamin E and vitamin C were purchased from Sigma. Monoclonal antibodies against β -actin, p50, and p65 were bought from Santa Cruz Biotechnology (Santa Cruz, CA). Recombinant human TNF- α , IgG₁ neutralizing antibody and monoclonal antibodies to human intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-Selectin were purchased from R&D Systems (Minneapolis, MN). Monoclonal antibody to human LOX-1 was kindly provided by Dr. Sawamura (National Cardiovascular Center Research Unit, Osaka, Japan). Calphostin C, BAY11-7085, N-acetyl-L-cysteine (NAC) and PD98059 were obtained from Calbiochem (La Jolla, CA). LY379196 was kindly provided by Eli Lilly (Indiana, USA).

Endothelial cells.

HAECs were grown to confluence in EGM under recommended conditions. The EGM was supplemented with 2% fetal bovine serum (FBS) containing 0.2 μ g/ml cupric sulfate, 0.01 μ g/ml human epidermal growth factor, 0.1% gentamicin sulfate

amphotericin-B, 1 μ g/ml hydrocortisone and 12 μ g/ml bovine brain extract protein content. Final LDL concentration in the EGM was around 40 μ g/ml. Determination of TBARS content in the EGM demonstrated that LDL oxidation occurred in the supernatants of HAECs cultured in normoglycemic (TBARS content: 1.4nmol/mg prot/ml) and hyperglycemic conditions (TBARS content:4.4nmol/mg prot/ml). Confluent cells were used in all experiments at passages 3-5. Evidence that these cells express Von Willebrand factor, adhesion molecules and cytokines and demonstrate PAI-1 activity supports the relevance of these cells as model for the study of native vascular endothelium.

Human monocytes.

Human monocytes were isolated as previously described (29). Briefly, peripheral blood mononuclear cells were isolated from healthy control subjects by density centrifugation using Ficoll, allowed to aggregate in the presence of FCS, then further purified by the rosetting technique. After density centrifugation, highly purified monocytes (85-90%) were recovered. Human monocyte purity was assessed by flow cytometry (FACScan, Becton Dickinson) using phyco-erythrin-conjugated anti-CD14 monoclonal antibody (Becton Dickinson).

Analysis of mRNA expression

Expression of the LOX-1 gene in human HAECs was measured by polymerase chain reaction (PCR) technique. Total RNA for use in the PCR reaction was extracted from cells by an improvement of the acid-phenol technique of Chomczynski (30). Briefly, cells were lysed with TRIzol reagent and chloroform was added to the solution. After centrifugation, the RNA present in the aqueous phase was precipitated and

resuspended in diethyl pyrocarbonate water. cDNA was synthesized from RNA by incubating total cellular RNA with 0.1 µg oligodT (Pharmacia) for 5 min at 98°C then by incubating the mixture with reverse transcription buffer for 1h at 37°C. The cDNA obtained was amplified by using 0.8µmol/L of two synthetic primers specific for human LOX-1 (5'-TTACTCTCCATGGTGGTGCC-3') (5'-AGCTTCTTCTDCTTGTTGCC-3') and human glyceraldehyde-3-phosphate deshydrogenase (GAPDH) (5'-CCCTTCATTGACCTCAACTACATGG-3') (5'-AGTCTTCTGGGTGGCAGTGATGG-3'), used as internal standard in the PCR reaction mixture. A 193-base pair human LOX-1 cDNA fragment and a 456-base pair human GAPDH cDNA fragment were amplified enzymatically by 30 and 20 repeated cycles, respectively. An aliquot of each reaction mixture was then subjected to electrophoresis on 1% agarose gel containing ethidium bromide. The intensity of the bands was measured by an image analysis scanning system (Alpha Imager 2000, Packard Instrument Company). Titrating the cDNA samples ensured that the signal lies on the exponential part of the standard curve. To achieve better quantification of LOX-1 mRNA expression, levels of LOX-1 mRNA were also measured in some experiments by Northern blot analysis. Ten millions HAECs were plated in plastic petri dishes (100x200mm) (Falcon, Lincoln Park, NJ). After treatment, cells were lysed with Trizol reagent. Total RNA was isolated and separated in a 1.2% agarose gel containing 2.2 mol/l formaldehyde. The blots were prehybridized for 6h. The mRNA expression was analyzed by hybridization with [³²P]dCTP-labeled human LOX-1 cDNA probe. Hybridization was detected by autoradiography with Kodak X-Omat-AR films (Rochester, NY). mRNA expression was quantified by high resolution optical densitometry (Alpha Imager 2000, Packard Instruments, Meriden, CT).

DNA binding assay

The isolation of the nuclei was performed as previously described (31). Briefly, 5×10^7 HAECs were collected, washed with cold phosphate-buffered salt solution (PBS), and lysed in 1 ml ice-cold buffer A (15 nmol/L KCL, 2 mmol/L $MgCl_2$, 10 mmol/L HEPES, 0.1 % PMSF, and 0.5% NP-40). After a 10-min incubation on ice, lysed cells were centrifuged, and the nuclei were washed with buffer A NP-40. The nuclei were then lysed in a buffer containing 2 mol/L KCL, 25 mmol/L HEPES, 0.1 mmol/L EDTA, and 1 mmol/L DTT. After a 15-min incubation period, a dialysis buffer (25 mmol/L HEPES, 1 mmol/L DTT, 0.1 % PMSF, 2 μ g/ml aprotinin, 0.1 mmol/L EDTA, and 11% glycerol) was added to the nuclei preparation. Nuclei were collected by centrifugation for 20 min at 13,000 rpm. Aliquots (50 μ l) of the supernatants were frozen at -70°C , and protein concentration was determined. DNA retardation (mobility shift) electrophoresis assays were performed as previously described by Fried and Crothers (32). Briefly, 5 μ g nuclear extracts were incubated for 15 min in the presence of 5 X binding buffer (125 mmol/L HEPES, pH 7.5, 50 % glycerol, 250 mmol/L NaCl, 0.25 % NP-40, and 5 mmol/L DTT) in the presence or absence of 200ng anti-p50 and anti-p65 antibodies. End-labeled double-stranded consensus sequences of the LOX-1 promoter AP-1-enhancing elements (20,000 cpm per sample) were then added to the samples for 30 min. Samples were analyzed on a 4% nondenaturing polyacrylamide gel (PAGE) containing 0.01% NP-40. The specificity of the nuclear protein binding was assessed by incubating the nuclear proteins isolated from HAECs with a labeled DNA probe in the presence of a 1,000-molar excess of unlabeled DNA probe.

DNA probes

Double-stranded oligonucleotides containing the NF- κ B (5'-CGTCTGCCCTTTCCCCCTCT-3'; 5'-GAGAAGAGGGGGAAAGG-3') consensus sequence of the human LOX-1 gene promoter (33) were synthesized with the aid of an automated DNA synthesizer. After annealing, the oligonucleotides were labeled with [γ - 32 P] ATP using the Boehringer-Mannheim 5' end-labeling kit (Indianapolis, IN).

Western-Blot

HAEC protein extracts (15 μ g) were applied to 10% SDS-PAGE and transferred to a nitrocellulose membrane using a Bio-Rad transfer blotting system at 100 V for 1h. Non-specific binding was blocked with 3% BSA for 1h at room temperature. After washing with PBS-Tween 0.1%, blots were incubated overnight at 4°C with anti-LOX-1 or anti- β -actin antibody. After further wash, membranes were incubated for 1h at room temperature with a horseradish peroxidase-conjugated donkey anti-mouse IgG (1/5000). Antigen detection was performed with an enhanced chemiluminescence detection system (Amersham).

Adhesion assay

Confluent HAECs were exposed for 72h to 5.6 or 30mmol/L glucose, then treated for 1h in the presence of antibodies to IgG₁, LOX-1, ICAM-1, VCAM-1 or E-Selectin. HAECs were then washed twice with HBSS and incubated for 2h with freshly purified human monocytes (280,000 cells/well) resuspended in serum-free RPMI medium. At the end of this incubation period, non-adherent monocytes were removed by washing the cells with PBS (pH 6.0). Monocyte adhesion to HAECs was quantitated by measuring monocyte myeloperoxidase (MPO) activity (34).

Statistical Analysis

All values were expressed as the mean \pm SEM. Data were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey test. A P value less than 0.05 was considered statistically significant.

RESULTS

Effect of D-glucose on endothelial cell LOX-1 mRNA expression.

Incubation of HAECs for 24 to 96h with D-glucose (5.6-30 mmol/L) enhanced, in a time-dependent manner, LOX-1 mRNA levels in these cells. Maximal effect was observed from 48 to 96h (Fig. 1A-a). Glucose-induced expression LOX-1 mRNA levels, normalized to the levels of GAPDH mRNA (Fig. 1A-b) are presented in Fig. 1A-c. Incubation of HAECs for 48h with increasing D-glucose concentrations (5.6-30 mmol/L) enhanced, in a dose-dependent manner, LOX-1 mRNA levels in these cells (Fig. 1B-a). Maximal effect was observed with a concentration of 30mmol/L glucose. LOX-1 mRNA levels, normalized to the levels of GAPDH mRNA (Fig. 1B-b) are presented in Fig. 1B-c. Incubation of HAECs with L-glucose or mannitol (30mmol/L) did not induce LOX-1 mRNA expression (LOX-1 mRNA expression [% of control values]: L-glucose: 102 ± 7 , mannitol: 97 ± 9). Quantification of LOX-1 mRNA levels by Northern blot analysis in HAECs exposed for 48h to 5.6 or 30mmol/L glucose is presented in Fig .1 C.

Effect of D-glucose on endothelial cell LOX-1 protein expression.

Treatment of HAECs for 24 to 96h with 5.6 or 30mmol/L glucose increased LOX-1 protein expression in these cells. Maximal effect was observed from 72 to 96h (Fig. 2A-a). LOX-1 protein levels normalized to the levels of β -actin (Fig.2A-b) are illustrated

in Fig. 2A-c. Incubation of HAECs for 72h with increasing D-glucose concentrations (5.6-30 mmol/L) enhanced, in a dose-dependent manner, LOX-1 protein expression in these cells (Fig. 2B-a). Maximal effect was observed with a concentration of 30mmol/L glucose. LOX-1 protein levels normalized to the levels of β -actin (Fig.2B-b) are illustrated in Fig. 2B-c. No stimulatory effect of mannitol (30mmol/L) on endothelial LOX-1 protein expression was observed (LOX-1 protein expression [% of control values]: mannitol: 105 ± 10).

Since $\text{TNF}\alpha$ is increased in diabetes and enhances endothelial LOX-1 expression in vitro, we next measured the levels of LOX-1 protein elicited by this cytokine in low or high glucose conditions. As shown in Fig.3, $\text{TNF}\alpha$ -treated endothelial cells cultured under normoglycemic conditions express similar LOX-1 levels than high glucose-treated cells. Levels of LOX-1 protein elicited by this cytokine were reduced in HAECs exposed to high glucose concentrations (Fig.3A). Under these experimental conditions, no modulation of the β -actin protein levels, used as internal control, was observed (Fig.3B). LOX-1 protein levels normalized to the levels of β -actin are illustrated in Fig. 3C.

Role of PKC, mitogen-activated protein kinases (MAPK) and $\text{NF-}\kappa\text{B}$ in the upregulation of endothelial cell LOX-1 gene expression by glucose.

To determine whether glucose induces endothelial LOX-1 gene expression via PKC- and/or MAPK-dependent pathways, HAECs were pretreated for 1h in the presence or absence of the pan specific PKC inhibitor, calphostin C (0.1 $\mu\text{g/ml}$), the PKC β inhibitor, LY379196 (30nM) or the MAPK inhibitor, PD98059 (50 μM) prior exposure to high glucose. As shown in Fig.4A-a, incubation of HAECs with these

inhibitors totally prevented the stimulatory effect of high glucose on LOX-1 mRNA expression. The inhibitory effect of LY379196 was still observed when cells were exposed to 20 mmol/l glucose (LOX-1 mRNA expression [% of control values]: Glucose (20mmol/l): 150 ± 9 ; glucose+LY379196: 113 ± 8 , $P < 0.05$). Under these experimental conditions, no modulation of the mRNA expression of GAPDH, used as internal control, was observed (Fig. 4A-b). LOX-1 mRNA levels, normalized to the levels of GAPDH mRNA are presented in Fig. 4A-c. Similar suppressive effect was observed when these cells were incubated with BAY 11-7085 ($40 \mu\text{M}$), an inhibitor of the oxidative stress-sensitive transcription factor NF- κ B (Fig.4A).

Role of oxidative stress in high glucose-stimulated endothelial cell LOX-1 gene expression.

Because vascular cells respond to high glucose by altering the intracellular redox state, we next determined the role of oxidative stress in glucose-induced LOX-1 mRNA expression. HAECs were pretreated for 1 h in the presence or absence of NAC (10mM) DMSO (0.5%), vitamin C ($10 \mu\text{M}$) or vitamin E ($50 \mu\text{M}$) and then incubated for 48h with 30 mmol/L glucose. As shown in Fig 4B-a, these antioxidants prevented the stimulatory effect of high glucose on HAECs LOX-1 gene expression. LOX-1 mRNA levels, normalized to the levels of GAPDH mRNA (Fig. 4B-b) are presented in Fig. 4B-c.

Effect of high glucose concentrations on the binding of nuclear proteins to the regulatory NF- κ B sequences of the LOX-1 gene promoter.

We next determined whether incubation of HAECs in the presence of high glucose concentrations might induce changes at the level of LOX-1 gene promoter binding proteins. We found that a 24-h exposure of HAECs to a high glucose

environment resulted in a dramatic increase in the binding of nuclear proteins to the NF- κ B consensus sequence of the human LOX-1 promoter. (Fig.5). This binding was specifically competed in the presence of a 1,000-fold molar excess of the unlabeled NF- κ B oligonucleotide and was totally abrogated by BAY 11-7085 and antibodies against p50 and/or p65 (Fig. 5). Inhibition of high glucose-induced NF- κ B binding was further observed after treatment of the cells with NAC, PKC and MAPK inhibitors (Fig.5).

Role for LOX-1 as mediator of the stimulatory effect of high glucose on monocyte binding to endothelial cells.

Treatment of HAECs for 72h with 30mmol/L glucose significantly increased monocyte adhesion to these cells (Fig.6). This effect was totally suppressed by preincubating HAECs with anti-LOX-1 antibody (Monocyte adhesion [% of control values]: 30 mmol/L glucose: 180 ± 16 $P < 0.05$, anti-LOX-1: 83 ± 10 $P < 0.001$, anti-IgG: 191 ± 24) (Fig.6). To test the involvement of selectins and integrins in glucose-induced monocyte adhesion to endothelium, HAECs were also preincubated with antibodies to ICAM-1, VCAM-1, and E-Selectin prior exposure to glucose. Our results demonstrate that under our experimental conditions, exposure of the cells to these antibodies did not inhibit glucose-induced monocyte adhesion to endothelial cells (Monocyte adhesion [% of control values]: 30 mmol/L glucose: 180 ± 16 $P < 0.05$, anti-ICAM-1: 167 ± 33 , anti-VCAM-1: 163 ± 16 , anti-E-Selectin: 156 ± 23).

DISCUSSION

Evidence that LOX-1 expression is increased in the vascular endothelium of diabetic rats (21) supports a role for this receptor in endothelial dysfunction associated with diabetes. The factors responsible for vascular LOX-1 upregulation in diabetes are unknown but may include atherogenic lipoproteins and AGE (21). In the present study, we demonstrated that high glucose enhances LOX-1 expression, both at the gene and protein levels, in cultured HAECs and that this effect requires glucose metabolism by endothelial cells since the nonmetabolized glucose isomer failed to produce similar effects. These results suggest that hyperglycemia per se may contribute to LOX-1 induction in diabetes. In contrast to our data, Chen et al recently reported that control rat serum, along with high glucose concentrations, did not enhance LOX-1 mRNA expression in cultured bovine aortic endothelial cells (21). Because both studies differ in many experimental aspects, including the cell type tested and the stimulatory conditions used, these conflicting results may only be apparent. Our findings that glucose-induced LOX-1 expression is comparable to that elicited by $\text{TNF}\alpha$ and that glucose and $\text{TNF}\alpha$ do not synergize for LOX-1 induction support the possibility that these factors may regulate LOX-1 expression through one major and possibly identical pathway. Glucose and $\text{TNF}\alpha$ are both well known activators of $\text{NF}\kappa\text{-B}$ (35-39) and the 5' flanking region of the LOX-1 gene contains a consensus $\text{NF}\kappa\text{-B}$ binding site-like sequence (33). While evidence has been provided that $\text{TNF}\alpha$ activates the transcription of the LOX-1 gene (23), we found that high glucose increases endothelial LOX-1 mRNA levels and enhances nuclear protein binding to the $\text{NF}\kappa\text{-B}$ regulatory sequence of the LOX-1 promoter. In agreement with previous studies showing that high glucose and $\text{TNF}\alpha$

stimulate the p50 and p65 subunits of NF- κ B in monocytic cells (39), we found that the p50 and p65 are critical components of the transacting NF- κ B complex stimulated by high glucose in endothelial cells. Taken together, these results suggest that transcriptional regulation of the LOX-1 gene mediated by p50 and p65 may be involved in LOX-1 induction by glucose and TNF α . Another interesting finding of this study is that culturing endothelial cells in high glucose medium reduces the responsiveness of these cells to further TNF- α stimulation. One possible explanation for this observation is that high glucose may stimulate the release of TNF- α by endothelial cells and that accumulation of this cytokine into the culture medium may contribute, at least in part, in decreasing TNF α -responsiveness of these cells. Although the effect of high glucose on TNF α secretion by endothelial cells has not been studied yet, evidence exists that these cells secrete TNF α in response to endotoxin and hydrogen peroxide (40-41). The possibility that glucose may stimulate TNF α release by endothelial cells is currently examined.

It is well demonstrated that vascular cells, including endothelial cells, respond to high glucose by altering the intracellular redox state (37,39, 42-43) and increasing PKC and MAPK activation (44). The clear link established between oxidative stress evoked by glucose and PKC/MAPK activation (37,39,45-46) as well as the documented ability of these kinases to regulate NF- κ B activity (47-48) suggest that glucose-stimulated LOX-1 gene expression in HAECs could involve increased oxidative stress and activation of PKC and MAPK-dependent pathways. Our results which demonstrate that antioxidants and inhibitors of PKC and MAPK prevented the activation of NF- κ B and the induction of endothelial LOX-1 mRNA expression by glucose confirm this possibility.

Evidence that the PKC β isoform-specific inhibitor, LY379196, effectively inhibits glucose-induced LOX-1 mRNA levels further indicate a role for PKC β in this effect. On the basis of these results, we elaborate a tentative model in which reactive oxygen species (ROS) generated by glucose metabolism induces the coordinate activation of PKC and MAPK as upstream kinases and NF- κ B as downstream transcription factor leading thereby to the induction of LOX-1 gene expression.

One major adverse vascular consequence associated with endothelial dysfunction in diabetes is increased monocyte adhesion to endothelium. Evidences that endothelial LOX-1 supports adhesion of monocytes and is upregulated in diabetes (19-21), suggest a role for this receptor in the enhanced binding of monocytes documented in patients with diabetes (28, 49-50). Previous studies have shown that glucose may contribute to the increased monocyte adherence to endothelium in diabetes (25-28). It has been proposed that the effect of glucose on monocyte adhesion may occur via mechanisms involving the upregulation of endothelial and/or leukocyte adhesion molecules (25, 26, 28, 51-52), the activation of NF- κ B and the generation of ROS (27). Our results showing that blockade of LOX-1 totally abolished glucose-induced monocyte adhesion to endothelial cells demonstrate a new role for LOX-1, that of mediating the stimulatory effect of glucose on monocyte adhesion. In contrast, our findings that blocking antibodies to ELAM-1, ICAM-1 and VCAM-1 did not significantly affect glucose-induced monocyte adhesion to HAECs clearly indicate that, under our experimental conditions, these adhesion molecules are not key determinants of glucose-induced monocyte binding to endothelium. Besides glucose, hypertriglyceridemia has been documented to enhance monocyte binding to endothelial cells in type 2 diabetes (49).

Because VLDL fractions of diabetic serum induce endothelial LOX-1 expression (21), a possible role for this receptor as mediator of hypertriglyceridemia-induced monocyte-endothelial adhesion may be proposed. Experiments aimed at testing this possibility are under way.

One limitation of this study is the use of high glucose concentrations. While plasma glucose concentrations around 20mmol/L are not seen in patients with diabetes chronically, 30mmol/L glucose is virtually incompatible with human life in chronic diabetes. In contrast, hyperglycemic excursions are frequent in patients with diabetes and peak glucose concentrations reaching 15 to 20mmol/L are documented in poorly controlled diabetic patients. Whether in vitro exposure of vascular cells to high glucose concentrations may replicate, to some extent, the conditions experienced by the cells of patients with diabetes during short-lived hyperglycemic excursions is unknown.

In conclusion, our data indicate that high glucose concentrations enhance LOX-1 expression in endothelial cells. They also demonstrate a new role for LOX-1 as mediator of glucose-induced monocyte adhesion. A better understanding of the mechanisms of hyperglycemia-induced endothelial dysfunction may unmask new strategies to reduce diabetic vascular complications.

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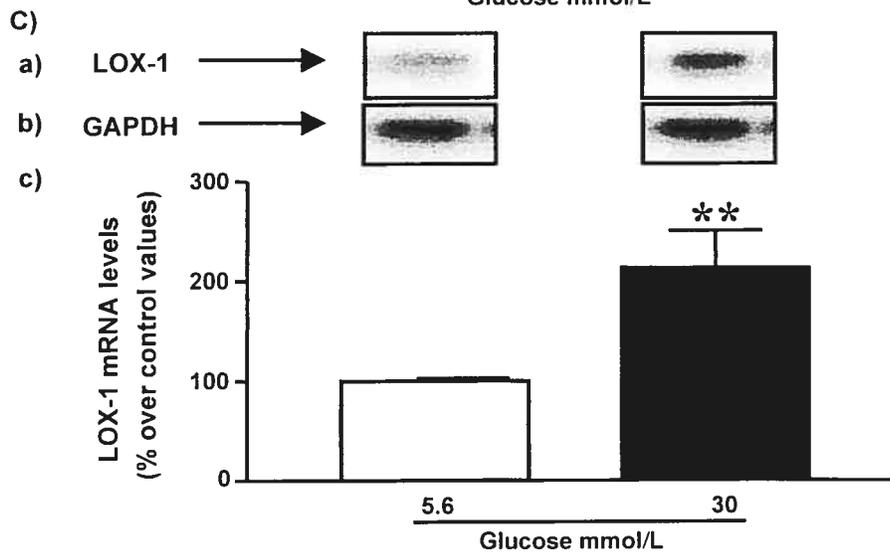
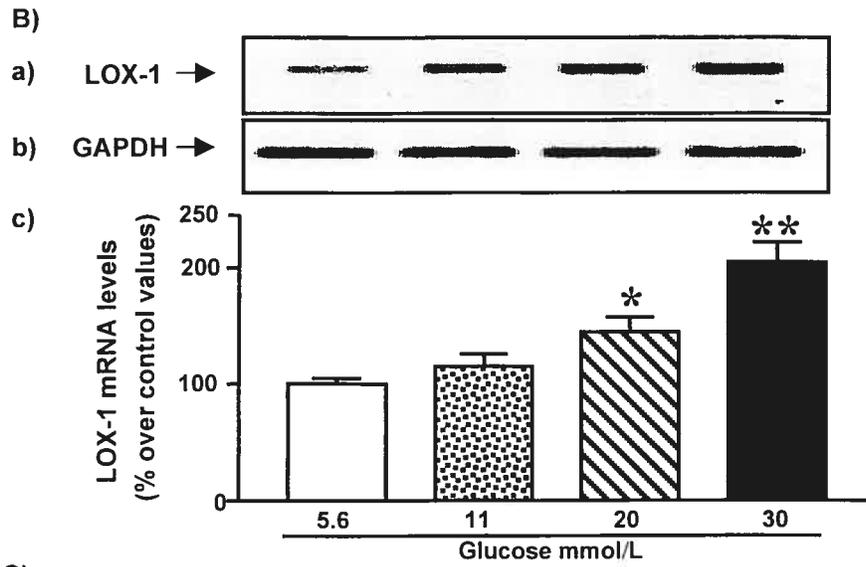
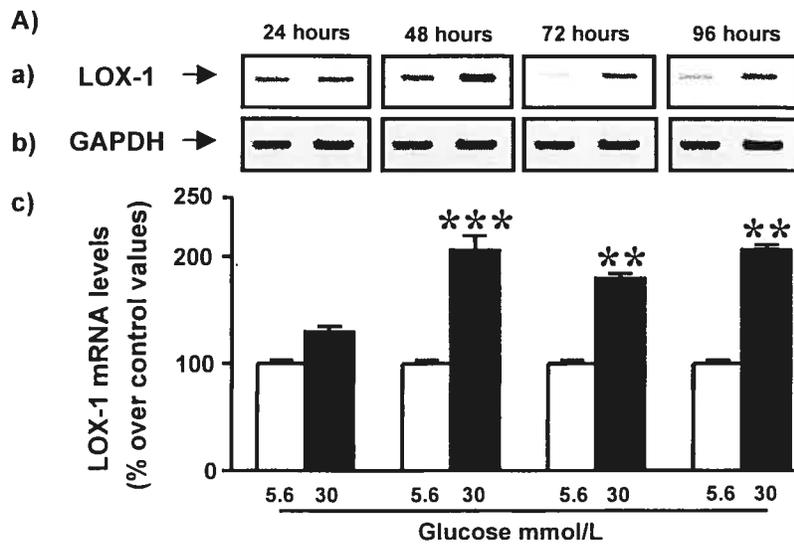


Fig.1. Time- and dose-dependent effect of high glucose on LOX-1 mRNA levels in HAECs. Cultured HAECs were incubated for 24 to 96h (A) or 48 hours (B, C) with 5.6 to 30 mmol/L glucose. At the end of the incubation period, cells were lysed and LOX-1 mRNA was analyzed by RT-PCR (A-B) or Northern Blot analysis (C). LOX-1 mRNA levels (a) were normalized to the levels of GAPDH mRNA (b). Data illustrated on the graph bar (c) represent the mean \pm SEM of 6 (A-B) or 3 (C) different experiments. *, $p < 0.05$, **, $p < 0.01$ ***, $p < 0.001$ vs 5.6 mmol/L glucose.

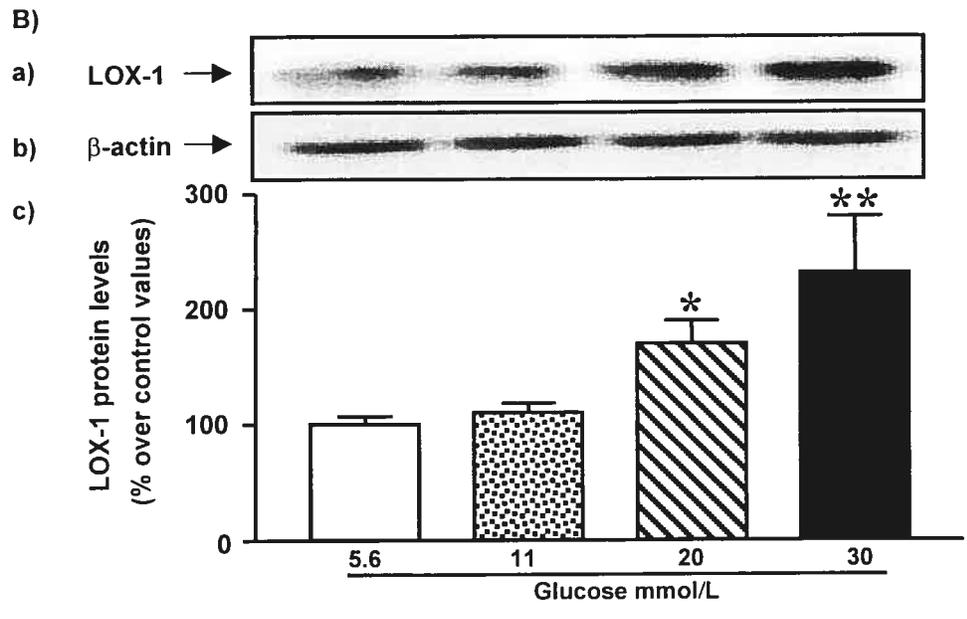
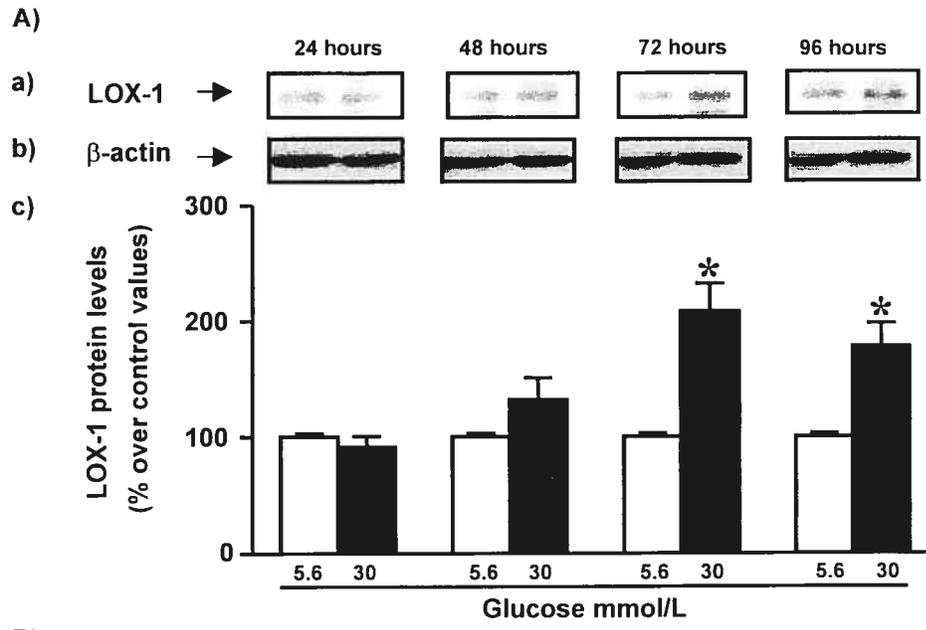


Fig.2. Time- and dose-dependent effect of high glucose on LOX-1 protein expression in HAECs. HAECs were cultured for 24 to 96h (A) or 72 hours (B) with 5.6 to 30 mmol/L glucose. At the end of the incubation period, cells were lysed and LOX-1 membrane protein expression was determined by Western blot analysis (a). LOX-1 protein levels were normalized to the levels of β -actin protein (b). Data illustrated on the graph bar represent the mean \pm SEM of 4 (A) and 3 (B) different experiments (c). *, $p < 0.05$, **, $p < 0.01$ vs 5.6 mmol/L glucose.

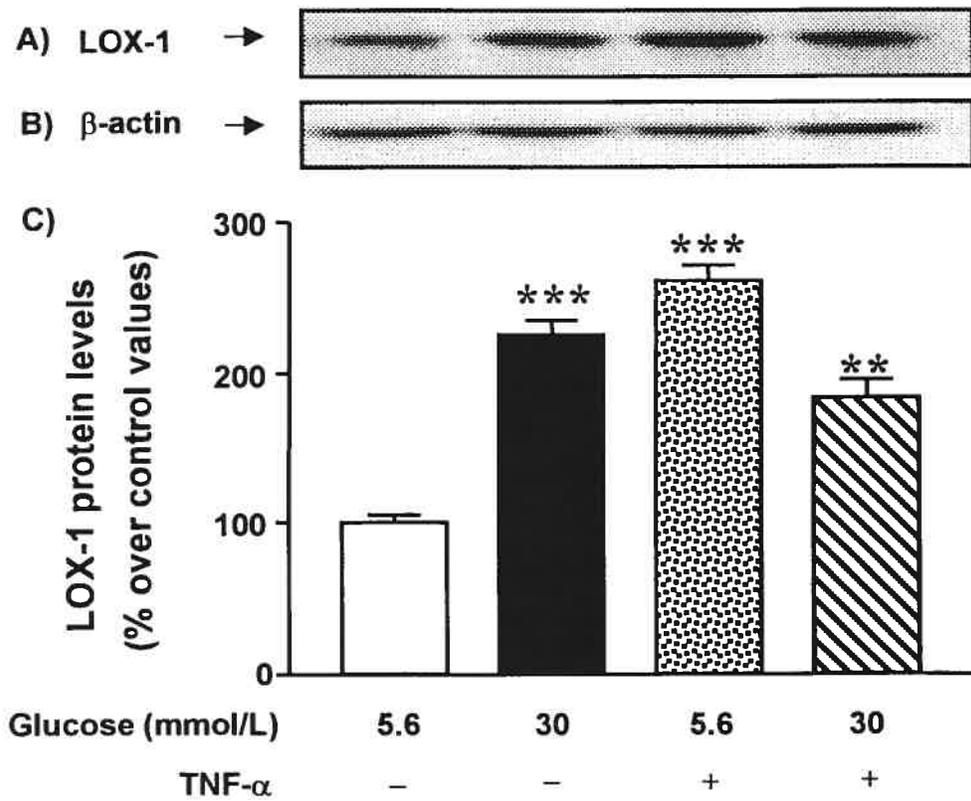


Fig.3. Effect of high glucose and TNF α on endothelial LOX-1 protein expression. HAECs were cultured for 48h in low (5.6mmol/L) or high (30mmol/L) glucose environment, then treated for an additional 24h time period with TNF- α (50ng/ml). At the end of the incubation period, cells were lysed and LOX-1 membrane protein expression was determined by Western blot analysis (a). LOX-1 protein levels were normalized to the levels of β -actin protein (b). Data illustrated on the graph bar represent the mean \pm SEM of 4 different experiments (c). **, $p < 0.01$, ***, $P < 0.001$ vs 5.6 mmol/L glucose.

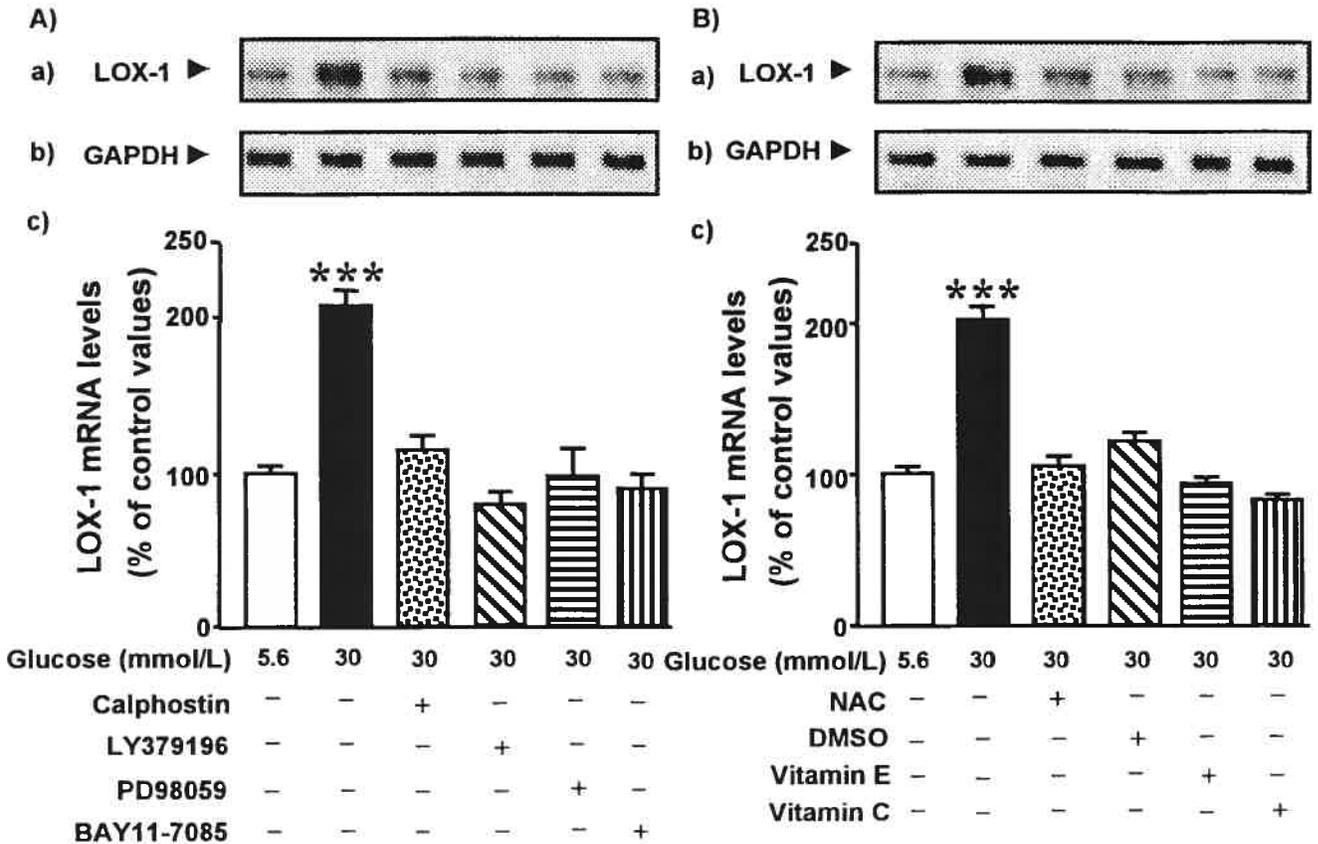


Fig.4. Effect of PKC, MAPK, NF- κ B inhibitors (A) and antioxidants (B) on glucose-induced LOX-1 mRNA levels. HAECs were pretreated for 1h with the pan specific PKC inhibitor, calphostin C (0.1 μ g/ml), the PKC β inhibitor, LY379196 (30nM) or the MAPK inhibitor, PD98059 (50 μ M), antioxidants, NAC (10mM), DMSO (0.5%), vitamin E (50 μ M) and vitamin C (10 μ M) or the NF- κ B inhibitor, BAY 11-7085 (40 μ M), then exposed to 30mmol/L glucose for 48h. At the end of the incubation period, cells were lysed and LOX-1 mRNA was analyzed by RT-PCR (a). LOX-1 mRNA levels were normalized to the levels of GAPDH mRNA (b). Data illustrated on the graph bar represent the mean \pm SEM of 7 different experiments (c). ***, $p < 0.001$ vs 5.6 mmol/L glucose.

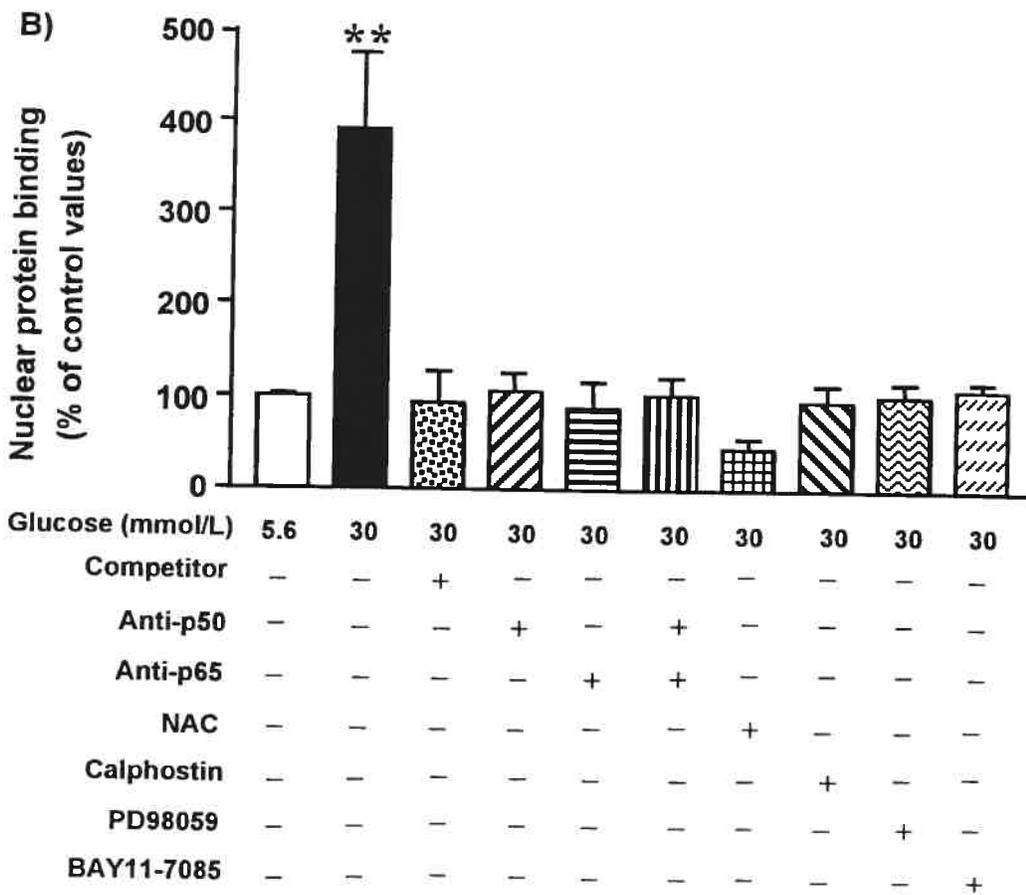
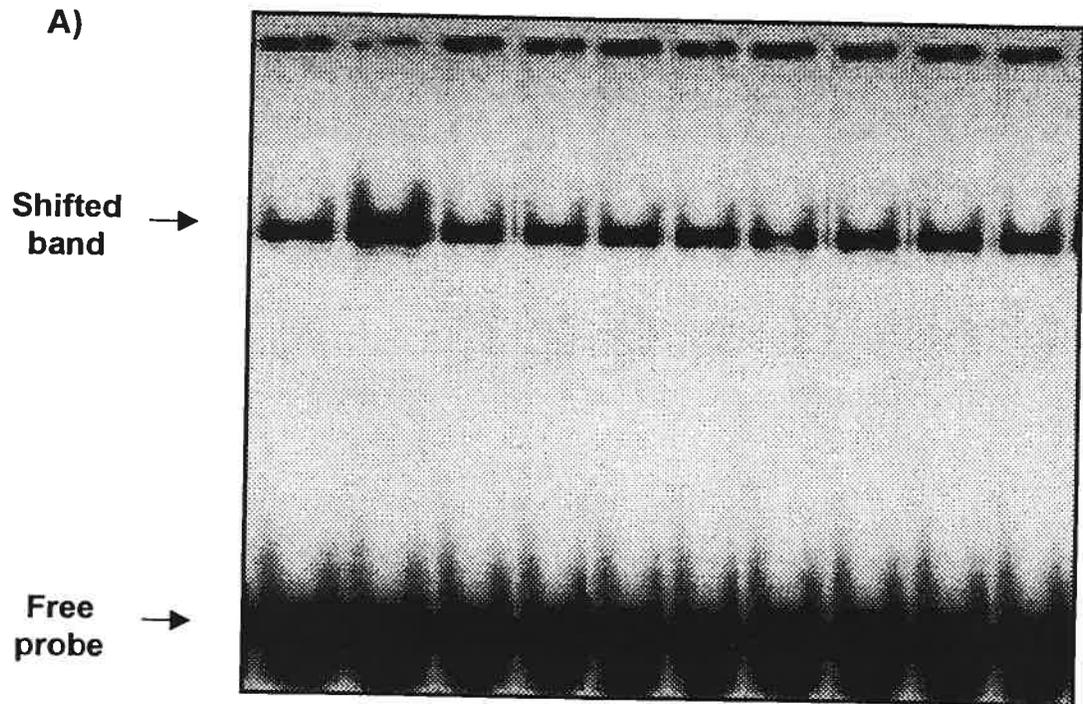


Fig.5. Effect of high glucose on the binding of nuclear proteins extracted from HAECs to the NF- κ B sequence of the LOX-1 gene promoter. HAECs were pretreated or not for 1h with NAC (10mM), calphostin C (0.1 μ g/ml), PD98059 (50 μ M) or BAY 11-7085 (40 μ M) then exposed for 24h to 5.6 or 30 mmol/L glucose. Nuclear proteins isolated from these cells were incubated with end-labeled double-stranded oligonucleotide containing the NF- κ B sequence of the LOX-1 promoter in the presence or absence of 1000-fold molar excess of unlabeled DNA probe (competitor). In some experiments, nuclear proteins were incubated in the presence of anti-p50 and/or anti-p65 antibodies. Retardation was assessed by gel electrophoresis. A. Data represent the result of one representative experiment out of 4. B. Graph bar showing the results of 4 independent experiments. **, $p < 0.01$ vs 5.6 mmol/L glucose.

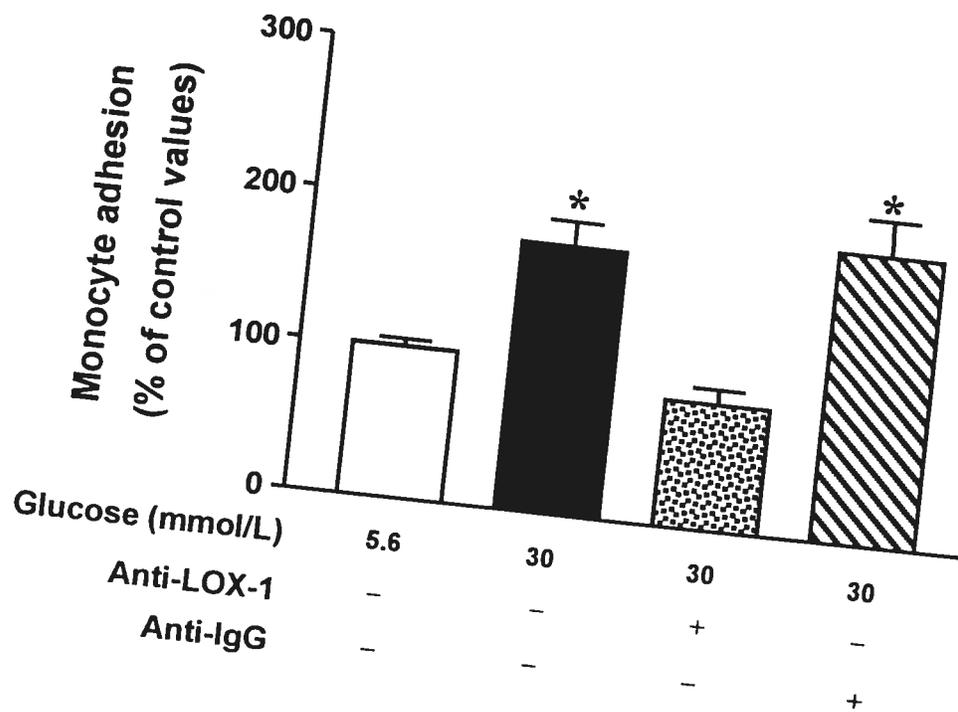


Fig.6. Effect of high glucose on human monocyte adhesion to endothelial cells. Confluent HAECs were exposed for 72h to 5.6 or 30 mmol/L glucose in the presence of anti-LOX-1 or anti-IgG₁ antibodies. At the end of this incubation period, cells were washed and monocytes were added to HAECs to determine monocyte adhesion. Data are expressed as percentage of adherent monocytes and represent the mean \pm SEM of 5 different experiments *, $p < 0.05$ vs 5.6 mmol/L glucose.

II.2. The second article

Glucose enhances human macrophage LOX-1 expression. Role for LOX-1 in glucose-induced macrophage foam cell formation.

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ABSTRACT

Lectin-like oxidized low-density lipoprotein (oxLDL) receptor-1 (LOX-1) is a newly identified receptor for oxLDL that is expressed by vascular cells. LOX-1 is upregulated in aortas of diabetic rats and thus may contribute to the pathogenesis of human diabetic atherosclerosis. In this study, we examined the regulation of human monocyte-derived macrophage (MDM) LOX-1 expression by high glucose and the role of LOX-1 in glucose-induced foam cell formation. Incubation of human MDM with glucose (5.6-30 mmol/L) enhanced, in a dose- and time-dependent manner, LOX-1 gene and protein expression. Induction of LOX-1 gene expression by high glucose was abolished by antioxidants, protein kinase C (PKC), mitogen-activated protein kinases (MAPK), NF- κ B and AP-1 inhibitors. In human MDM cultured with high glucose, increased expression of PKC β 2 and enhanced phosphorylation of extracellular signal-regulated protein kinase (ERK) 1/2 was observed. Activation of these kinases was inhibited by the antioxidant, N-acetyl-L-cysteine (NAC) and by the PKC β inhibitor, LY379196. High glucose also enhanced the binding of nuclear proteins extracted from human MDM to the NF- κ B and AP-1 regulatory elements of the LOX-1 gene promoter. This effect was abrogated by NAC and PKC/MAPK inhibitors. Finally, high glucose-induced human macrophage-derived foam cell formation through a LOX-1-dependent pathway. Overall, these results demonstrate that high glucose concentrations enhance LOX-1 expression in human MDM and that this effect is associated with foam cell formation. Pilot data showing that MDM of patients with type 2 diabetes overexpress LOX-1 support the relevance of this work to human diabetic atherosclerosis.

INTRODUCTION

The prevalence, incidence and mortality from all forms of cardiovascular diseases are increased in diabetic patients.¹ Among the cardiovascular risk factors documented in diabetes, hyperglycemia appears as an independent risk factor for diabetic macrovascular complications.² Mechanisms through which hyperglycemia may promote the development of diabetic cardiovascular disease include glycooxidation and lipoxidation, increased oxidative stress and PKC activation.³⁻⁸ One of the earliest events in atherogenesis is the accumulation of oxidized low-density lipoprotein (oxLDL) in the intima and the subsequent uptake of this modified lipoprotein by macrophages leading to foam cell formation.⁹ One limiting factor for oxLDL uptake by endothelial cells is lectin-like oxLDL receptor-1 (LOX-1), a newly identified vascular receptor for oxLDL.¹⁰⁻¹² Accumulating evidence indicates a key role for LOX-1 in atherogenesis. First, uptake of oxLDL by endothelial cells through LOX-1 induces endothelial dysfunction. Second, the two main LOX-1 ligands, oxLDL and advanced glycation end product (AGE), are implicated in the pathogenesis of atherosclerosis.^{5,9} Third, expression of LOX-1 by vascular cells, including endothelial cells and macrophages is enhanced by pro-atherogenic factors.¹³⁻¹⁸ Finally, LOX-1 is expressed in vivo in the aortas of animals with pro-atherogenic settings^{16,19-20} and is upregulated in early human atherosclerotic lesions.²¹

LOX-1 expression is increased in the endothelium and aortas of diabetic rats¹⁶ and thus may play a role in atherogenesis associated with diabetes. Evidence that AGE induce LOX-1 expression in cultured endothelial cells¹⁶ and macrophages²² supports a primary role for these products in modulating vascular LOX-1 expression in diabetes.

On the basis of these results and given the key role for macrophages as precursors of foam cells in the vascular wall,⁹ the present study was aimed at investigating the regulation of human macrophage LOX-1 expression by hyperglycemia and the role of this receptor in glucose-induced macrophage foam cell transformation.

MATERIALS AND METHODS

Reagents

(see "Online Data Supplements" for details).

Cell culture

Freshly isolated human monocytes²³ or THP-1 monocytes were differentiated into macrophages *in vitro* and treated with high glucose (see "Online Data Supplements" for details).

Analysis of mRNA expression

-Northern Blot Analysis:

LOX-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression in THP-1 monocyte-derived macrophages (MDM) (10×10^6 /ml) was analyzed by hybridization with [³²P] dCTP-labeled human LOX-1 and GAPDH cDNA probes. (see "Online Data Supplements" for details).

-Polymerase Chain Reaction (PCR) Analysis:

Total RNA for use in the PCR reaction was extracted from human MDM (2×10^6 /ml) by an improvement of the acid-phenol technique of Chomczynski.²⁴ cDNA was synthesized from RNA and amplified by synthetic primers specific for human LOX-1 and GAPDH (see "Online Data Supplements" for details).

Western blot

LOX-1, MAPK and PKC- β 2 expression in human MDM was analyzed by western blot analysis using specific antibodies (see "Online Data Supplements" for details).

DNA binding assay

Nuclear proteins were isolated from THP-1 MDM and their binding to consensus sequences of the LOX-1 promoter NF- κ B and AP-1-enhancing elements was assessed by DNA retardation electrophoretic mobility shift assay (EMSA)²⁵⁻²⁶ (see "Online Data Supplements" for details).

DNA probes

(see "Online Data Supplements" for details).

Uptake of Dil-oxLDL by human MDM

Native LDL (density, 1.019 to 1.063) was isolated from plasma obtained from healthy donors by sequential ultracentrifugation²⁷ and extensively dialyzed for 24h at 4°C against 5 mmol/L Tris/50 nmol/L NaCl to remove EDTA. Oxidation of LDL was performed by incubating native LDL (2mg prot/ml) at 37°C for 20h in serum-free RPMI 1640 containing 7.5 μ g/ml CuSO₄. Oxidation of LDL was monitored by measuring the amount of thiobarbituric acid-reactive substances and by electrophoretic mobility on agarose gel. OxLDL was labeled with Dil as described previously.²⁸ Uptake of Dil-oxLDL by human MDM was assessed by fluorescence microscopy and determination of fluorescence at 520/564 nm (see "Online Data Supplements" for details). Results were normalized to total cell protein concentrations.²⁹

Quantification of cytosolic AGE in MDM

The total AGE content present in the cytosolic extracts of glucose-treated MDM was determined by competitive ELISA. Results were expressed as B/Bo (see "Online Data Supplements" for details).

Patients

The study group comprised 7 patients with type 2 diabetes and 12 healthy control subjects. The diabetic patients were recruited from the Notre-Dame Hospital outpatient clinic and gave written consent to participate in this study. Their mean (\pm SE) age was 65 ± 3 years, BMI 32 ± 2 kg/m², fasting glucose 9.4 ± 1.2 mmol/L, triglyceride 3.26 ± 1.21 mmol/L, LDL cholesterol 3.08 ± 0.31 mmol/L and serum glycated hemoglobin 0.072 ± 0.006 . All the patients were treated with glyburide and metformin. None of the patients was primarily insulin-dependent. One patient was hypertensive and was treated with enalapril, one had macroangiopathy and microalbuminuria. Control subjects were recruited from the hospital staff and relatives. Their mean (\pm SE) age was 38 ± 4 years, BMI 23 ± 1 kg/m², fasting glucose 5.0 ± 0.1 mmol/L, triglyceride 1.30 ± 0.20 mmol/L, LDL cholesterol 3.50 ± 0.40 mmol/L. Subjects who had infectious or inflammatory conditions or cardiac, renal or pulmonary decompensated diseases or who were treated with anti-inflammatory or antioxidant drugs were excluded from the study.

Determination of cell viability

Cell viability after treatment with the different agents under study was assessed by trypan blue exclusion and was consistently found to be $> 90\%$.

Statistical Analysis

Values were expressed as mean \pm SEM. Data were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey test. A P value less than 0.05 was considered statistically significant.

RESULTS

Effect of D-glucose on human MDM LOX-1 mRNA expression.

Incubation of human MDM for 24 to 72h with 5.6 or 30 mmol/L D-glucose increased, in a time-dependent manner, macrophage LOX-1 gene expression. Maximal effect was observed from 48 to 72h (Fig. 1A-a). Under these experimental conditions, no modulation of the mRNA expression of GAPDH, used as internal control, was observed (Fig. 1A-b). LOX-1 mRNA levels, normalized to the levels of GAPDH mRNA are shown in Fig. 1A-c. The stimulatory effect of D-glucose on human MDM LOX-1 mRNA expression was dose-dependent with maximal effect occurring between 20 and 30mM glucose (Fig. 1B-a). Under these experimental conditions, no modulation of the mRNA expression of GAPDH was observed (Fig. 1B-b). LOX-1 mRNA levels, normalized to the levels of GAPDH mRNA are shown in Fig. 1B-c. Incubation of human MDM with L-glucose or mannitol (30 mmol/L) did not enhance LOX-1 mRNA expression (LOX-1 mRNA expression [% of control values]: L-glucose: 98 ± 5 , mannitol: 107 ± 6).

Effect of D-glucose on human MDM LOX-1 protein expression.

Treatment of human MDM with 30 mmol/L D-glucose enhanced LOX-1 protein expression in these cells. This effect was observed from 72 to 96 h (Fig. 2A-a). Under these experimental conditions, no modulation of β -actin, used as internal control, was observed (Fig. 2A-b). LOX-1 protein levels normalized to the levels of β -actin protein are illustrated in Fig. 2A-c. Incubation of human MDM for 72h with increasing D-glucose concentrations (5.6-30 mmol/L) enhanced, in a dose-dependent manner, LOX-1 protein expression in these cells (Fig. 2B-a). LOX-1 protein levels normalized to the levels of β -actin (Fig.2B-b) are illustrated in Fig. 2B-c. No stimulatory effect of mannitol (30 mmol/L)

on macrophage LOX-1 protein expression was observed (LOX-1 protein expression [% of control values]: mannitol: 109 ± 9).

Role for AGE in the induction of MDM LOX-1 by high glucose.

To evaluate whether intracellular AGE formation may play a role in the induction of MDM LOX-1 expression by high glucose, the levels of cytosolic glycated proteins present in MDM exposed to high glucose for 24 to 48h were determined. Regardless of the glucose concentrations used, levels of glycated proteins in MDM consistently fell below the minimum concentration of AGE detected by this assay, i.e. less than 0.25ng AGE/ μ g protein. (B/Bo: Glucose 24h (mmol/L) 5.6: 2.5 ± 0.7 ; 10: 3.9 ± 1.3 ; 20: 3.4 ± 0.8 ; 30: 3.9 ± 0.7 ; Glucose 48h (mmol/L) 5.6: 3.7 ± 1.7 ; 10: 2.9 ± 1.2 ; 20: 3.3 ± 0.3 ; 30: 2.3 ± 0.5). While non glycated BSA (50ng/ml), used as negative control, failed to inhibit antiserum binding (B/Bo: 1.1), competition for antibody binding was observed with methylglyoxal- and glucose-derived AGE-BSA (50ng/ml) used as positive controls. (B/Bo: 0.53 and 0.57, respectively).

Effect of high glucose on TNF α -induced MDM LOX-1 expression.

One pathophysiological stimulus relevant to atherosclerosis in diabetes is tumor necrosis factor alpha (TNF α).³⁰⁻³¹ Because this cytokine stimulates LOX-1 expression in vascular cells^{13,17} and is released by monocytic cells in response to high glucose and AGE,³²⁻³⁵ we determined the modulatory effect of TNF- α on human MDM LOX-1 expression under normal and high glucose conditions. As shown in Fig. 3A and B, TNF α -treated human MDM cultured under normoglycemic conditions express similar levels of LOX-1 gene and protein expression than high glucose-treated cells. Levels of LOX-1 expression elicited by this cytokine did not differ when human MDM were

cultured in high glucose conditions. The effect of TNF α alone on LOX-1 protein expression was blocked by anti-TNF- α antibodies (Fig.3B).

Signaling pathways involved in glucose-induced human MDM LOX-1 gene expression.

To identify the signaling pathways involved in the stimulatory effect of high glucose on LOX-1 gene expression, human MDM were pretreated for 2h with PKC, MAPK, tyrosine kinase, NF- κ B or AP-1 inhibitors, prior exposure to glucose. As shown in Fig.4A, the pan specific PKC inhibitor, calphostin C (0.1 μ g/ml) and the PKC β inhibitor, LY379196 (30 nmmol/L) totally abrogated glucose-induced macrophage LOX-1 gene expression. A similar effect was observed when the cells were pre-incubated with the MAPK inhibitor, PD98059 (50 μ mol/L), the AP-1 inhibitor, curcumin (10 μ mol/L) or the NF- κ B inhibitor, BAY 11-7085 (40 μ mol/L)³⁶ (Fig.4A). In contrast, tyrosine kinase inhibitors did not affect this parameter (data not shown). Under these experimental conditions, no modulation of the mRNA expression of GAPDH was observed (Fig. 4A-b). LOX-1 mRNA levels, normalized to the levels of GAPDH mRNA are presented in Fig. 4A-c. Because diabetes and high glucose induce increased oxidative stress,³⁷ we next determined the role of oxidative stress in the regulation of LOX-1 gene expression by glucose. As shown in Fig 4B, preincubation of human MDM with various antioxidants, including NAC (10 mmol/L), vitamin E (50 μ mol/L), vitamin C (10 μ mol/L) and DMSO (0.5%), totally prevented the stimulatory effect of high glucose on LOX-1 gene expression. Involvement of these signaling events was confirmed in THP-1 MDM by demonstrating that PKC and MAPK inhibitors as well as antioxidants abolished glucose-induced LOX-1 mRNA expression in these cells (Fig.4C,D). Having established

the relevance of THP-1 cells to human MDM, we next assessed using these cells the sequential events leading to glucose-induced PKC/MAPK activation. As shown in Fig 5, treatment of THP-1 cells for 48h with high glucose induced PKC β 2 (Fig.5A) and ERK1/2 (Fig.5B) activation as assessed by western blot analysis. Glucose-induced activation of these kinases was abrogated by NAC (Fig.5A,B). Furthermore, ERK1/2 activation in glucose-treated macrophages was inhibited by LY379196 (Fig.5B), thereby identifying MAPK as downstream targets of PKC.

Effect of high glucose concentrations on the binding of nuclear proteins to the regulatory NF- κ B and AP-1 sequences of the LOX-1 gene promoter.

Exposure of THP-1 MDM to a high glucose environment increased the binding of nuclear proteins to the NF- κ B (Fig.6) and AP-1 (Fig.7) consensus sequences of the human LOX-1 promoter. These binding complexes were specifically competed in the presence of a 1,000-molar excess of the unlabeled NF- κ B or AP-1 oligonucleotides and were significantly decreased by BAY 11-7085 (Fig.6) or curcumin (Fig.7). Nuclear protein binding was further inhibited in presence of antibodies against p50 and p65 (Fig.6) or c-fos and c-Jun (Fig.7). In contrast, irrelevant antibodies or competitors did not alter glucose-induced NF- κ B and AP-1 activation, thus confirming the specificity of the inhibition documented in these EMSA assays (Fig.6,7). Preincubation of THP-1 cells with NAC, PKC and MAPK inhibitors also suppresses the nuclear binding to the NF- κ B and AP-1 sequences of the LOX-1 gene promoter (Fig.6,7).

Role of LOX-1 in mediating glucose-induced human MDM foam cell formation.

To evaluate whether increased expression of LOX-1 by high glucose resulted in enhanced uptake of oxLDL by human MDM, these cells were treated for 48h with 5.6 or

30 mmol/L glucose, then incubation was pursued for an additional 24h period in the presence of saturating amounts (20 μ g/ml) of antibodies to CD36, SR-A, LOX-1 or IgG₁. At the end of the incubation period, cells were exposed for 3h to Dil-oxLDL (80 μ g/ml) in the presence or absence of excess unlabeled oxLDL. Incubation of human MDM with high glucose in the presence of anti-CD36 and anti-SR-A antibodies led to enhanced uptake of oxLDL by these cells as assessed by fluorescence microscopy (Fig 8A) and measurement of extracted Dil-oxLDL (Fig8B). This effect was abrogated by incubating human MDM with excess unlabeled oxLDL or with anti-LOX-1 antibody. In contrast, exposure of these cells to anti-IgG₁ did not affect glucose-induced MDM foam cell formation (Fig.8A, B).

Levels of LOX-1 mRNA in MDM of diabetic patients.

MDM of patients with type 2 diabetes demonstrated a significant increase in LOX-1 mRNA levels compared with those isolated from control subjects. (LOX-1 mRNA levels (%): controls: 100 ± 7 ; diabetic patients: 169 ± 25 , $P < 0.01$).

DISCUSSION

Despite the recent evidence linking experimental diabetes with increased vascular LOX-1 expression,¹⁶ only few studies have examined the regulation of LOX-1 by metabolic factors dysregulated in diabetes. The present study demonstrates, for the first time, that high glucose increases human macrophage LOX-1 expression, both at gene and protein levels. These results together with our preliminary observations that MDM of diabetic patients exhibit increased LOX-1 gene expression suggest a role of hyperglycemia in the regulation of vascular LOX-1 in human diabetes. In macrophages which do express multiple scavenger receptors,³⁸ over 50% of the uptake of oxLDL seems to occur via CD36,³⁹ whereas SR-A shares the rest with several other scavenger receptors, including LOX-1. Because high glucose enhances macrophage CD36 expression,⁴⁰ it is tempting to postulate that upregulation of macrophage scavenger receptors in response to glucose may play a role in the pathogenesis of atherosclerosis in human diabetes.

It has been previously shown that AGE enhance LOX-1 mRNA expression in cultured aortic endothelial cells and human macrophages.^{16,22} On the basis of the time course and concentration of glucose required to modulate macrophage LOX-1 expression, we speculated that generation of AGE might be responsible for LOX-1 induction in glucose-treated MDM. However, arguing against this hypothesis, we did not ascertain the presence of AGE in these cells over the time course required to modulate LOX-1 gene expression. Considering the short incubation period of macrophages with high glucose, lack of intracellular AGE detection may be related to this in vitro variable. Alternatively, characteristics relating to the sensitivity of the ELISA and the specificity of

the anti-AGE-Rnase antiserum used in this assay⁴¹ may account for these negative results.

Interestingly, we found that the extent of stimulation of macrophage LOX-1 expression achieved by glucose was comparable to that elicited by TNF- α and that these two stimuli did not synergize for macrophage LOX-1 induction. Because glucose and AGE stimulate TNF α secretion,³²⁻³⁵ one possible explanation for this observation is that induction of LOX-1 by glucose involves TNF α . However, this hypothesis is not supported by our finding that immunoneutralization of TNF α does not affect glucose-induced LOX-1 expression. Alternatively, glucose and TNF α may regulate macrophage LOX-1 through one major and possibly identical pathway. Like TNF α , glucose is a well-known activator of NF- κ B and AP-1⁴²⁻⁴⁶ and may therefore induce, through the activation of these factors, the transcription of the LOX-1 gene. Consistent with this, we found that glucose increases the LOX-1 mRNA levels in macrophages and enhances the binding of nuclear proteins to the NF- κ B and AP-1 regulatory sequences of the LOX-1 promoter.⁴⁷ Although final proof for a role for NF- κ B and AP-1 as functional responsive elements involved in the transcriptional activation of the LOX-1 gene would require promoter-reporter gene assays, these data support a role for these transcriptional factors in the regulation of LOX-1 gene expression by glucose.

Regulation of LOX-1 gene expression is redox sensitive.¹⁴ Therefore, reactive oxygen species (ROS) generated by glucose in vascular cells^{6,7,46} may represent key intermediates in the regulation of LOX-1 gene expression by this metabolic factor. Evidence linking glucose-induced oxidative stress with activation of PKC and MAPK in vascular cells^{6-8,48-49} further supports a role of these kinases in the control of LOX-1

expression by hyperglycemia. In line with these hypotheses, we found that antioxidants and PKC/MAPK inhibitors abolish glucose-induced macrophage LOX-1 mRNA levels, thus implicating ROS and kinases as signaling molecules in this effect. Our findings that antioxidants suppressed glucose-induced PKC/MAPK activation and that PKC inhibition abolished glucose-induced MAPK activation, support the hypothesis that glucose-induced kinase activation involves oxidative stress and that MAPK act in this signaling cascade as intermediate molecules transducing signal from PKC to macrophage LOX-1. Convincing data also indicate a role of oxidative stress and kinases in NF- κ B and AP-1 activation.^{7,42,46,50-52} In accordance with these results, we found that antioxidants as well as PKC/MAPK inhibitors block glucose-induced NF- κ B and AP-1 activation, thus identifying these transcriptional factors as downstream ROS and kinase targets. Taken together, these results indicate that increased production of intracellular ROS and activation of PKC/MAPK pathways are initial signaling events in the regulation of LOX-1 gene by glucose that are required for subsequent activation of NF- κ B and AP-1.

Accumulation of cholesterol-loaded foam cells in the arterial intima is a hallmark and key event of early atherogenesis. Evidence that incubation of macrophages in high glucose conditions leads to increased intracellular accumulation of cholesterol ester⁵³ suggests a role for hyperglycemia in foam cell formation. Like other scavenger receptors, LOX-1 is highly expressed in macrophages present in human atherosclerotic lesions²¹ and thus may play a role in macrophage foam cell formation. The present study demonstrates, for the first time, that increased LOX-1 surface expression in glucose-treated macrophages is associated with enhanced uptake of oxLDL by these cells, suggesting thereby a new role for LOX-1, that of mediating glucose-induced foam

cell formation. Importantly, such a role for LOX-1 in foam cell formation was only evident after functional blockade of CD36. It is widely believed that much of the oxLDL uptake by human macrophages occurs via CD36.³⁹ Although the quantitative contribution of CD36 in glucose-induced foam cell formation is unknown, it has been shown that glucose-induced macrophage CD36 expression correlates with a 10-fold increase in CD36-mediated oxLDL uptake,⁴⁰ thus suggesting a major role of this receptor in glucose-induced foam cell formation. In the present study, we reported a 2-fold increase in non-CD36/non-SR-A-mediated oxLDL uptake in glucose-treated macrophages that was only partly reduced by an anti-LOX-1 antibody. While these results demonstrate a role of LOX-1 in glucose-induced foam cell formation, they do not argue for a major contribution of LOX-1 in this process. Consistent with this idea, one recent study failed to demonstrate a key role of LOX-1 in the progression of macrophages to foam cells in vitro.⁵⁴ Nevertheless, extrapolation of in vitro results to the in vivo situation is hazardous and further studies are needed to assess the functional significance of increased LOX-1 expression on foam cell formation in vivo.

In summary, the present study demonstrates that high glucose enhances human MDM LOX-1 expression in vitro and that this effect is associated with foam cell formation. Our preliminary results showing increased MDM LOX-1 expression in human diabetes support the relevance of this work to the human setting.



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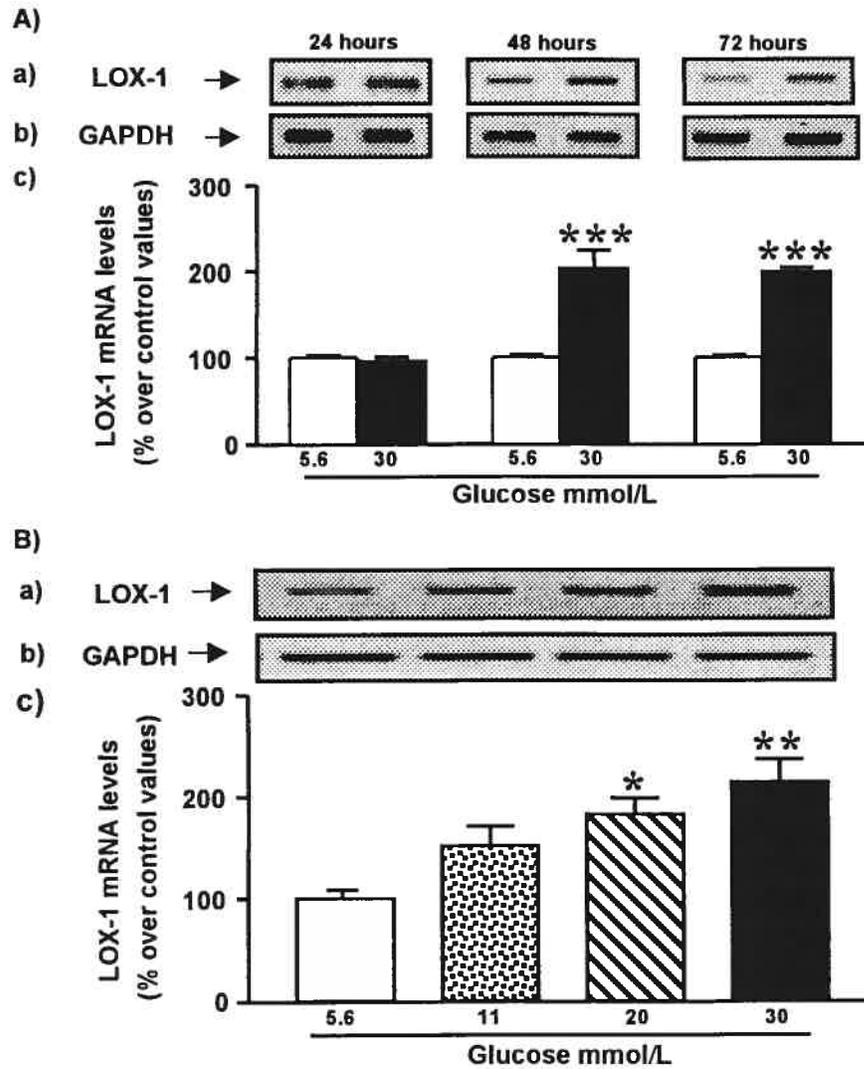


Fig.1. Time- and dose-dependent effect of high glucose on LOX-1 mRNA levels in human MDM. Cultured human MDM were incubated for 24 to 72h (A) or 48 hours (B) with 5.6 to 30 mmol/L glucose. At the end of the incubation period, cells were lysed and LOX-1 mRNA was analyzed by RT-PCR (A-B). LOX-1 mRNA levels (a) were normalized to the levels of GAPDH mRNA (b). Data illustrated on the graph bar (c) represent the mean \pm SEM of 6 (A-B) different experiments. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ vs 5.6 mmol/L glucose.

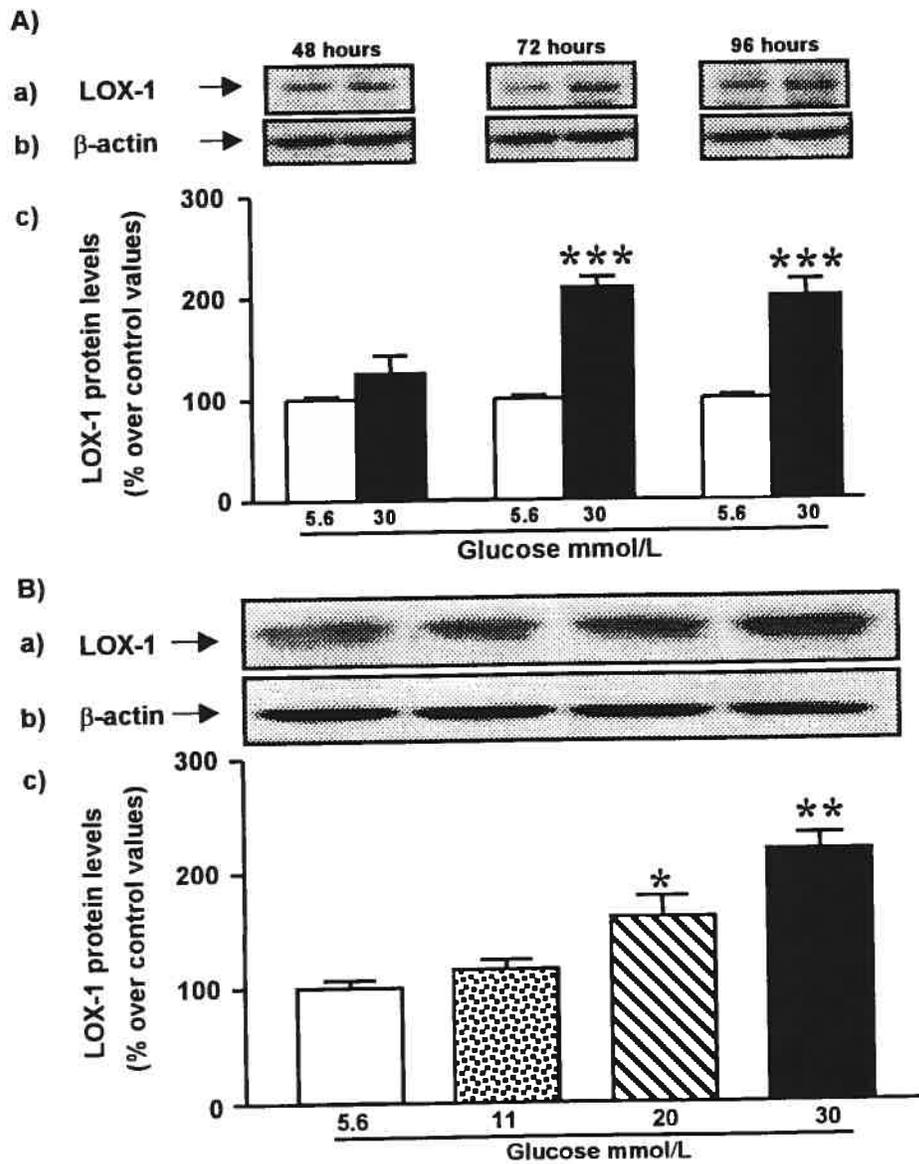


Fig.2. Time- and dose-dependent effect of high glucose on LOX-1 protein expression in human MDM. Human MDM were cultured for 48 to 96h (A) or 72 hours (B) with 5.6 to 30 mmol/L glucose. At the end of the incubation period, cells were lysed and LOX-1 membrane protein expression was determined by Western blot analysis (a). LOX-1 protein levels were normalized to the levels of β-actin protein (b). Data illustrated on the graph bar (c) represent the mean ± SEM of 6 (A) and 3 (B) different experiments. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ vs 5.6 mmol/L glucose.

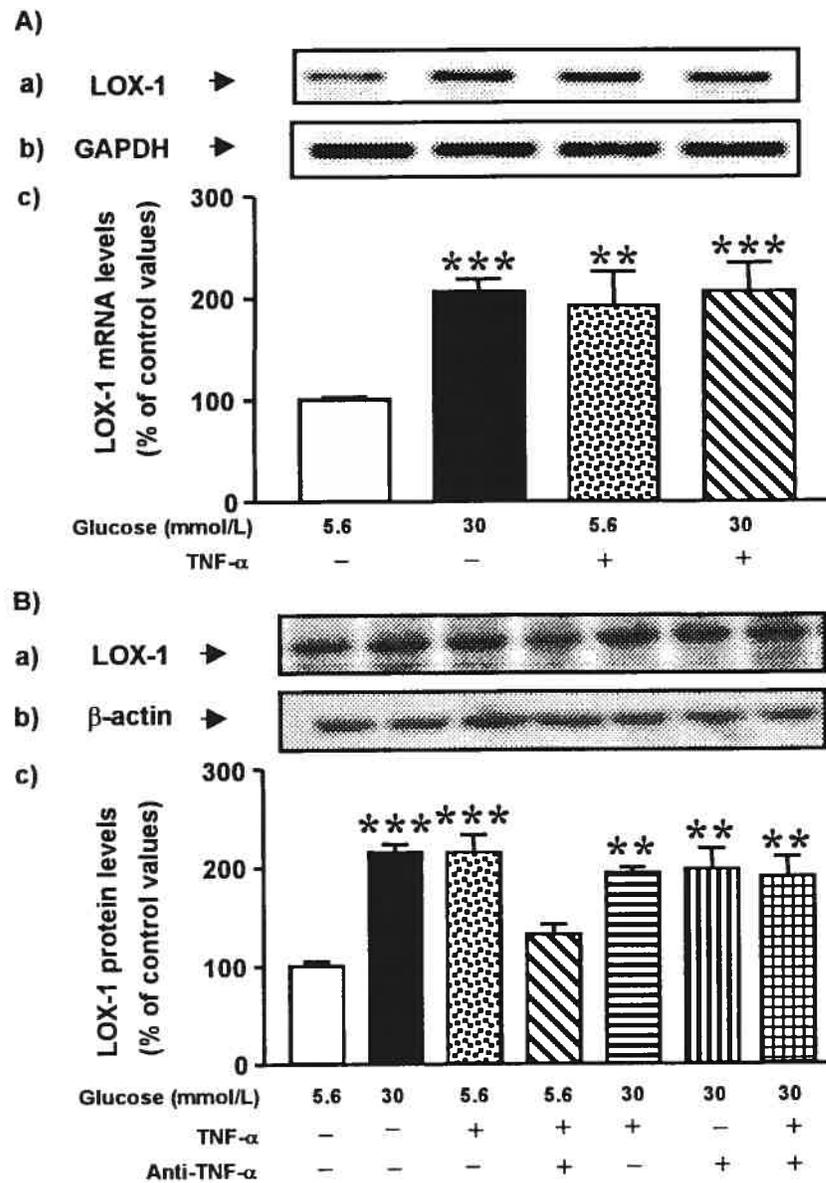


Fig.3. Effect of high glucose on TNF- α -induced macrophage LOX-1 mRNA and protein expression. Human MDM were cultured for 24h (A) or 48h (B) in 5.6 mmol/L or 30 mmol/L glucose environment, then treated for an additional 24h-time period with TNF- α (5 ng/ml) in the presence or absence of anti-TNF α antibodies (10 μ g/ml) (B). At the end of the incubation period, cells were lysed and LOX-1 mRNA (A) and membrane protein expression (B) were determined by RT-PCR and Western blot analysis, respectively. LOX-1 mRNA and protein levels were normalized to the levels of GAPDH mRNA (A-b) or β -actin protein (B-b). Data illustrated on the graph bar (A-c and B-c) represent the mean \pm SEM of 4 different experiments. **, $p < 0.01$, ***, $P < 0.001$ vs 5.6 mmol/L glucose.

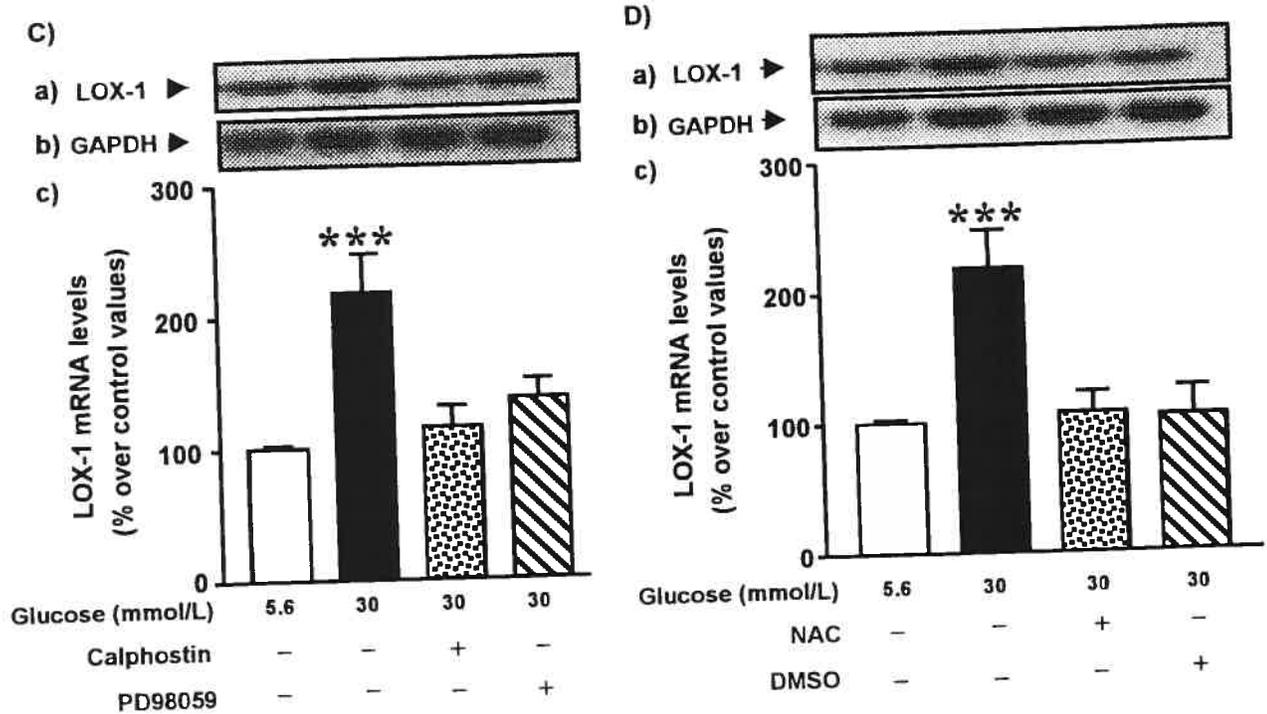
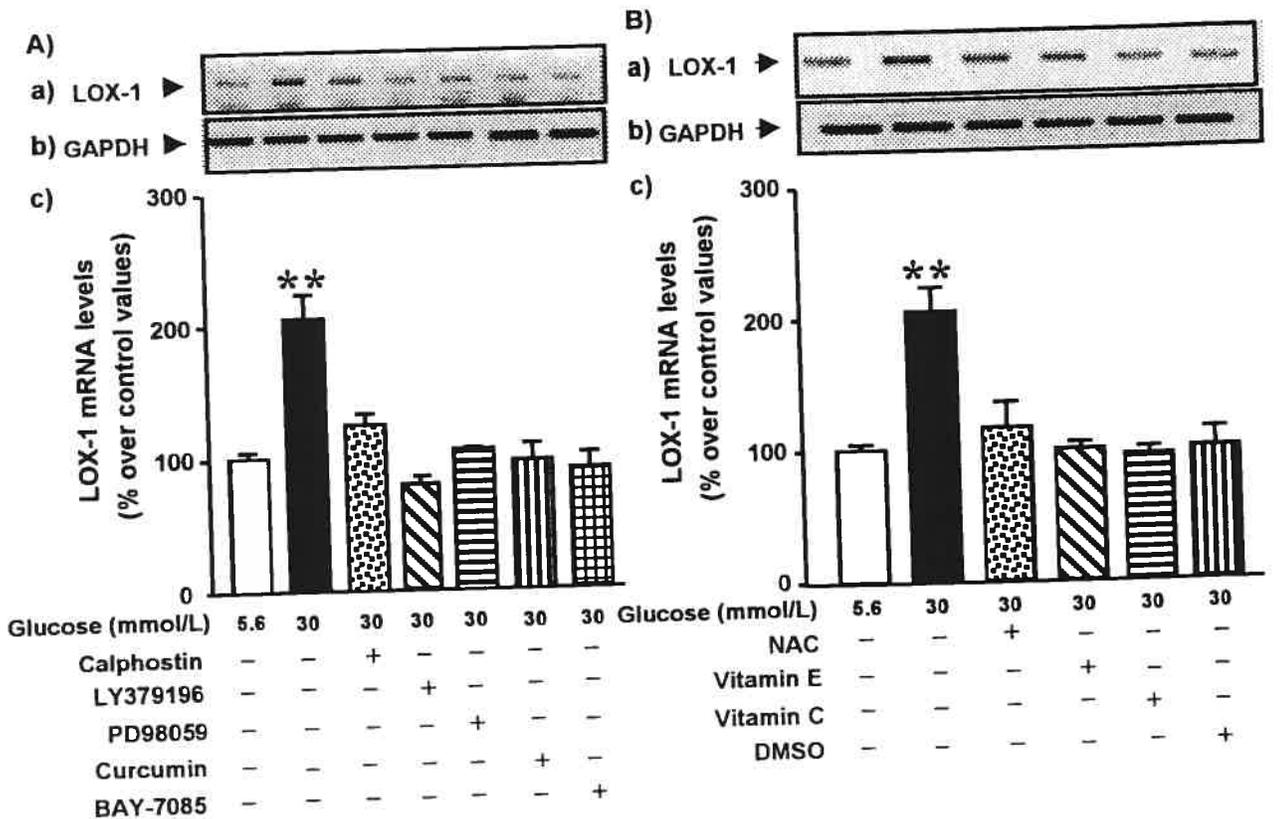


Fig.4. Effect of PKC, MAPK, NF- κ B, AP-1 inhibitors (A and C) and antioxidants (B and D) on glucose-induced LOX-1 mRNA levels. Human (A and B) and THP-1 (C and D) MDM were pretreated for 1h with the PKC inhibitor calphostin C (0.1 μ g/ml), the specific PKC β inhibitor LY379196 (30 nmmol/L), the MAPK inhibitor PD98059 (50 μ mol/L), the NF- κ B inhibitor BAY 11-7085 (40 μ mol/L), the AP-1 inhibitor curcumin (10 μ mol/L) or the antioxidants NAC (10 mmol/L), vitamin E (50 μ mmol/L), vitamin C (10 μ mmol/L) and DMSO (0.5 %), then exposed for 48h to 30 mmol/L glucose. At the end of the incubation period, cells were lysed and LOX-1 mRNA was analyzed by RT-PCR (A-a and B-a) or Northern-Blot analysis (C-a and D-a). LOX-1 mRNA levels were normalized to the levels of GAPDH mRNA (b). Data illustrated on the graph bar represent the mean \pm SEM of 7 (A-c and B-c) and 6 (C-c and D-c) different experiments . **, $p < 0.01$, ***, $p < 0.001$ vs 5.6 mmol/L glucose.

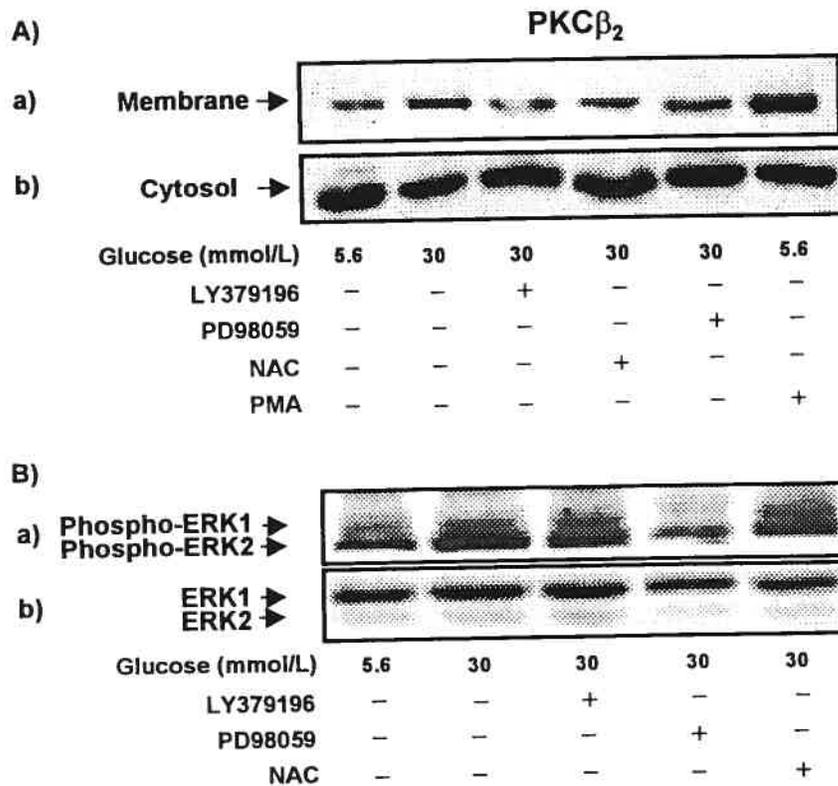


Fig.5. Effect of high glucose on PKC and MAPK activation in THP-1 MDM. Modulatory effect of NAC and PKC/MAPK inhibitors. THP-1 MDM were pretreated for 1h with NAC (10 mmol/L), LY379196 (30 nmmol/L), or PD98059 (50 μ mol/L) then incubated for 48h with 30 mmol/L glucose. A. Membrane (a) and cytosolic (b) fractions were assayed for PKC β_2 expression by western blot analysis. Cells stimulated with PMA (0.5 μ mol/L) for 30 min were used as positive control. B. Phosphorylation of ERK1/2 was assessed by western blot using phospho-specific ERK1/2 antibody (a) or specific ERK1/2 antibody (b). Representative blots are shown.

A)

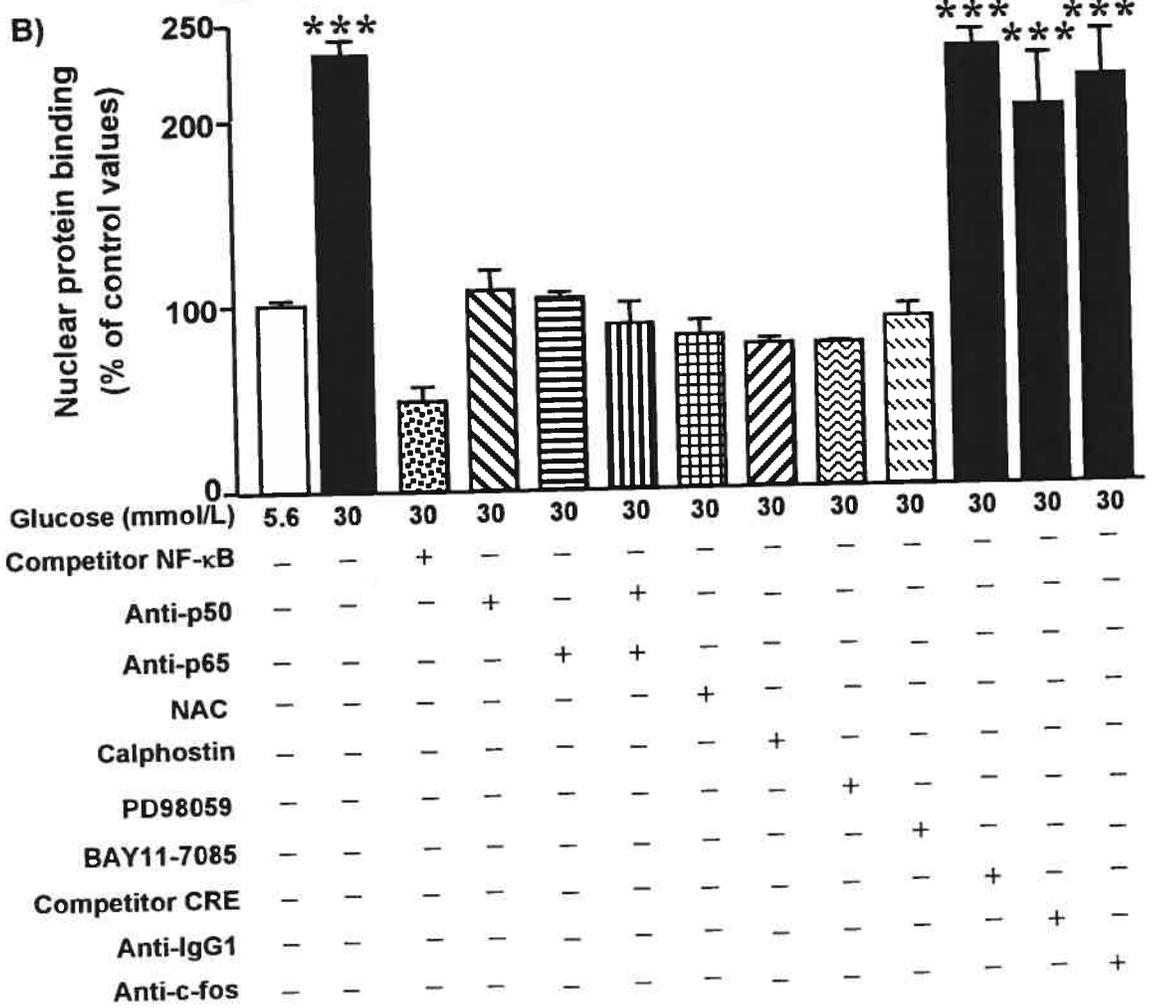
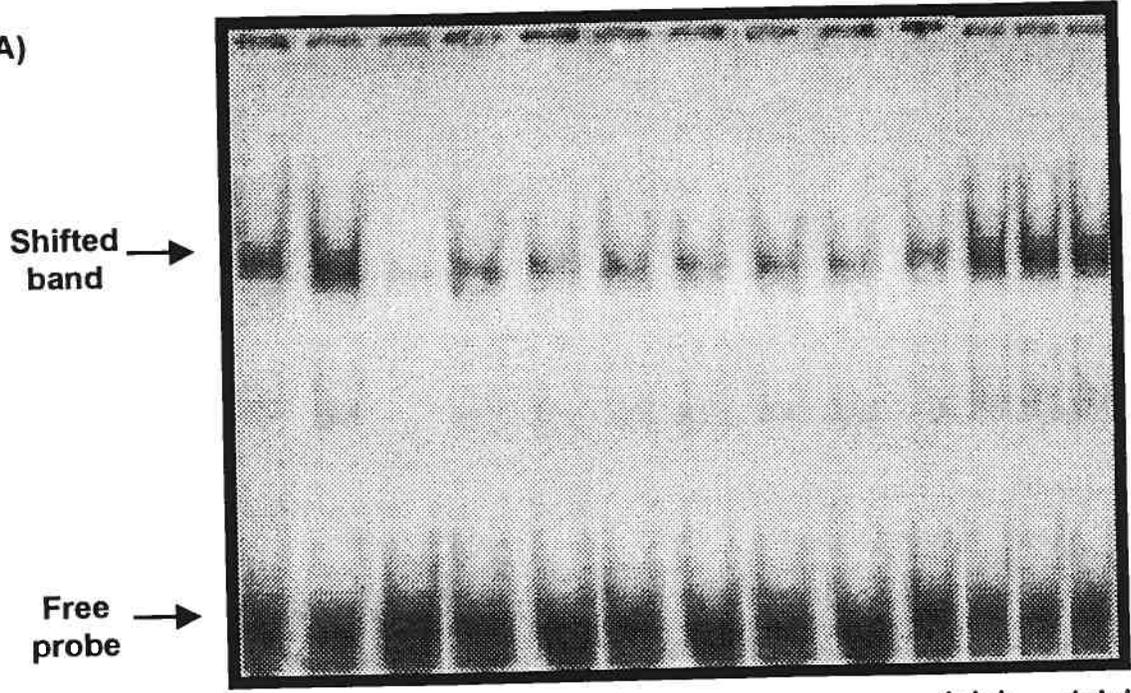


Fig.6. Effect of high glucose on the binding of nuclear proteins extracted from THP-1 MDM to the NF- κ B sequence of the LOX-1 gene promoter. THP-1 MDM were pretreated or not for 1h with NAC (10 mmol/L), calphostin C (0.1 μ g/ml), PD98059 (50 μ mol/L), BAY 11-7085 (40 μ mmol/L), then exposed for 24h to 5.6 or 30 mmol/L glucose. Nuclear proteins isolated from these cells were incubated with end-labeled double-stranded oligonucleotide containing the NF- κ B sequence of the LOX-1 promoter in the presence or absence of 1000-fold molar excess of unlabeled NF- κ B or CRE DNA probe (competitor). In some experiments, nuclear proteins were incubated in the presence of anti-p50, -p65, -IgG₁ or -c-fos antibodies. Retardation was assessed by gel electrophoresis. A. Data represent the result of one representative experiment out of 4. B. Graph bar showing the results of 4 independent experiments. ***, p<0.001 vs 5.6 mmol/L glucose.

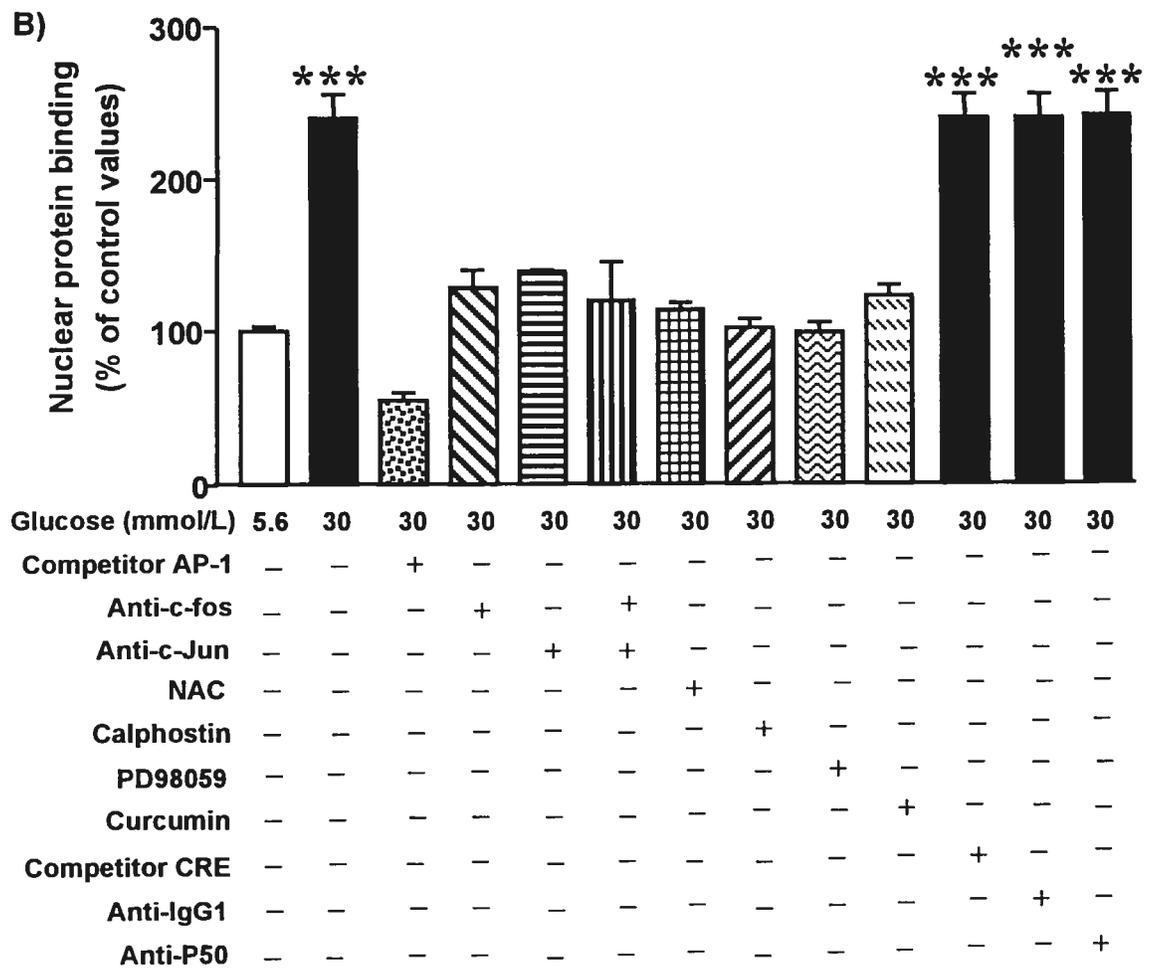
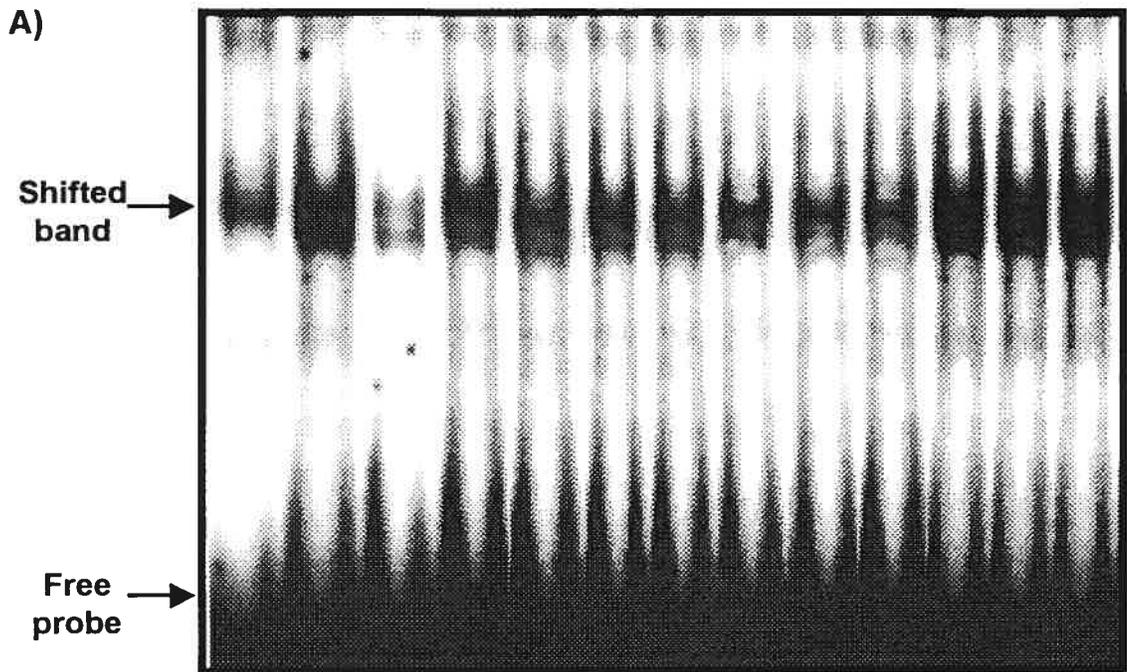
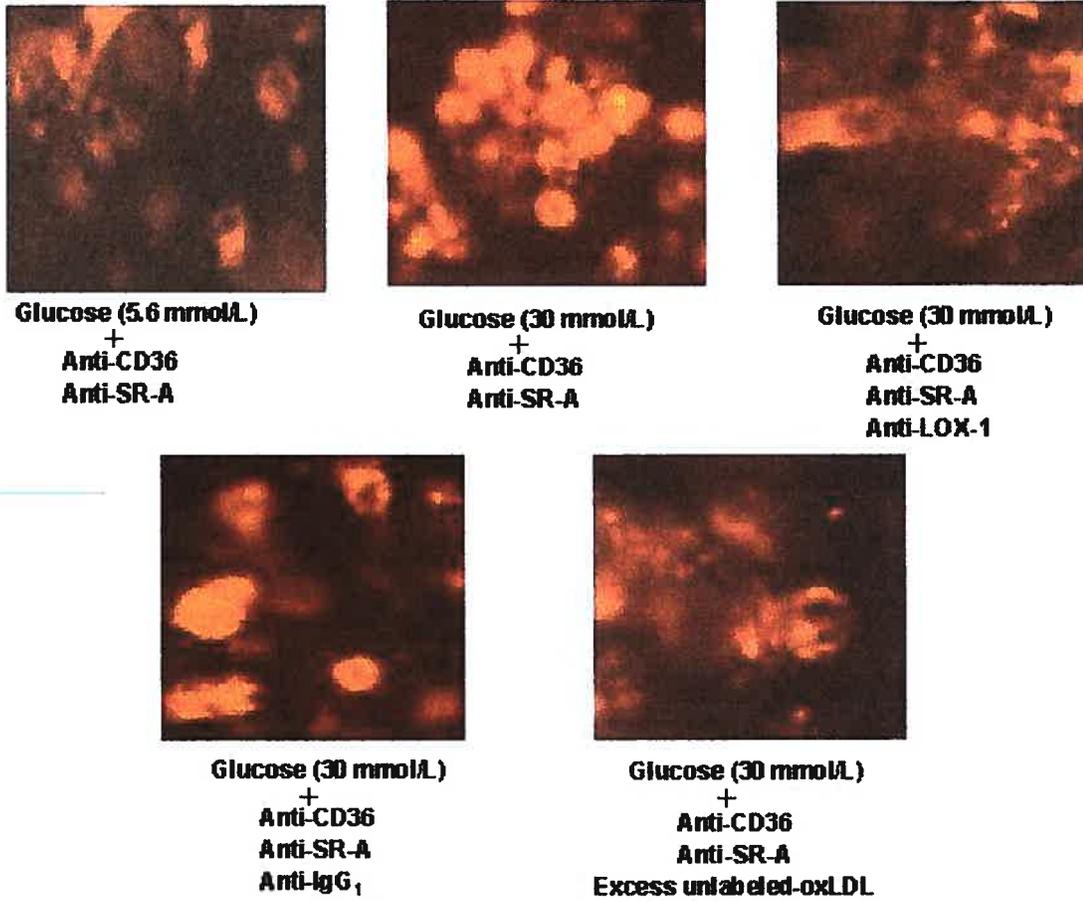


Fig.7. Effect of high glucose on the binding of nuclear proteins extracted from THP-1 MDM to the AP-1 sequence of the LOX-1 gene promoter. THP-1 MDM were pretreated or not for 1h with NAC (10 mmol/L), calphostin C (0.1 μ g/ml), PD98059 (50 μ mol/L), or curcumin (10 μ mmol/L), then exposed for 24h to 5.6 or 30 mmol/L glucose. Nuclear proteins isolated from these cells were incubated with end-labeled double-stranded oligonucleotide containing the AP-1 sequence of the LOX-1 promoter in the presence or absence of 1000-fold molar excess of unlabeled AP-1 or CRE DNA probe (competitor). In some experiments, nuclear proteins were incubated in the presence of anti-c-fos, c-Jun, -IgG₁ or -p50 antibodies. Retardation was assessed by gel electrophoresis. A. Data represent the result of one representative experiment out of 4. B. Graph bar showing the results of 4 independent experiments. ***, $p < 0.001$ vs 5.6 mmol/L glucose.

A)



B)

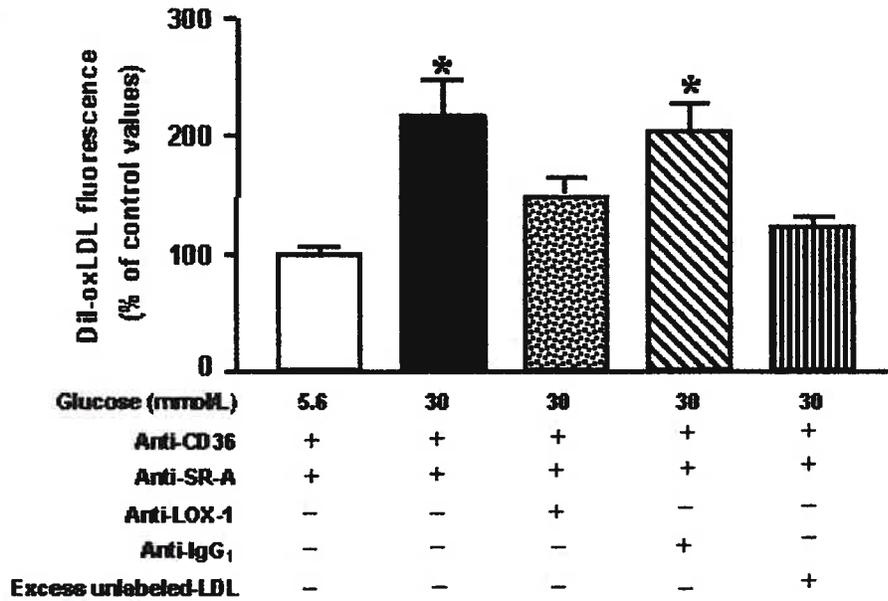


Fig.8. Effect of high glucose on oxLDL uptake by human MDM. Role for LOX-1. Human MDM were treated for 48h with 5.6 or 30 mmol/L glucose, then incubation was pursued for an additional 24h period in the presence of saturating amounts (20 μ g/ml) of antibodies to CD36, SR-A, LOX-1 or IgG₁. At the end of the incubation period, cells were exposed for 3h to Dil-oxLDL (80 μ g/ml) in the presence or absence of excess unlabeled oxLDL. After washing, fluorescence of Dil was detected in cytoplasm of MDM by fluorescence microscopy (A) or measured at 520/564nm (B). Data illustrated on the graph bar represent the mean \pm SEM of 4 independent experiments. *, P<0.05 vs 5.6 mmol/L glucose.

II.3. The third article

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C-reactive protein (CRP) enhances lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) expression in human aortic endothelial cells. Relevance of LOX-1 to CRP-induced endothelial dysfunction.

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ABSTRACT

Background— C-reactive protein (CRP), a characteristic inflammatory marker, is a powerful predictor of cardiovascular events. Recent data suggest that CRP may also promote atherogenesis through inducing endothelial dysfunction. Lectin-like oxidized low-density lipoprotein (oxLDL) receptor-1 (LOX-1) is a newly identified endothelial receptor for oxLDL that plays a pivotal role in oxLDL-induced endothelial dysfunction. Whether CRP may regulate endothelial LOX-1 and induce endothelial dysfunction through this receptor is unknown.

Methods and Results—In the present study, we studied the *in vitro* effect of CRP on LOX-1 expression in human aortic endothelial cells (HAECs) and the role of LOX-1 in CRP-induced human monocyte adhesion to endothelium and oxLDL uptake by endothelial cells. Incubation of HAECs with CRP enhanced, in a dose- and time-dependent manner, LOX-1 mRNA and protein levels. Induction of LOX-1 protein was already present at 5 $\mu\text{g/ml}$ CRP and reached a maximum at 25 $\mu\text{g/ml}$. This effect was reduced by antibodies against CD32/CD64, endothelin-1 and interleukin-6 (IL-6). The extent of stimulation of LOX-1 achieved by CRP was comparable to that elicited by high glucose and IL-6 and remained unchanged in presence of these factors. Finally, CRP increased, through LOX-1, both human monocyte adhesion to endothelial cells and oxLDL uptake by these cells.

Conclusions—We conclude that CRP enhances endothelial LOX-1 expression and propose a new mechanism by which CRP may promote endothelial dysfunction, that of inducing LOX-1.

INTRODUCTION

Atherosclerosis is an inflammatory process that takes place in the arterial wall and is accompanied by a systemic response. Since the concept of an inflammatory soil of atherosclerosis has been validated¹⁻³, serum markers of inflammation have been identified as risk markers for cardiovascular (CV) disease. Among these, C-reactive protein (CRP) has been proven to be the strongest predictor of CV events.^{4,5} Besides being a risk marker, CRP may further play a pivotal role in promoting atherogenesis. Arguing for this hypothesis, it has been shown that CRP increases the release of inflammatory cytokines⁶, enhances the binding of monocytes to endothelium⁷ and favors macrophage foam cell formation.⁸ CRP also decreases endothelial nitric oxide synthase activation while increasing the expression of endothelial cell adhesion molecules, chemokines, endothelin-1 (ET-1), and plasminogen activator inhibitor-1 (PAI-1).⁹⁻¹³

Unregulated uptake of oxidized low-density lipoprotein (oxLDL) by vascular cells is a crucial step in atherogenesis. Endothelial LOX-1 is the major receptor of oxLDL¹⁴ and accumulating evidences indicate that oxLDL uptake through this receptor induces endothelial dysfunction. Indeed, oxLDL binding to endothelial LOX-1 generates superoxide anions, decreases nitric oxide (NO) production and activates nuclear factor- κ B (NF- κ B).¹⁵⁻¹⁶ Furthermore, inhibition of LOX-1 reduces ox-LDL-mediated up-regulation of monocyte chemoattractant protein-1 (MCP-1) and monocyte adhesion to endothelial cells.¹⁷ Endothelial LOX-1 expression¹⁷ is induced by various pro-inflammatory cytokines, such as tumor necrosis factor α (TNF α)¹⁸ and transforming growth factor β (TGF β)¹⁹ as well as by pro-atherogenic factors, such as oxLDL and advanced glycation end products (AGEs) in vitro.²⁰ This receptor is expressed in the

aortas of hypertensive,²¹ diabetic,²⁰ and hyperlipidemic²² animals and is upregulated in early human atherosclerotic lesions.²³ In the present study, we hypothesized that CRP-induced endothelial dysfunction may be mediated in part via LOX-1. Thus we tested the effect of CRP on endothelial LOX-1 expression and the role of this receptor in CRP-induced monocyte adhesion and oxLDL uptake by endothelial cells.

MATERIALS AND METHODS

Reagents

Phenylmethylsulfonyl fluoride (PMSF), Hank's balanced salt solution (HBSS), penicillin-streptomycin, glycine, sodium dodecyl sulfate (SDS) and Trizol reagent were obtained from Invitrogen life technologies (Burlington, Ontario, Canada). Human aortic endothelial cells (HAECs), endothelial growth culture medium (EGM) and EGM bullet kit were obtained from Cedarlane Laboratories Limited (Hornby, Ontario, Canada). D-glucose, bovine serum albumin (BSA) fraction V, dianisidine dihydrochloride, hexadecyltrimethylamine ammonium bromide (HTAB), isopropanol and E-TOXATE kit were purchased from Sigma. END-X B15 endotoxin removal affinity resin kit was obtained from Seikagaku America (Falmouth, MA02540, USA). Ficoll and horseradish peroxidase-conjugated anti-mouse IgG were obtained from Amersham Biosciences. Monoclonal antibody against β -actin was bought from Santa Cruz Biotechnology (Santa Cruz, CA). Recombinant human interleukin-6 (IL-6), IgG₁ neutralizing antibody, and antibodies against intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), E-Selectin, IL-6, ET-1 were purchased from R&D Systems (Minneapolis, MN). Monoclonal antibody to human CD32 was purchased from MEDICORP (Montreal, QC, Canada). Monoclonal antibodies to human LOX-1 and human CD64 were kindly provided by Dr. Sawamura (National Cardiovascular Center Research Unit, Osaka, Japan) and Dr. Sarfati (CHUM Research Center, Montreal, Canada), respectively. Recombinant human CRP and actinomycin D were obtained from Calbiochem (La Jolla, CA). 1,1'-dioctadecyl-3,3',3',3'-tetramethylindocarbocyanine perchlorate (DiI) was bought from Molecular Probes.

Cells

HAECs were grown to confluence in EGM under recommended conditions. The EGM was supplemented with 2% fetal bovine serum (FBS) containing 0.01 $\mu\text{g/ml}$ human epidermal growth factor, 0.1% gentamicin sulfate amphotericin-B, 1 $\mu\text{g/ml}$ hydrocortisone and 12 $\mu\text{g/ml}$ bovine brain extract protein content. Cells were used at passages 3 to 5. Confluent HAECs were exposed for different time periods to various concentrations of endotoxin-free CRP. Endotoxin was removed from CRP using END-X B15 endotoxin removal affinity resin kit at 4°C overnight. After removal of endotoxin, CRP contained less than 3 pg/ml endotoxin, as detected by E-TOXATE kit. In some experiments, cells were pretreated with antibodies against CD32, CD64, ET-1 or IL-6 or co-incubated with CRP in the presence or absence of glucose (30mmol/L), or IL-6.

Human monocytes were isolated as previously described.²⁴ Briefly, peripheral blood mononuclear cells were isolated from healthy control subjects by density centrifugation using Ficoll, allowed to aggregate in the presence of fetal calf serum (FCS), then further purified by the rosetting technique. After density centrifugation, highly purified monocytes (85-90%) were recovered. Human monocyte purity was assessed by flow cytometry (FACScan, Becton Dickinson) using phyco-erythrin-conjugated anti-CD14 monoclonal antibody (Becton Dickinson).

Analysis of mRNA expression

Expression of the LOX-1 gene in human HAECs was measured by polymerase chain reaction (PCR) technique. Total RNA for use in the PCR reaction was extracted from cells by an improvement of the acid-phenol technique of Chomczynski.²⁵ Briefly, cells were lysed with TRIzol reagent and chloroform was added to the solution. After

centrifugation, the RNA present in the aqueous phase was precipitated and resuspended in diethyl pyrocarbonate water. cDNA was synthesized from RNA by incubating total cellular RNA with 0.1 µg oligodT (Pharmacia) for 5 min at 98°C then by incubating the mixture with reverse transcription buffer for 1h at 37°C. The cDNA obtained was amplified by using 0.8 µmol/L of two synthetic primers specific for human LOX-1 (5'-TTACTCTCCATGGTGGTGCC-3') (5'-AGCTTCTTCTDCTTGTTGCC-3') and human glyceraldehyde-3-phosphate deshydrogenase (GAPDH) (5'-CCCTTCATTGACCTCAACTACATGG-3') (5'-AGTCTTCTGGGTGGCAGTGATGG-3'), used as internal standard in the PCR reaction mixture. A 193-base pair human LOX-1 cDNA fragment and a 456-base pair human GAPDH cDNA fragment were amplified enzymatically by 30 and 25 repeated cycles, respectively. An aliquot of each reaction mixture was then subjected to electrophoresis on 1% agarose gel containing ethidium bromide. The intensity of the bands was measured by an image analysis scanning system (Alpha Imager 2000, Packard Instrument Company). Titrating the cDNA samples ensured that the signal lies on the exponential part of the standard curve.

Western-Blot

HAEC protein extracts (12 µg) were applied to 10% SDS-PAGE and transferred to a nitrocellulose membrane using a Bio-Rad transfer blotting system at 100 V for 1 hour. Non-specific binding was blocked with 5% BSA for 1 hour at room temperature. After washing with PBS-Tween 0.1%, blots were incubated overnight at 4°C with anti-LOX-1 or anti-β-actin antibodies. After further wash, membranes were incubated for 1 hour at room temperature with a horseradish peroxidase-conjugated donkey anti-mouse

IgG (1/5000). Antigen detection was performed with an enhanced chemiluminescence detection system (Amersham).

Adhesion assay

Confluent HAECs were exposed for 15 hours to 25 µg/ml CRP, then treated for 1 hour in the presence of antibodies to IgG₁ or LOX-1. HAECs were then washed twice with HBSS and incubated for 2 hours with freshly purified human monocytes (280,000 cells/well) re-suspended in serum-free RPMI medium. At the end of this incubation period, non-adherent monocytes were removed by washing the cells with PBS (pH 6.0). Monocyte adhesion to HAECs was quantified by measuring monocyte myeloperoxidase (MPO) activity.²⁶

Determination of endothelial cell associated adhesion molecule expression

Endothelial cell surface expression of ICAM-1, VCAM-1 and E-Selectin was determined by the cellular-enzyme-linked immunosorbent assay (ELISA) method. After treatment with CRP for 15 hours, HAECs were washed with PBS. To block non-specific binding, endothelial cells were treated for 1 hour at room temperature with PBS-3% BSA. Ten µg/ml of monoclonal antibodies against ICAM-1, VCAM-1, E-Selectin, and control IgG₁ were then added to the cells for 2 hours at 37°C. After washing, endothelial cells were incubated for 90 minutes with horseradish conjugated anti-mouse IgG (1/1000) (Bio-Rad). The peroxidase substrate, o-phenylenediamine dihydrochloride, was then added to the cells. The reaction was stopped by addition of 50µl of H₂SO₄ (0.5M) and the optical density was read at 490 nm.

Uptake of Dil-oxLDL

Native LDL (density, 1.019 to 1.063) was isolated from plasma obtained from healthy donors by sequential ultracentrifugation using potassium bromide for density adjustment.²⁷ Native LDL was extensively dialyzed for 24 hours at 4°C against 5 mmol/L Tris/50 nmol/L NaCl to remove EDTA. Oxidation of LDL was performed by incubating native LDL (2mg prot/ml) at 37°C for 20 hours in serum-free RPMI 1640 containing 7.5 µg/ml CuSO₄. Oxidation of LDL was monitored by measuring the amount of thiobarbituric acid-reactive substances (approximately 10 nmol malondialdehyde equivalent/mg protein) and by electrophoretic mobility on agarose gel (data not shown). Labeling of oxLDL with Dil was performed as described previously.²⁸ To examine cellular uptake of oxLDL, HAECs were seeded in 8-well cultureslides (FALCON®) and incubated for 3 hours in medium containing 5% of lipoprotein-deficient serum with Dil-labeled oxLDL (80 µg/ml) in the presence or absence of a 500-fold excess of unlabeled oxLDL. At the end of the incubation period, cells were washed, mounted on coverslips with mounting medium and examined by fluorescence microscopy. To measure amounts of Dil-oxLDL accumulated in cells, HAECs were seeded in 12-well plates receiving the same treatment as mentioned above. At the end of the incubation period, Dil was extracted by isopropanol and the fluorescence was determined at 520/564 nm. Results were normalized to total cell protein concentrations.²⁹

Statistical Analysis

All values were expressed as the mean ± SEM. Data were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey test. A P value less than 0.05 was considered statistically significant.

RESULTS

Effect of CRP on endothelial LOX-1 mRNA expression.

Incubation of HAECs for 6 hours with 25 $\mu\text{g/ml}$ CRP enhanced LOX-1 mRNA levels. This effect was sustained up to 24 hours (Fig. 1A-a). LOX-1 mRNA levels, normalized to the levels of GAPDH mRNA (Fig. 1A-b) are presented in Fig. 1A-c. The stimulatory effect of CRP on LOX-1 was already present at a concentration of 5 $\mu\text{g/ml}$ and was maximal with CRP concentrations ranging between 10 and 25 $\mu\text{g/ml}$. (Fig. 1B-a). LOX-1 mRNA levels, normalized to the levels of GAPDH mRNA (Fig. 1B-b) are presented in Fig. 1B-c. Preincubation of the cells with actinomycin D (5 $\mu\text{g/ml}$) prevented this effect (LOX-1 gene expression (% of control values): controls:100 \pm 13; CRP(25 $\mu\text{g/ml}$):215 \pm 45; actinomycin D+CRP:95 \pm 13). Interestingly, an increase in LOX-1 mRNA expression was also observed when cells were treated for 7 days with 5 $\mu\text{g/ml}$ CRP (LOX-1 gene expression (% of control values): CRP (5 $\mu\text{g/ml}$):143 \pm 8, $P < 0.05$ vs controls).

Effect of CRP on endothelial LOX-1 protein expression.

Treatment of HAECs for 15 hours with 25 $\mu\text{g/ml}$ CRP significantly increased LOX-1 protein levels. This effect was sustained up to 48 hours (Fig. 2A-a). LOX-1 protein levels normalized to the levels of β -actin (Fig.2A-b) are illustrated in Fig. 2A-c. CRP-induced LOX-1 protein expression was dose-dependent with maximal effect being observed between 10 and 25 $\mu\text{g/ml}$ CRP. (Fig. 2B-a). LOX-1 protein levels normalized to the levels of β -actin (Fig.2B-b) are illustrated in Fig. 2B-c. To determine whether the CRP effect on LOX-1 is receptor-mediated, HAECs were pre-incubated in the presence of antibodies to CD32 and/or CD64 prior treatment with CRP. CRP effect on LOX-1 was

reduced in part by anti-CD32 and anti-CD64 antibodies and coincubation of the ECs with both antibodies totally abrogated this effect (Fig. 2C).

Effect of glucose and IL-6 on CRP-induced endothelial LOX-1 protein expression.

Because diabetes is associated with inflammatory processes with an increase in the levels of CRP³⁰⁻³¹ and pro-inflammatory cytokines³²⁻³³, we studied the effect of CRP on LOX-1 expression in high glucose and IL-6-treated HAECs. As reported earlier³⁴, incubation of HAECs in a high glucose environment led to increased endothelial LOX-1 protein expression. Addition of CRP did not increase additionally LOX-1 expression under hyperglycemic conditions (controls: 100 ± 1 ; CRP: 173 ± 8 ; glucose: 209 ± 13 ; CRP+glucose: 161 ± 5). Treatment of HAECs with IL-6 (0-200ng/ml) resulted in a dose-dependent increase in LOX-1 protein expression with maximal effect at 100 ng/ml (Fig.3) CRP did not potentiate this effect (LOX-1 protein expression (% of control values): controls: 100 ± 1 ; CRP: 173 ± 8 ; IL-6: 168 ± 15 ; CRP+IL-6: 169 ± 12). CRP-induced LOX-1 expression was reduced by ET-1 and IL-6 inhibition. Coincubation of endothelial cells with anti-ET-1 and IL-6 antibodies totally suppressed this effect (LOX-1 protein expression (% of control values): controls: 100 ± 1 ; CRP: 178 ± 8 ; CRP+anti-IL-6: 120 ± 19 ; CRP+anti-ET-1: 142 ± 22 ; CRP+anti-IL-6+anti-ET-1: 88 ± 6).

CRP enhances monocyte binding to HAECs. Role of LOX-1

Treatment of HAECs with CRP (25 μ g/ml) or lipopolysaccharide (10ng/ml), used in these experiments as positive control (data not shown), increased monocyte adhesion to these cells (Fig.4). Anti-LOX-1 antibody suppressed this effect, while anti-IgG1 antibody was ineffective (Fig.4). Under our experimental conditions, CRP did not enhance cell associated ICAM-1, VCAM-1 or E-Selectin (adhesion molecule protein

expression (% over control values): ICAM-1: 115 ± 7 ; VCAM-1: 97 ± 5 ; E-Selectin: 90 ± 9) and incubation of HAECs with antibodies to ICAM-1, VCAM-1, and E-Selectin failed to affect CRP-induced monocyte adhesion (Fig.4).

CRP stimulates oxLDL uptake in endothelial cells via LOX-1.

To evaluate whether induction of LOX-1 by CRP results in enhanced uptake of oxLDL by endothelial cells, HAECs were treated for 14 hours with CRP ($25 \mu\text{g/ml}$), then incubation was pursued for 1 hour in the presence of saturating amounts ($20 \mu\text{g/ml}$) of antibodies to LOX-1 or IgG₁. At the end of the incubation period, cells were exposed for 3 hours to Dil-oxLDL ($80 \mu\text{g/ml}$) in the presence or absence of excess unlabeled oxLDL. Incubation of HAECs with CRP led to enhanced uptake of oxLDL by these cells as assessed by fluorescence microscopy (Fig 5A) and measurement of extracted Dil-oxLDL (Fig 5B). This effect was abrogated by incubating HAECs with anti-LOX-1 antibody or with excess unlabeled oxLDL. In contrast, exposure of these cells to anti-IgG₁ did not affect CRP-induced oxLDL uptake by endothelial cells (Fig 5A and B).

DISCUSSION

Accumulating evidences indicate that chronic low-grade inflammation is a major pathogenic component of endothelial dysfunction.¹ Consistent with this concept, elevated serum CRP levels have been found to be associated with blunted endothelium-dependent vasodilation in vivo.³⁵ and a direct proinflammatory effect of CRP on endothelial cell has been documented in vitro.⁹⁻¹³ The present study which demonstrates that CRP enhances the expression of LOX-1, a limiting factor for oxLDL uptake by endothelial cells, further stresses the potential key role of CRP in endothelial dysfunction. Induction of LOX-1 by CRP is receptor-mediated and appears to be exerted at the transcriptional level, as reflected by the parallel increase in LOX-1 gene and protein expression and the inhibitory effect of actinomycin D on CRP-induced LOX-1 gene expression. Transcriptional activation of the LOX-1 gene by CRP may theoretically involve NF- κ B. Supporting this possibility, a NF- κ B responsive element has been located in the promoter of the LOX-1 gene³⁶ and increased transcriptional activity of this factor has been documented in CRP-treated endothelial cells.³⁷ CRP predicts incident type 2 diabetes^{38,39} and is increased in subjects with diabetes.⁴⁰ Thus, hyperglycemia, in states of high CRP, may exaggerate the deleterious effects of CRP on endothelial cell activation. In contrast to previous observations showing that high glucose potentiates the proatherogenic effects of CRP in endothelial cells^{13,41}, we found that induction of LOX-1 by CRP remained unchanged by hyperglycemia, suggesting that CRP and glucose may operate through common molecular mechanisms to induce LOX-1. Because glucose induces endothelial LOX-1 through NF- κ B activation³⁴ and because CRP activates this transcription factor in endothelial cells³⁷, it is tempting to

speculate that NF- κ B may represent a common signal mediator of CRP and glucose effect on LOX-1.

CRP and glucose are well known activators of ET-1 release by endothelial cells^{11,42} and this peptide mediates the proinflammatory effects of CRP¹¹. Interestingly, ET-1 is also a LOX-1 stimulatory factor⁴³, and thus may mediate CRP effect on LOX-1. In support for this possibility, our data demonstrate that inhibition of ET-1 attenuates CRP effect on LOX-1. ET-1 is one of the upstream activators of IL-6 secretion⁴⁴ and recent data suggest that CRP may stimulate the release of this cytokine by endothelial cells.¹¹ Our results which demonstrate that IL-6 induces endothelial LOX-1 and that inhibition of IL-6 reduces CRP-induced LOX-1 expression clearly identify IL-6 as one mediator of CRP effect on LOX-1. Our finding that attenuation of CRP-induced LOX-1 expression was greater during coincubation with anti-ET-1 and IL-6 antibodies support the concept that CRP induces LOX-1 via stimulating the production of ET-1 and IL-6 concurrently.

One major pathophysiological event associated with endothelial dysfunction is increased monocyte adhesion to endothelial cells. Recent studies have demonstrated that CRP increases monocyte-endothelium interaction^{7,45} by inducing endothelial and monocyte adhesion molecules.^{7,9} Our findings that CRP increases the expression of LOX-1, a well identified cell-adhesion molecule⁴⁶, and that blockade of LOX-1 abolished CRP-induced monocyte adhesion demonstrate a new role for LOX-1, that of mediating the stimulatory effect of CRP on monocyte adhesion. In contrast to one previous study showing that CRP increases the expression of E-selectin, ICAM-1 and VCAM-1 in endothelial cells⁸, we did not observe an upregulation of these antigens in CRP-treated

HAECs. Several differences in the experimental conditions used in the two studies, including the time points at which adhesion molecule expression was determined and the concentrations of human serum present in the incubation medium are likely to provide a good explanation for this discrepancy.

In contrast to other vascular cells that do express multiple oxLDL receptors, LOX-1 is definitively the limiting factor for oxLDL uptake by endothelial cells and uptake of oxLDL by this receptor induces endothelial dysfunction. Our results showing that CRP enhances, through LOX-1, the uptake of oxLDL by endothelial cells suggest that CRP may trigger the toxic effect of oxLDL on vascular endothelium.

In conclusion, the present study demonstrates that CRP, at concentrations applicable to both acute and low-grade chronic inflammation, increases endothelial LOX-1 expression. Whether this potentially proatherogenic effect of CRP has clinical relevance remains to be evaluated.

ACKNOWLEDGMENTS

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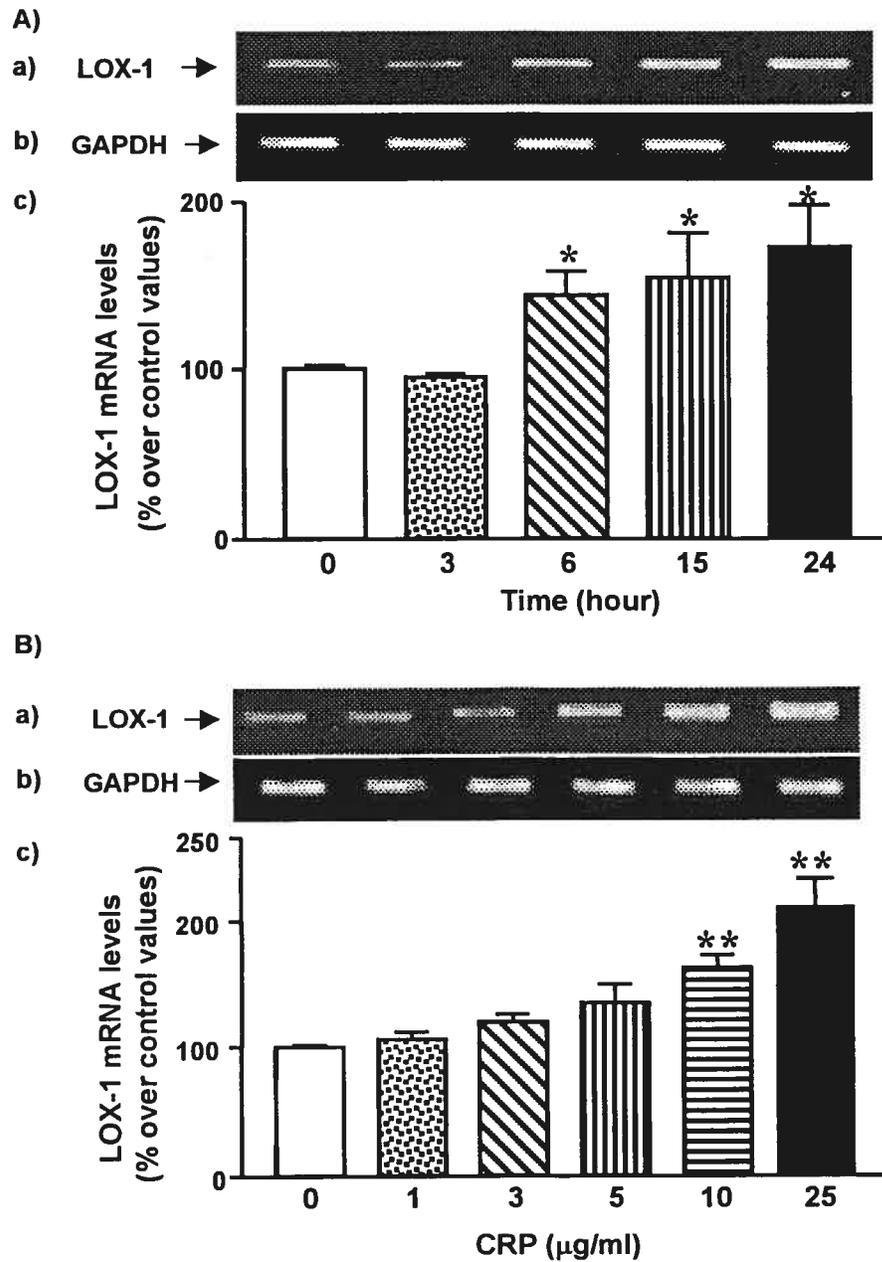


Fig.1. Time- and dose-dependent effect of CRP on LOX-1 mRNA levels in HAECs. HAECs were incubated for 3 to 24 hours with 25 µg/ml CRP (A) or for 6 hours with 1 to 25 µg/ml CRP (B). At the end of the incubation period, cells were lysed and LOX-1 mRNA was analyzed by RT-PCR. LOX-1 mRNA levels (a) were normalized to the levels of GAPDH mRNA (b). Data illustrated on the graph bar (c) represent the mean \pm SEM of 6 different experiments. *, $p < 0.05$ vs control.

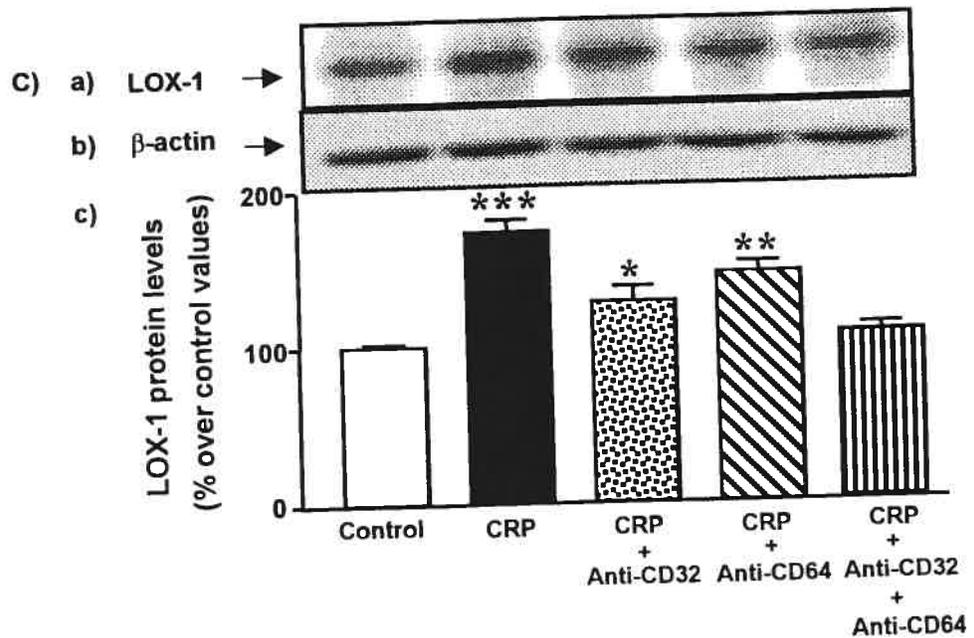
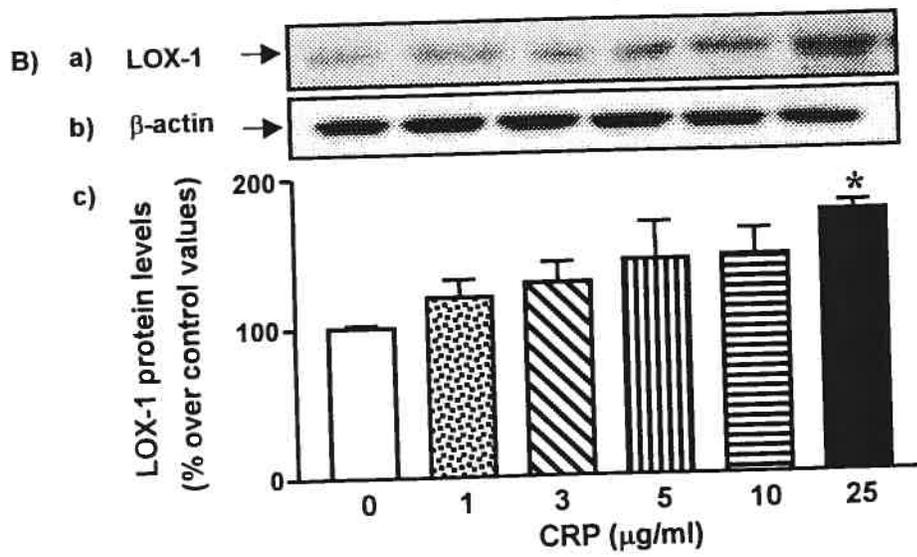
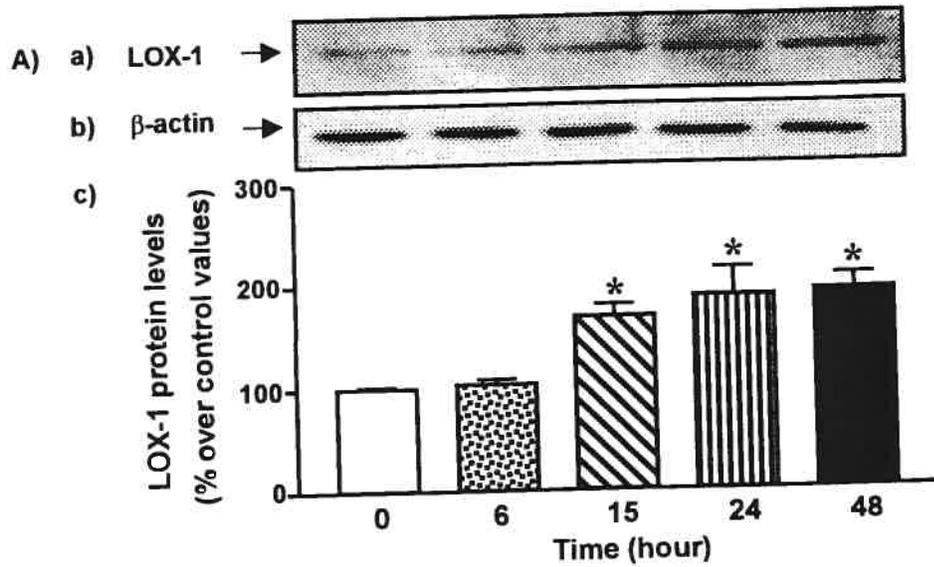


Fig.2. Time- and dose-dependent effect of CRP on endothelial LOX-1 protein expression. HAECs were cultured for 6 to 48 hours with 25 $\mu\text{g/ml}$ CRP (A) or for 15 hours with 1-25 $\mu\text{g/ml}$ CRP (B). In some experiments, HAECs were preincubated with anti-CD32 and/or anti-CD64 antibodies prior exposure for 15 hours with 25 $\mu\text{g/ml}$ CRP (C). At the end of the incubation period, cells were lysed and LOX-1 membrane protein expression was determined by Western blot analysis (a). LOX-1 protein levels were normalized to the levels of β -actin protein (b). Data illustrated on the graph bar represent the mean \pm SEM of 5 (A) and 6 (B-C) different experiments (c). *, $p < 0.05$, **, $p < 0.01$, ***, $P < 0.001$ vs control.

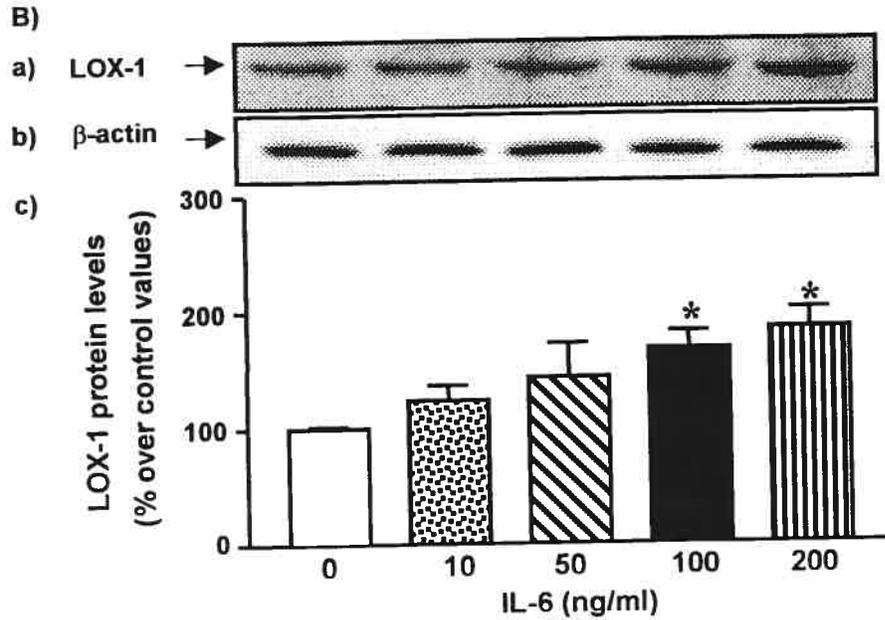


Fig.3. Dose-dependent effect of IL-6 on endothelial LOX-1 protein expression. HAECs were incubated for 15 hours with IL-6 (10-200 ng/ml). At the end of the incubation period, cells were lysed and LOX-1 membrane protein expression was determined by Western blot analysis (a). LOX-1 protein levels were normalized to the levels of β -actin protein (b). Data illustrated on the graph bar represent the mean \pm SEM of 4 different experiments (c). *, $p < 0.05$, vs control.

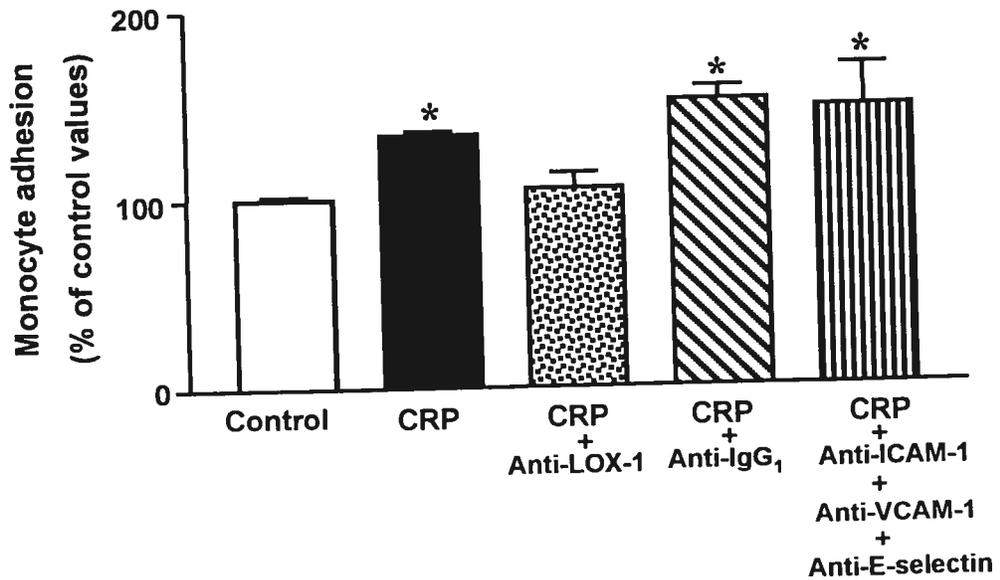
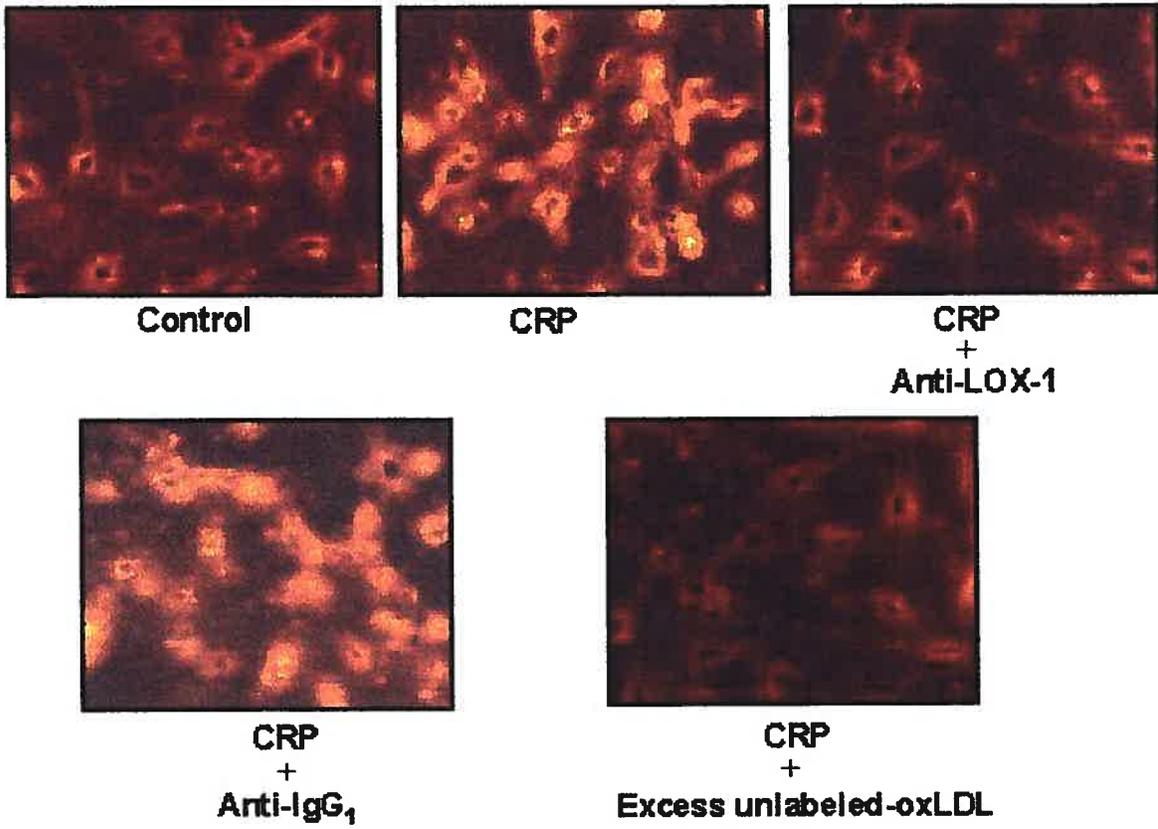


Fig.4. Effect of CRP on human monocyte adhesion to endothelial cells. Confluent HAECs were exposed for 15 hours to CRP (25 $\mu\text{g/ml}$) in the presence of saturating amounts (20 $\mu\text{g/ml}$) of antibodies to LOX-1, IgG₁, ICAM-1, VCAM-1 and E-selectin. At the end of this incubation period, cells were washed and monocytes were added to HAECs to determine monocyte adhesion. Data are expressed as percentage of adherent monocytes to HAECs and represent the mean \pm SEM of 6 different experiments *, $p < 0.05$ vs control.

A)



B)

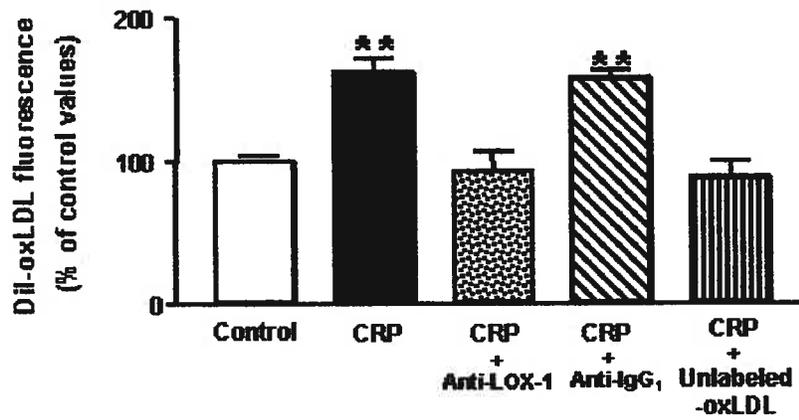


Fig.5. Effect of CRP on oxLDL uptake by HAECs. HAECs were treated for 14 hours with CRP (25 $\mu\text{g/ml}$), then incubation was pursued for an additional 1 hour period in the presence of saturating amounts (20 $\mu\text{g/ml}$) of antibodies to LOX-1 or IgG₁. At the end of the incubation period, cells were exposed for 3 hours to Dil-oxLDL (80 $\mu\text{g/ml}$) in the presence or absence of excess unlabeled oxLDL. After washing, fluorescence of Dil was detected in cytoplasm of HAECs by fluorescence microscopy (A) or measured at 520/564nm (B). Data illustrated on the graph bar represent the mean \pm SEM of 6 independent experiments. **, $p < 0.01$ vs control.

III. DISCUSSION

Diabetes mellitus is one of the most costly and burdensome chronic diseases. The number of diabetic patients is increasing worldwide and the WHO predicts that between 1997 and 2025, the number of diabetics will double from 150 to about 300 million. The incidence of type 2 diabetes in the US has increased by about 33% over the past decade and is expected to increase further. The most important factors contributing to the development of type 2 diabetes are obesity and physical inactivity. During the past decade, the percentage of Americans defined as obese has increased by about 33%. It is not a coincidence that these figures mirror the dramatic rise in diabetes over the same 10-year period.

Type 2 diabetes, accounts for 80-90% of diabetes mellitus worldwide [Simpson et al. 2003]. It is a major risk factor for CVD [McGill et al. 1998, Khamaisi et al. 2003] and is associated with a two- to eight-fold increase in the risk of CVD morbidity and mortality [Harris et al. 1998]. The pathogenesis of CVD in type 2 diabetes is multifactorial. Although conventional CVD risk factors such as dyslipidemia and hypertension, are operative in type 2 diabetes, these risk factors only partly account for the excessive risk of developing CVD in the diabetic population. Recognition of this fact has recently led to the identification of novel risk factors specific to individuals with diabetes. These factors include insulin resistance, post-prandial hyperglycemia, inflammation, AGE formation, endothelial dysfunction and oxidative stress. Today evidence exists that much of the increased risk of developing CVD in diabetes is related to insulin resistance. Insulin resistance is a hallmark of type 2 diabetes that precedes and predicts the

disease for several years. It is related to both traditional and nontraditional CV risk factors, including hyperglycemia, dyslipidemia, hypertension and obesity, linking all these abnormalities to the development of CV disease. For such reasons, insulin resistance might be regarded as an accomplice in the pathogenesis of CV disease in type 2 diabetes. It has been recently proposed that insulin resistance might also contribute to CV disease in a direct and independent pathway, i.e. independently of classic risk factors [Bonora et al. 2002]. Insulin resistance is associated with endothelial dysfunction and inflammation, thus supporting the concept that the atherosclerotic process may actually begin earlier in the spectrum of insulin resistance. These data suggest that detection of insulin resistance relatively early in life may offer the opportunity to identify those persons likely to develop dyslipidemia, hypertension and ultimately, diabetes and CVD. Improvement of insulin resistance might have beneficial effects not only on glucose control but also on CV disease in patients with type 2 diabetes.

Type 2 diabetes is preceded by a long period of asymptomatic hyperglycemia. While glucose is the driving force in microvascular complications of diabetes, yet the extent to which hyperglycemia contributes to diabetic macrovascular complications is still controversial. Although some epidemiological and intervention studies have reported a correlation among glucose, atherosclerotic plaque burden, CV events and increased morbidity and mortality [Laakso et al. 1996, Tominaga et al, 1999, Ledru et al. 2001] as well as a reduction of CV risk by glycemetic control [Laakso et al. 1999, Gerstein et al. 1996,

Fuller et al. 1983, Fuller et al. 1980, Singer et al. 1992], other studies have demonstrated that hyperglycemia is not a particularly strong determinant for developing CVD in type 2 diabetic patients. For example the UKPDS has shown that intensive blood-glucose control by either sulphonylureas or insulin does not statistically reduce the risk of developing macrovascular disease in patients with type 2 diabetes, although treatment sufficient to lower median HbA_{1c} to 7% over 10 years reduced the risk of myocardial infarction by 16% in these subjects [UKPDS Group 1998]. There are two possible explanations for the weak association between glycemia and CVD. The first possible explanation is that postprandial glycemia may be more importantly related to CVD than fasting glycemia. Supporting this possibility, epidemiological data have demonstrated that postprandial plasma glucose is an independent risk factor for CVD in diabetic subjects [The DECODE Study Group 1999, Bonora et al 2000, 2001, Haffner et al. 2003] Another possible explanation for the weak influence of glycemia on CVD in diabetes is the existence of an atherogenic prediabetic state. Numerous studies have shown that subjects before diabetes have an adverse pattern of dyslipidemia and hypertension, with increased levels of inflammatory markers and PAI-1 [Medalie et al. 1975, McPhillips et al. 1990, Mykkanen et al. 1993, Haffner et al. 1990, Fagot-Campagna et al. 1997]. This provides possible mechanisms for the common basis of type 2 diabetes and CVD as initially suggested by Stern in the "common soil" hypothesis [Stern 1995],.

As mentioned above, patients with type 2 diabetes present elevated serum levels of inflammatory markers and cytokines, such as CRP, fibrinogen,

TNF α and IL-6, thus suggesting a low-grade inflammatory condition in type 2 diabetes. Because atherosclerosis is an inflammatory-mediated disease [Ross 1999], it has been proposed that diabetes and atherosclerotic CV disease might share a common inflammatory basis. Epidemiological evidence showing that inflammatory markers predict incident diabetes mellitus, metabolic syndrome in addition to CV events supports this hypothesis.

Two viewpoints exist on the factors that initiate the acute phase response in insulin-resistant and diabetic patients. The first hold that the acute phase response is activated by ongoing intraarterial inflammation in which arterial wall resident inflammatory cells secrete cytokines in response to multiple stimuli. According to the second view, extravascular stimuli, including obesity, aging, infections, smoking, and stress induce a chronic, low-level activation of the acute phase response. In this regard, it is now well accepted that obesity constitutes a low-grade inflammatory state and that adipose tissue is the source of many biologically active proinflammatory secretory molecules such as TNF α , IL-6 , PAI-1 and CRP The final result of both views would be the triggering of the inflammatory cascade leading to atherosclerosis.

As discussed before, much of the increased risk of developing CVD in diabetes is related to insulin resistance. Evidence that insulin resistance is closely associated with chronic subclinical inflammation [Pradhan et al. 2001, Barzilay et al. 2001, Hanley et al. 2002, Pradhan et al. 2003] and in particular with increased plasma CRP levels [Yudkin et al. 1999, Hak et al. 1999, Lemieux et al. 2001], supports the notion that proinflammatory processes may contribute

to atherogenesis by inducing insulin resistance [Nesto 2004]. For example, expression of $\text{TNF}\alpha$ and IL-6 at high levels in the adipose tissue may function locally and systematically to decrease insulin release and action. Although there is good evidence that these mediators may contribute to insulin resistance in animal models, the exact molecular mechanisms that lead to the proinflammatory changes resulting in insulin resistance in humans are however unclear. Conversely, defects in insulin action on the main insulin-sensitive tissues, i.e. adipose tissue, muscle and liver, may also lead to a worsening of the chronic, low-grade inflammatory state. For example, in the liver, insulin seems to be one of the main inhibitors of the cytokine-associated acute-phase response. Thus, the lack of significant insulin action, as found in type 2 diabetes, would not be able to block $\text{TNF}\alpha$, IL-1 and IL-6 actions. From this, it is tempting to conclude that the relationship is bidirectional: any process linked to chronic inflammation will decrease insulin action and insulin resistance will lead to worsening of inflammation in a vicious cycle.

There is little doubt that one important link between insulin resistance and diabetic vasculopathy is endothelial dysfunction. Indeed, endothelial dysfunction is a key early event in atherogenesis, predicts CV events and evidence exists that endothelial dysfunction and insulin resistance closely coexist throughout the natural history of type 2 diabetes [Steinberg et al. 1996, Balletshofer et al. 2000]. Indeed, endothelial dysfunction is detected in obese patients, in relatives without diabetes as well as in the spectrum of insulin resistance, before the detection of any distinct carbohydrate intolerance. Insulin resistance may contribute to

endothelial dysfunction through systemic defect of the PI3 kinase pathway and decreased insulin-mediated endothelial NO production. In the same way insulin resistance may contribute to endothelial dysfunction, evidence also exists that defects in NO-mediated vasodilation may contribute to insulin resistance. Multiple interrelated mechanisms likely contribute to endothelial dysfunction in insulin resistance. Virtually all components of the metabolic syndrome, such as hypertension, dyslipidemia and hyperglycemia have been shown to induce endothelial dysfunction. Hyperglycemia may induce endothelial dysfunction through multiple mechanisms including protein glycation, oxidative stress, activation of the PKC and polyol pathways as well as activation of the coagulation cascade.

Two potential mechanisms that may link insulin resistance with endothelial dysfunction are oxidative stress and inflammation. Supporting this possibility, it has been shown that insulin resistance is associated with increased oxidative stress and inflammation and that agents with antioxidant and antiinflammatory properties improve endothelium-dependent vasodilation and insulin sensitivity in diabetic patients. While the causal role of inflammation on endothelial dysfunction is well demonstrated, the possibility that the mechanistic basis for the powerful predictive value of CRP may reside at the level of the endothelium has only been recently investigated. In 2000, Fichtlcherer et al have reported that elevated CRP serum levels are associated with blunted systemic endothelial vasodilation, suggesting that identification of elevated CRP levels as an independent risk factor for endothelial dysfunction might provide an important clue to link a

systemic marker of inflammation to atherosclerotic disease development. More recently, *in vitro* studies have suggested a direct causal role for CRP in endothelial dysfunction. These studies have demonstrated that CRP increases the expression of ET-1, MCP-1 and adhesion molecules, activates NF- κ B and decreases NO release [Verma et al. 2002, Szmitko et al 2003]. From these data it appears that CRP is not merely an inflammatory marker but may be directly involved in atherogenesis. These findings may have important implications for future cardiovascular research.

LOX-1 is a newly identified endothelial receptor for oxLDL, which is also expressed by other vascular cell types, including macrophages and SMCs [Sawamura et al. 1997]. Numerous studies support a role of LOX-1 as a potential mediator of endothelial dysfunction. Indeed, evidence exists that oxLDL binding to LOX-1 in endothelial cells generates superoxide anions, decreases NO, activates NF- κ B pathway [Cominacini et al. 2000, Cominacini et al. 2001], and induces endothelial apoptosis [Li et al. 2000]. LOX-1 also acts as an adhesion molecule favoring monocyte adhesion to endothelium [Honjo et al. 2003, Li et al. 2002]. Of most importance, we and others have reported that metabolic factors potentially involved in endothelial dysfunction associated with diabetes upregulate endothelial LOX-1. For example, Chen et al [2001] have demonstrated that diabetic rat serum and AGEs induce endothelial LOX-1 expression, and we have recently generated preliminary data showing that FFAs increase endothelial LOX-1 expression both at gene and protein levels. In the present study, we obtained evidence that high glucose concentrations enhance

endothelial LOX-1 expression. Because increased serum concentrations of AGEs in patients with type 2 diabetes is associated with endothelial dysfunction [Tan et al. 2002] and because AGEs induce endothelial LOX-1 expression in vitro, this effect of glucose may theoretically be related to AGE formation in endothelial cells. Studies by Brownlee et al have characterised the time course for intracellular glycoxidation in vascular cells cultured in a high glucose environment. These investigators have shown that the levels of cytosol AGE-proteins increase in a time-dependent manner upon exposure of endothelial cells to high glucose, being significantly increased by 2- to 12-fold after 5 and 7 day - exposure to 30mmol/L glucose, respectively [Giardino et al. 1994, Du et al. 2003]. Since one week was not sufficient to reach a steady state even of early protein glycation products with glucose, these authors suggested that the unexpected magnitude of the increase of AGE proteins in high glucose-treated endothelial cells most likely results from glycosylation by more highly reactive intracellular sugars and glucosones. The correctness of this hypothesis was later demonstrated by studies showing the critical role for intracellular accumulation of 3-deoxyglucosone synthesized via the Maillard reaction and the polyol pathway in intracellular AGE formation [Niwa T et al. 2001]. From these data, it appears that length of incubation time is critical for AGE formation in cultured vascular cells and that this process occurs over a period of week. Considering the short incubation period of endothelial cells with high glucose in our study, it seems unlikely that, under our experimental conditions, AGE formation may be

responsible for the stimulatory effect of high glucose on endothelial LOX-1 expression.

As discussed above, high glucose per se is believed to play a key role in endothelial dysfunction associated with diabetes. In accordance with this possibility, acute hyperglycemia has been shown to induce endothelial dysfunction in both healthy subjects [Title et al. 2000] and patients with type 2 diabetes [Lee et al. 2002]. On the basis of these results and on the potential key role of LOX-1 in endothelial dysfunction, our data demonstrating that hyperglycemia enhances endothelial LOX-1 expression suggest a role for this receptor as mediator of the in vivo effect of hyperglycemia on endothelial dysfunction.

Although the signaling events underlying hyperglycemia-induced endothelial dysfunction are not well known, recent studies have strongly pointed to a decisive role of the diacylglycerol (DAG)-PKC pathway in this alteration [Ishii et al. 1998]. PKC and its activating cofactor DAG are elevated in normal tissues exposed to high glucose [Koya et al. 1998] as well as in several organs in diabetes [Shiba et al. 1993, Craven et al. 1989]. In particular, the PKC isoform β is more selectively activated in the heart and the aorta of diabetic rats [Inoguchi et al. 1992]. Importantly, inhibition of PKC attenuates vascular dysfunction in diabetes and glucose-induced endothelial dysfunction [Tesfamariam et al. 1991]. Recent studies investigating the role of PKC in glucose-induced endothelial dysfunction have proposed that PKC reduces the activity of NOS III protein by inducing the phosphorylation of this enzyme [Hirata et al. 1995] and activates

vascular NADPH oxidase, thereby leading to increased superoxide production and NOS III uncoupling in diabetic vessels [Hink et al. 2001]. Conversely, inhibition of PKC activity increases the availability of endothelium-derived NO and attenuates leukocyte-endothelium interaction in microvessels of diabetic animals [Nonaka et al. 2000], probably through the anti-inflammatory and inhibitory effects of NO on endothelial cell adhesion molecules [Altman 2003]. Glucose-induced activation of PKC and more particularly of the PKC β and δ isoforms also leads to enhanced ET1 production [Park et al. 2000]. Furthermore, PKC activation increases the production of growth factors by the endothelium, such as VEGF, EGR, and TGF β [Xia et al. 1996].

Previous studies have demonstrated that activation of LOX-1 decreases NO released from endothelial cells and induces the expression of ET-1 and cell adhesion molecules. It has been postulated that activation of LOX-1 decreases the release of NO not only by enhancing the catabolism of NO through the generation of ROS but also by attenuating eNOS activity [Mehta et al. 2002, 2003]. Our in vitro study which demonstrates that high glucose upregulates endothelial LOX-1 expression suggests that this effect may lead to enhanced binding of oxLDL and/or AGEs to LOX-1 thus resulting in further functional changes in endothelial cells in response to these factors. Future experiments should evaluate whether incubation of endothelial cells in a hyperglycemic milieu potentiates the deleterious effect of LOX-1 ligands on several biological events associated with endothelial dysfunction such as inactivation of NO, increase in ET-1 and chemokine expression and NF- κ B activation.

Diabetes is associated with changes in endothelial function that augur the development of atherosclerosis. Endothelial dysfunction mainly results from an imbalance in the local production and/or activity of potent vasoactive agents including NO and ET-1 in the vessel wall [Clement et al. 1990, Haynes et al. 1994, Martin et al. 1996]. Endothelium-derived NO is the most potent endogenous vasodilator, which inhibits leukocyte and platelet adherence to the vessel wall [Kubes et al. 1991, Tsao et al. 1994]. ET-1, produced directly by vascular endothelial cells, is the most important locally produced vasoconstrictor, acting principally through type A receptors on vascular smooth muscle cells [Levin et al. 1995]. NO and ET-1 not only function as mutual antagonists in the determination of vascular tone, but also act in net as antiatherosclerotic (NO) or proatherosclerotic (ET-1) factors through modulation of platelet activity, lipid oxidation, leukocyte chemotaxis, and local production of thrombotic factors as well as growth and proliferation of vascular smooth muscle cells and fibroblasts [Levin et al. 1995, Moncada et al. 1993, De Meyer et al. 1997]. Experimental evidence supports the notion that hyperglycemia can directly decrease endothelium-derived NO [Teschfariam et al. 1991] and alter ET-1 system [King et al. 1996]. Besides PKC activation [Teschfariam et al. 1991, Nishio et al. 1996, Ishii et al. 1996], potential mechanisms through which hyperglycemia may decrease NO include CRP and LOX-1. Indeed, elevated plasma levels of CRP have been shown to be associated with hyperglycemia and in vitro studies have provided evidence that CRP decreases NO release [Verma et al. 2002, Szmítko et al. 2003]. Furthermore, decreased NO production by endothelial cells has been

closely related to increased LOX-1 expression through NF κ B activation [Cominacini et al. 2000, 2001, Morawietz et al. 2001, 2002]. Endothelial LOX-1 expression is upregulated by its ligands, oxLDL and AGE [Chen et al. 2001], whose increased formation in diabetes results at least partly from oxidative stress [Creager et al. 2003]. Because high glucose induces oxidative stress, one may postulate that this metabolic factor by favoring oxLDL and AGEs formation may increase LOX-1 activation, thereby decreasing NO. Alternatively, hyperglycemia may directly, by generating oxidative stress and NF- κ B activation in endothelial cells, enhance LOX-1 expression. Induction of LOX-1 by high glucose may provide a new mechanism by which hyperglycemia reduces NO and induces endothelial dysfunction in type 2 diabetes.

As mentioned above, endothelial cells respond to high glucose by increasing PKC activation and, activation of this signaling pathway plays a critical role in hyperglycemia-induced endothelial dysfunction. In accordance with these observations, we found that glucose-induced upregulation of endothelial LOX-1, a key mediator of oxLDL-induced endothelial dysfunction, requires PKC activation, as reflected by the complete inhibition of LOX-1 induction by glucose in endothelial cells pretreated with the pan specific PKC inhibitor, calphostin C as well as by the specific PKC β 2 inhibitor, LY379196. Because activation of LOX-1 induces the generation of superoxide anion that activates NF- κ B and inactivates NO and enhances the expression of vasoconstrictive molecules such as ET-1, adhesion molecules and MCP-1, upregulation of LOX-1 through PKC activation may represent a new mechanism by which PKC induces endothelial dysfunction.

While there are no other reports in the literature showing a role for PKC as signal transduction molecule involved in the regulation of endothelial LOX-1 expression, previous studies have demonstrated that LOX-1 is upregulated by oxidative stress as well as by molecules that induce oxidative stress [Halvorsen et al. 2001]. Oxidative stress is increased in patients with diabetes [Wolff et al. 1991, Oberley 1988, Mullarkey et al. 1990, Bayes 1991, Williamson et al. 1993, Sano et al. 1998]. and may represent a key mechanism underlying endothelial dysfunction in diabetes mellitus. Although there is no evidence that antioxidants improve endothelium dysfunction in chronic studies, this possibility is supported by short-term studies demonstrating the acute beneficial effect of ascorbic acid on vascular function in type 2 diabetic patients [Ting et al. 1996]. It is well accepted that high glucose leads to intracellular changes in the redox state resulting in depletion of the cellular NADPH pool [Williamson et al. 1993] and generates superoxide production [Ohara et al. 1993]. Consistent with these findings and accordingly to the redox sensitive regulation of LOX-1, we demonstrated that antioxidants such as NAC, vitamin E, vitamin C, and DMSO, significantly decrease glucose-induced endothelial LOX-1 expression. Based on the link established between oxidative stress and PKC activation [Pricci et al. 2003], it is tempting to speculate that ROS generated by glucose metabolism may induce the activation of PKC thereby leading to the induction of LOX-1. Because glucose activates different members of the MAPK family in endothelial cells [Liu et al. 2000] and that co-activation of PKC and MAPK occurs in vascular cells maintained in high glucose [Haneda et al. 1995], our observation that MAPK

inhibitor completely attenuates glucose-induced endothelial LOX-1 gene expression is not surprising. On the basis of the growing literature showing that PKC can activate MAPK and that PKC inhibitors can prevent their activation by a range of stimuli, one may postulate that under our experimental conditions, MAPK are activated downstream of a cascade initiated by PKC activation. This may explain our finding that inhibitors of PKC totally abrogate glucose-induced LOX-1 expression.

The MAPK pathway consists of a group of interconnecting protein kinase cascades that link signals at the plasma membrane to nuclear events. Four different families of MAPKs that are linked to different signals and have different substrate specificities have been described, including ERK-1 and -2, c-jun NH₂-terminal kinases (JNKs), p38 kinases and big MAPK1 or ERK 5 [Tomlinson 1999]. JNKs respond to several forms of cellular stress and ERKs are primarily regarded as growth factor signaling kinases. Activation of the sorbitol pathway, increased oxidative stress and AGE formation, and other biochemical anomalies arising from hyperglycemia in diabetes, share the capacity to activate MAPK, thus indicating that these enzymes function as glucose transducers. [Tomlinson 1999]. By phosphorylating transcription factors such as NF κ B and AP-1, these kinases alter the balance of gene expression, leading to a spectrum of changes from cellular proliferation, altered production of extracellular materials or an altered phenotype [Tomlinson 1999, Haneda et al. 1997, Igarashi et al. 1999]. Many studies have indeed demonstrated that proinflammatory cytokines, mitogens, ROS, and shear stress trigger signal molecules to initiate the activation

of multiple intracellular pathways, which often converge at MAPK activation and endothelial dysfunction [Bogatcheva et al. 2003]. Importantly, several studies have documented that MAPK activation plays a role in endothelial dysfunction associated with diabetes. For example, studies have shown that glucose inhibits endothelial growth and induces endothelial apoptosis through activation of MAPK pathway [Liu et al. 2000, Nakagami et al. 2001]. In addition, a recent study has reported that hyperglycemia accelerates MCP-1 production by vascular endothelial cells through MAPK activation [Takaishi et al. 2003]. Despite evidence supporting a role of MAPK in several biological events associated with LOX-1 activation [Li et al. 2000, Iwai-kanai et al. 2001, Li et al. 2003], a role for MAPK in the regulation of endothelial LOX-1 expression has not been reported. Our study which demonstrates that glucose-induced endothelial LOX-1 expression occurs through the activation of the MAPK pathway further stress the critical importance of MAPK in endothelial dysfunction associated with diabetes [Li et al. 2003].

Previous studies have shown that the signaling pathways involved in oxLDL-induced endothelial dysfunction include the activation of the transcription factor NF κ B [Li et al. 2000, Cominacini et al. 2000]. Glucose is a well known activator of this transcription factor and a number of transcription-factor-binding sites including a NF κ B element have been found in the promoter of the LOX-1 gene [Aoyama et al. 1999]. NF κ B is a well known oxidative stress-sensitive transcription factor [Ho et al. 1999] that is activated by glucose and proinflammatory cytokines [Lavrotsky et al. 2000]. Five NF- κ B subunits that form

dimers have been identified in mammalian cells, including Rel A (p65), p50, RelB, c-Rel, and p52. The most common and best-characterized form of NF- κ B is the p65-p50 heterodimer [Delfino et al. 1999]. In the present study, we found that glucose induces LOX-1 gene expression and that antibodies to P65 and P50 significantly attenuate glucose-enhanced nuclear proteins binding to the NF κ B regulatory element present in the promoter of endothelial LOX-1 gene [Li et al. 2003]. This effect was abrogated by antioxidants, and inhibitors of PKC and MAPK. Taken together, these results suggest that glucose induces LOX-1 expression at the transcriptional level and that induction of LOX-1 gene expression in glucose-treated endothelial cells involves oxidative stress and activation of PKC/MAPK and NF κ B.

The transcription factor NF- κ B has been proposed as a critical bridge between oxidant stress and gene expression [Barnes et al. 1997]. Translocation of activated NF- κ B into the nucleus results in gene expression of NF- κ B controlled target genes such as adhesion molecules, cytokines eNOS, MCP-1 endothelin-1 and TF. Thus, this signal transduction system appears capable of transforming the appearance and disappearance of short-lived oxygen species into more sustained signals for cellular activation. While poor glycemic control has been shown to induce activation of NF- κ B in ex vivo isolated peripheral blood mononuclear cells of patients with type 1 diabetes [Levin 1998], a long-lasting sustained activation of NF- κ B has recently been documented in mononuclear cells from patients with diabetes [Bierhaus et al. 2001]. While ROS produced by the mitochondrial respiratory chain have been described as one

major mediator of hyperglycemia-dependent NF- κ B activation [Giugliano et al. 1996], monocytes do not contain mitochondria and thus additional routes of ROS production seem to be involved. In this regard, a recent study has demonstrated that upon AGE engagement of RAGE, NADPH oxidase is one major source of ROS in endothelial cells [Wautier et al. 2001], thus suggesting that multiple sources of oxidative stress may contribute to NF- κ B activation in diabetes.

That NF- κ B activation can occur by a variety of signals, including oxidative stress, raises the possibility that the diverse risk factors associated with the initiation of atherosclerosis, may be linked by a final common pathway of induced endothelial gene expression. The agents associated with activated or dysfunctional endothelium may act through second messenger systems to activate specific members of the NF- κ B family. Transcriptional activation of specific sets of endothelial genes will result in the generation of numerous endothelial products, including adhesion proteins, cytokines, growth factors and chemokines contributing to endothelial dysfunction and activation. In diabetes, hyperglycemia, AGE, oxidative stress and oxLDL can lead to the activation of NF- κ B and products of the NF- κ B controlled target genes are believed to be directly involved in the pathogenesis of diabetic vasculopathies. Our study, which demonstrates that high glucose enhances LOX-1 expression through a NF- κ B dependent pathway, identifies a new NF- κ B target gene potentially involved in endothelial dysfunction in diabetes. Whether induction of endothelial LOX-1 by other metabolic or inflammatory factors is mediated by NF- κ B remains to be evaluated.

Besides metabolic factors, inflammatory mediators may contribute to endothelial dysfunction in diabetes. Indeed, diabetes constitutes a low grade inflammatory state and is associated with overproduction of various proinflammatory cytokines and AAs, including CRP, TNF α and IL-6 [Pradhan et al. 2001]. It has been shown that proinflammatory cytokines such as TNF α and IL-6 induce endothelial activation/dysfunction. For example, TNF α increases procoagulant activity of endothelial cells, enhances endothelial cell adhesion molecule expression and stimulates the release of MCP-1 and IL-6 by endothelial cells, suggesting that this cytokine may, alone or in concert with other cytokines, alter endothelial function. Despite these in vitro observations, it is not yet known to what extent chronic, moderate elevations of this cytokine levels induce endothelial dysfunction in diabetes [Bilsborough et al. 2002]. In a similar manner, elevated levels of IL-6 may also contribute to endothelial dysfunction in diabetes. Supporting this possibility, it has been recently shown that IL-6 causes endothelial barrier dysfunction via the PKC pathway [Desai et al. 2002] and induces endothelial dysfunction by increasing the expression of AT1 [Wassmann et al. 2004]. Alternatively, this cytokine may alter endothelial cell function through its stimulatory effect on CRP release from the liver.

CRP concentrations correlate with indirect markers of endothelial function and recent data suggest that this acute phase protein per se may affect endothelial function. Indeed, it has been shown that CRP exerts direct pro-inflammatory effects on human endothelial cells by increasing the expression of adhesion molecules and MCP-1 and decreasing the production of NO. Several

mechanisms have been proposed by which CRP may contribute to endothelial dysfunction, including increased expression of ET-1, NF- κ B activation, and decreased NO release [Verma et al. 2002, Szmitko et al 2003].

Our study which demonstrates that CRP enhances the expression of LOX-1 in endothelial cells supports a new mechanism by which CRP may promote endothelial dysfunction. As discussed before, LOX-1 is a key mediator of oxLDL-induced endothelial injury, activation and dysfunction [Chen et al. 2002, Mehta et al. 2002]. Since endothelial dysfunction is one of the earliest events in atherogenesis, induction of LOX-1 in response to CRP may play an essential role in the initiation of the atherosclerotic process. Further support for this hypothesis comes from our observation that CRP-induced LOX-1 expression is associated with increased monocyte adhesion to endothelium. LOX-1 has been recently identified as an adhesion molecule [Honjo et al. 2003] and blocking LOX-1 mRNA using an antisense has been shown to inhibit oxLDL-mediated monocyte adhesion to endothelial cells [Li et al 2000]. While previous studies have documented a stimulatory effect of CRP on monocyte-endothelium interaction through the induction of endothelial cell adhesion molecules, including VCAM-1, ICAM-1, and E-selectin [Pasceri et al. 2000], we found that CRP induces monocyte adhesion through LOX-1 and that this effect was independent of VCAM-1, ICAM-1, and E-selectin. Pasceri et al reported that CRP's effect on E-selectin was evident at 6 but not 24 hours, whereas induction of ICAM-1 and VCAM-1 was observed at 24 hours. Importantly, these investigators noted that CRP effect was dependent on presence of human serum, being achieved only in

the presence of 15% human serum. Under our experimental conditions, endothelial cells were exposed to CRP for 15 hours and incubation was performed in the presence of 2% FBS. Such differences in the experimental conditions used in the two studies are likely to provide a good explanation for these discrepancies.

Because endothelial LOX-1 is the major receptor for oxLDL and that infiltration of this lipoprotein in the intimal space is a key event in the pathogenesis of atherosclerosis, increased expression of LOX-1 in the endothelium may favor lipoprotein accumulation in the vessel wall. It is well known that LDL particles either pass between the endothelial cells or cross the endothelium in transcytotic vesicles [Vasile et al. 1983, Kao et al. 1994, Kao et al. 1995] and evidence exists that CRP binds to aggregated lipoproteins and favors macrophage-derived foam cell formation. Our finding that CRP enhances oxLDL uptake by HAECs through LOX-1 receptor supports a role for this protein in arterial lipid infiltration. These results and the recent observations that endothelial LOX-1 expression is increased in diabetic animals and that lipoprotein flux into the arterial wall is increased in patients with type 2 diabetes [Kornerup et al. 2002] further stress a role for LOX-1 in transvascular LDL transport in diabetes. Further in vivo studies using CRP and LOX-1 transgenic animals are required to address this important issue.

Among the proinflammatory cytokines that predict and are increased in type 2 diabetes, IL-6 is a central mediator of the acute-phase response and the primary determinant of CRP production [Heinrich et al. 1990]. IL-6 circulates in

plasma of diabetic subjects at high concentration and one third of circulating IL-6 concentrations seem to originate from the adipose tissue. Increased IL-6 levels have been shown to predict cardiovascular mortality and future myocardial infarction [Miyao et al. 1993, Libby et al. 1999, Ridker et al. 2000, Bennet et al. 2003]. IL-6 may promote coronary disease through a number of metabolic, endothelial and procoagulant mechanisms [Yudkin et al. 2000, Woods et al. 2000]. For example, high secretion rate of this cytokine is associated with decreased insulin release and action and in vitro studies demonstrate that IL-6 is correlated with resistance of insulin action of glucose uptake in adipose tissue [Bastard et al. 2002, Sopasakis et al. 2004]. Exposure of endothelial cells to IL-6 induces expression of adhesion molecules and enhances binding of lymphocytes [Watson et al. 1996]. In addition, binding of IL-6 to its receptor stimulates leukocyte recruitment and promotes endothelial cell inflammatory responses [Modur et al. 1997, Romano et al. 1997]. Our finding that IL-6 enhances endothelial LOX-1 expression further supports the notion that this cytokine may contribute to endothelial dysfunction associated with diabetes.

The mechanisms through which IL-6 upregulates endothelial LOX-1 are unknown. On the basis of our and previous data showing that PKC is involved in endothelial LOX-1 regulation by glucose and mediates IL-6-induced endothelial barrier dysfunction [Desai et al. 2002], one may postulate a role of PKC in IL-6-induced LOX-1 expression. Because activation of PKC in response to many stimuli enhances NF κ B activity [Ghosh et al. 1990, Li et al. 2000], a critical transcription factor in the regulation of endothelial LOX-1 gene expression [Li et

al. 2002], a role for this factor as downstream target of PKC may further be proposed. Interestingly, we found that IL-6 and CRP did not synergize for LOX-1 induction, suggesting that these factors may act through common signaling events to induce endothelial LOX-1 expression. Importantly, it has recently been documented that CRP stimulates the release of IL-6 by endothelial cells [Verma et al. 2002] and we found that inhibition of IL-6 reduces CRP-induced LOX-1 expression. Taken together, these results strongly support a role for IL-6 as mediator of CRP effect on LOX-1.

The monocytes/macrophages play a critical role in the initiation and progression of atherogenesis. The macrophage functions both as a scavenger and an immune mediator cell and as a source of chemotactic molecules, proinflammatory cytokines such as $\text{TNF}\alpha$, IL-6, and IL-1 [Linton et al. 2003], and APPs such as CRP [Yasojima et al. 2001]. Macrophages contribute to the local inflammatory responses through production of cytokines, free oxygen radicals, proteases, and complement factors. By serving as antigen-presenting cells, macrophages participate in the immune response by activating T-cells. The uptake of modified lipoproteins by macrophages leads to the accumulation of cholesterol esters and formation of macrophage-derived foam cells, the hallmark of the fatty streak in atherosclerosis. This process of lipoprotein internalization by macrophages involves scavenger receptors expressed on the surface of these cells. In contrast to endothelial cells which do not express or express in very small amounts the classic scavenger receptors [Bickel et al. 1992], macrophages do express multiple oxLDL receptors, including SR-A, SR-B1, CD36, CD68, and

LOX-1 [Yamada et al. 1998]. In these cells, over 50% of the uptake of oxLDL seems to occur via CD36, whereas SRA has to share the rest with the other scavenger receptors, including LOX-1. It has recently been proposed that accelerated foam cell formation may occur in the diabetic state [Dobrian et al. 2000]. Inflammatory and metabolic factors dysregulated in diabetes, including CRP, hyperglycemia and AGEs, may contribute to this alteration. Indeed, it has been shown that CRP is expressed on the surface of foam cells [Torzewski et al. 1998], and mediates LDL uptake by macrophages in vitro [Bharadwaj et al. 1999, Zwaka et al. 2001]. Furthermore, it has been documented that high glucose and AGEs upregulate in vitro macrophage scavenger receptors [Griffin et al. 2001, Iwashima et al. 2000] and that monocytes of patients with type 2 diabetes show increased CD36 expression. Increased monocyte CD36 expression after monocyte entry into the subendothelial space, or increased expression of CD36 in the earliest stages of macrophage transformation could be highly atherogenic.

To further substantiate the role of high glucose on macrophage scavenger receptors, we evaluated LOX-1 expression in macrophages cultured in a high glucose environment. We demonstrated that high glucose concentrations significantly increase macrophage LOX-1 expression both at the gene and protein levels. As observed in endothelial cells, induction of macrophage LOX-1 gene expression by glucose was totally inhibited by pre-incubating the cells with PKC and MAPK inhibitors and appeared to involve NF κ B activation. However, in contrast to our observations in endothelial cells, we found that glucose also

enhanced the binding of nuclear proteins binding to the AP-1 regulatory element present in the promoter of the LOX-1 gene, suggesting that regulation of LOX-1 gene expression in vascular cells may involve different transcription factors.

While many data have been generated showing that uptake of modified lipoproteins by macrophages is in large part mediated by CD36 and SR-A, no studies have examined the contribution of LOX-1 in foam cell formation. Because we assumed that the contribution of LOX-1 in this process may be weak, we assessed the relative role of LOX-1 vs other scavenger receptors in oxLDL-induced foam cell formation by preincubating the cells with anti-CD36, anti-SR-A and anti-LOX-1 antibodies. Using this approach we successfully demonstrated that glucose-induced LOX-1 expression was associated with foam cell formation. These results together with the observation that high glucose increases CD36 further stress the role for hyperglycemia in foam cell formation. Although the contribution of LOX-1 in lipoprotein uptake by macrophages may be weak, evidence that LOX-1 is highly expressed in macrophages present in human atherosclerotic lesions [Kataoka et al. 1999] supports a role for this receptor in macrophage foam cell formation in vivo. To address this possibility and evaluate the relevance of our work to the diabetic human setting, we measured the levels of LOX-1 expression in monocyte-derived macrophages isolated from control and type 2 diabetic patients. Our results showing increased expression of macrophage LOX-1 in human diabetes further stress the potential role of macrophage LOX-1 expression in atherogenesis.

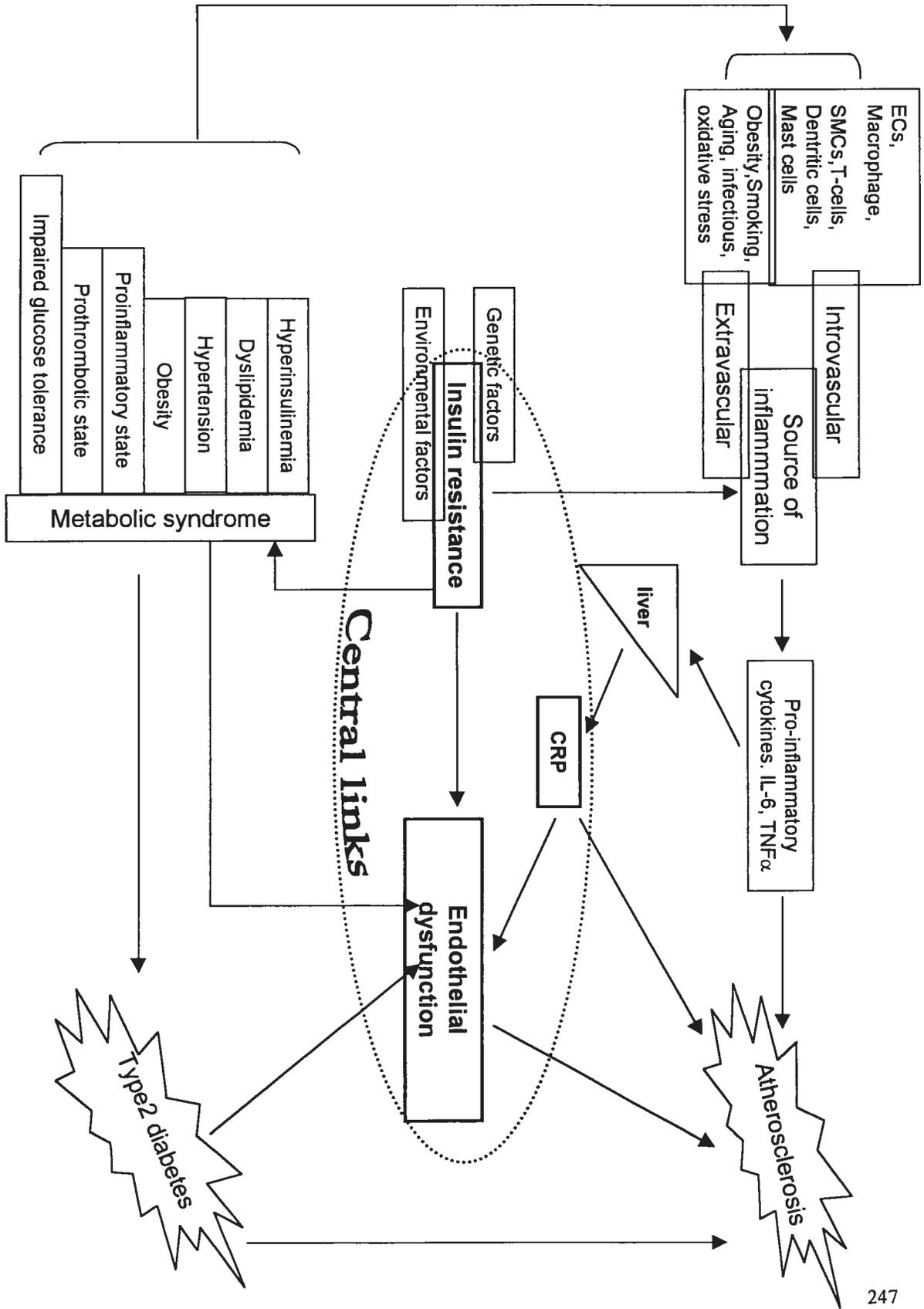


Figure 16. Summary of Discussion

IV. CONCLUSIONS AND PERSPECTIVES

IV.1. Conclusion

Despite advances in our understanding of the pathogenesis of atherosclerosis, the mechanisms underlying the increased risk of CVD in type 2 diabetes remain incompletely delineated. Endothelial dysfunction and foam cell formation are early and key potentially reversible events in atherogenesis and better understanding of the relationship between these features and risk factors in diabetes is required. Diabetes, like atherosclerosis is an inflammatory disease. Importantly, chronic subclinical inflammation precedes overt diabetes and may represent, in the prediabetic state, a triggering factor for endothelial dysfunction and atherosclerosis. Because endothelial dysfunction predicts CV events, precedes the detection of any distinct carbohydrate intolerance and links insulin resistance syndrome with atherosclerosis, determination of markers of endothelial dysfunction may be part of the strategies aimed at preventing the development of atherosclerosis associated with diabetes. Based on the stimulatory effect of CRP on endothelial LOX-1 and on the identification of soluble forms of LOX-1, determination of the plasma levels of this receptor in subjects at risk of developing diabetes may represent a novel diagnostic and therapeutic tool for vascular diseases associated with human diabetes.

In overt diabetes, three major mechanisms have emerged that encompass most of the diabetic vasculopathies. These mechanisms include oxidative stress, PKC activation, and AGE formation. Evidence has been provided that high glucose operates through these mechanisms to induce endothelial dysfunction. In accordance with this notion, we found that glucose-induced LOX-1 expression

involves oxidative stress and PKC activation. Although we were unable to document the formation of AGE in the cytosolic extracts of high glucose-treated human macrophages, a role for these factors in the induction of LOX-1 cannot be ruled out.

In conclusion, our results demonstrate that metabolic and inflammatory factors dysregulated in diabetes upregulate vascular LOX-1 expression. These data and our observation that macrophages of patients with type 2 diabetes overexpress LOX-1 support a contributive role for this receptor in the accelerated atherosclerosis associated with diabetes. Detailed understanding of the role of hyperglycemia and inflammation in the regulation of vascular LOX-1 expression could provide new strategies in the prevention and treatment of diabetic atherosclerosis.

IV.2. Perspectives

Although we have demonstrated that CRP and hyperglycemia upregulate vascular LOX-1 expression *in vitro*, the functional relevance of these observations in diabetic patients remains unclear. To address this question, we have generated some preliminary data showing that macrophages isolated from several patients with diabetes show increased LOX-1 gene expression. Future *ex vivo* studies are needed to a) confirm these data in a larger study population, b) determine macrophage LOX-1 protein levels in human diabetes and c) study the impact of LOX-1 inhibitory agents such as statins and PPAR gamma ligands on macrophage foam cell formation in human diabetes.

In a similar manner, future experiments will need to evaluate the functional relevance of the upregulation of endothelial LOX-1 by CRP. The question of whether the relationship between CRP and LOX-1 holds in vivo is of critical importance and will be examined by determining endothelial LOX-1 expression in CRP transgenic mice and by measuring in these animal models monocyte-endothelium interaction and several parameters of endothelial function.

Finally, experiments aimed at determining the molecular mechanisms involved in the induction of endothelial LOX-1 gene expression by CRP will also be conducted.

Chronic inflammation is a promising candidate mechanism underlying the common soil hypothesis linking type 2 diabetes and atherosclerotic CVD. While impressive evidence has accumulated over the past years that chronic subclinical inflammation is a risk factor for both diabetes and CVD, little is known about the potentially unique features of the inflammatory process associated with CVD in diabetes. Is the inflammatory process in diabetic patients different from those in nondiabetic individuals? The release of inflammatory markers may be greater in diabetes because some of the processes known to activate macrophages and therefore the release of cytokines, such as oxidation and glycoxydation of proteins and lipids and increased formation of immune complexes, are enhanced in diabetes. Further elucidation of whether there is an unique role for inflammation in CVD in diabetes and states of insulin resistance is required and if so the cellular and molecular mechanisms should be determined.

Also, not much is known about plaque differences in subjects with versus those without diabetes. Pathologic and angiographic studies have shown that diabetes favors diffuse and accelerated progression of atherosclerosis. Diabetic plaques are also commonly complicated and at greater risk of subsequent complications. Moreover diabetics who die suddenly show an increased number of fissured atherosclerotic plaques as compared with nondiabetics. In fact, diabetic patients with unstable angina have a higher incidence of plaque ulceration and intracoronary thrombus formation than nondiabetic patients and diabetic plaques usually have a greater lipid core burden, an increased content of macrophages and are more commonly complicated by overlying thrombosis. Furthermore, cell-rich and necrotic areas with large lipid core seem to be features of coronary atherosclerosis in persons with diabetes. The relationship between plaque content and risk factors in type 2 diabetes requires more insight.

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