Mechanisms for Oxidized or Glycated LDL-induced Oxidative Stress and Upregulation of Plasminogen Activator Inhibitor-1 in Vascular Cells

by

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Abstract

Atherosclerotic cardiovascular disease is the leading cause of death of adults in North America. Diabetes is a classical risk factor for atherosclerotic cardiovascular disease. Plasminogen activator inhibitor-1 (PAI-1), the major physiological inhibitor of fibrinolysis, has been implicated in thrombogenesis, atherogenesis, tissue remodeling and inflammation. Elevated levels of PAI-1, oxidized low-density lipoprotein (oxLDL) and glycated LDL (glyLDL) were detected in patients with diabetes. Increased oxidative stress is associated with diabetic cardiovascular complications. Previous studies in our laboratory demonstrated that oxLDL or glyLDL increased the production of PAI-1 and reactive oxygen species (ROS) in vascular endothelial cells (EC). This study was undertaken to investigate transmembrane signaling mechanisms involved in oxLDL or glyLDL-induced upregulation of PAI-1 in cultured vascular EC. Further, we examined the mechanism for oxLDL or glyLDL-induced oxidative stress in EC.

Lectin-like oxLDL receptor-1 (LOX-1) antibody, Farnesyltransferase inhibitor (FTI-277), and small interference RNA (siRNA) against H-Ras each significantly reduced oxLDL-induced expression of H-Ras and PAI-1 in EC. Raf-1 inhibitor blocked Raf-1 phosphorylation and the elevation of PAI-1 mRNA level in EC induced by oxLDL. Treatment with an ERK-1/2 inhibitor blocked oxLDL-induced ERK-1/2 phosphorylation and PAI-1 expression in EC. These results suggest that LOX-1, H-Ras, and Raf-1/ERK-1/2 are involved in PAI-1 expression induced by oxLDL in cultured EC.

GlyLDL may activate EC via a distinct transmembrane signaling pathway. Receptor for advanced glycation end products (RAGE) antibody prevented glyLDL-induced increase in the abundance of PAI-1 in EC. FTI-277 and siRNA against H-Ras

inhibited glyLDL-induced increase in heat shock factor-1 (HSF1) and PAI-1 in EC. NADPH oxidase (NOX) is a source of ROS in EC. Treatment with NOX inhibitor (Diphenyleneiodonium chloride) blocked glyLDL-induced H-Ras translocation, the release of H_2O_2, and increased abundance of HSF-1 and PAI-1 from EC respectively. SiRNA for $p22^{phox}$, an essential subunit of NOX complex, prevented glyLDL-induced expression of NOX2, HSF1 and PAI-1 in EC. Raf-1 inhibitor suppressed glyLDL-induced increase of PAI-1 mRNA in EC. The levels of RAGE, H-Ras, NOX4, HSF1 and PAI-1 were increased in hearts of streptozotocine (STZ)-induced diabetic mice and positively correlated with plasma glucose. The results indicate that RAGE, NOX and H-Ras/Raf-1 are implicated in the upregulation of HSF-1 and PAI-1 in vascular EC under diabetes-associated metabolic stress.

We also investigated additional mechanisms for oxLDL or glyLDL-induced ROS production in EC. Treatment with oxLDL or glyLDL significantly impaired oxygen consumption and the activities of electron transport chain (ETC) enzymes in mitochondria of EC. The abundance of mitochondria-associated ROS was significantly increased following oxLDL or glyLDL treatment. These findings suggest that oxLDL or glyLDL attenuated activity of ETC and increased ROS generation in EC, which potentially contributes to oxidative stress in vasculature.

In conclusion, diabetes-associated lipoproteins may upregulate stress response mediators and PAI-1 production via distinct transmembrane signaling pathways. OxLDL or glyLDL may increase ROS production via NOX activation and the impairment of mitochondrial ETC enzyme activity in EC. The understanding and identification of the

regulatory mechanisms involved in lipoprotein-induced signaling may help pharmacological design for the management of diabetic cardiovascular complications.

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I dedicate this work

To my parents

To my wife Swapnali

&

To my sweet daughter SEJAL

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AGE	advanced glycation end products
A-II	angionetsin II
ANOVA	one-way analysis of variance
ANT	adenine nucleotide translocase
apoB	apolipoprotein B-100
ATP	adenosine triphosphate
BHT	butylated hydroxytoluene
CAD	coronary artery disease
cAMP	cyclic adenosine monophosphate
CS	citrate synthase
$CuSO_4$	copper sulphate
cyt *c*	cytochrome *c*
DM	diabetes mellitus
DMEM	dulbecco's modified eagle medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DPI	diphenyleneiodonium
e	electron
EC	endothelial cells
ECL	enhanced chemical luminescence

EDTA	ethylenediaminotetraacetic acid
ELISA	enzyme-linked immunosorbent assay
eNOS	endothelial NO synthase
ET-1	endothelin-1
ERK-1/2	extracellular signal-regulated kinase-1/2
ETC	electron transport chain
FAD	flavoprotein or flavin adenine dinucleotide
$FADH_2$	reduced FAD
FBS	fetal bovine serum
FCCP	carbonylcyanide p-trifluoromethoxyphenylhydrazone
F-oLDL	$FeSO_4$-modified LDL
FTI-277	Farnesyltransferase inhibitor
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GDP	guanine dinucleodite phosphate
GEF's	guanidine nucleotide exchange factors
glyLDL	glycated LDL
GPx	glutathione peroxidase
GTP	guanine trinucleotide phosphate
H^+	proton
H_2O_2	hydrogen peroxide
HCAEC	human coronary artery EC
HDL	high-density lipoprotein
HPLC	high performance liquid chromatography

HRP	horseradish peroxidase
HSEs	heat shock elements
HSF1	heat shock factor-1
HSF2	heat shock factor-2
HSF4	heat shock factor-4
Hsp	heat shock proteins
HSP27	heat shock proteins-27
HSP60	heat shock proteins-60
HSP70	heat shock proteins-70
HSP90	heat shock proteins-90
HUVEC	human umbilical vein EC
i.p.	intra peritoneal
ICAM-1	intercellular adhesion molecule-1
IgG	immunoglobin G
IL-1	interleukin-1
IL-6	interleukin-6
IM	inner membrane
JNK	c-Jun-N-terminal kinase
KCl	potassium chloride
KCN	potassium cyanide
kDa	kilo-dalton
KH_2PO_4	potassium dihydrogen phosphate
LDL	low-density lipoprotein

LDLR	LDL receptor
LOX-1	lectin-like oxLDL receptor-1
LP(a)	lipoprotein (a)
LPS	lipopolysaccharide
MAPK	mitogen activated protein kinase
MEK	MAP kinase kinases ½
mg/kg	milligram per kilogram
mg/ml	miligram per mililitre
$MgCl_2$	magnesium chloride
mM	milimolar
mmol/L	milimolar per litre
moLDL	mildly oxidized LDL
mtDNA	mitochondrial DNA
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAD^+	nicotinamide adenine dinucleotide
ND	NADH-ubiquinone dehydrogenase
NF-κB	nuclear factor κB
ng/ml	nano gram per mililitre
nM	nano-molar
NO	nitric oxide
NOS	NO synthase
NOX	nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) oxidase
NOXA1	NOX activator 1

NOXO1	NOX organizer 1
OM	outer membrane
oxLDL	oxidized LDL
OXPHOS	oxidative phosphorylation
PAEC	porcine aortic EC
PAI-1	plasminogen activator inhibitor-1
pERK-1/2	phosphorylated extracellular-signal regulated kinase-1/2
PI3K	phosphatidylinositol 3-kinase
PKC	protein kinase c
PKC-β	protein kinase c-beta
PPAR-γ	peroxisome proliferator-activated receptor gamma
pRaf-1	phosphorylated Raf-1
RAGE	receptor for advanced glycation end products
RCI	respiratory control index
RNA	ribonucleic acid
RNS	reactive nitrogen species
ROS	reactive oxygen species
RT-PCR	reverse transcription-polymerase chain reaction
SAPK	stress-activated protein kinase
SCCR	succinate cytochrome *c* reductase
SDS	sodium dodecyl sulfate
SEM	standard error mean
siRNA	small interference RNA

SMC	smooth muscle cells
SOD	superoxide dismutase
SR	scavenger receptors
STZ	streptozotocine
T2DM	Type 2 diabetes mellitus
TBARS	thiobarbituric acid-reactive substance
TGF-β	transforming growth factor-beta
TMPD	N,N,N',N'-tetramethyl-p-phenylendiamine dihydrochloride
TMRM	tetramethylrhodamine methyl ester
TNF-α	tumor necrosis factor α
tPA	tissue-plasminogen activator
Ubi	ubiquinone
UCCR	ubiquinone cytochrome *c* reductase
UCPs	uncoupling proteins
uPA	urokinase-plasminogen activator
µg/ml	microgram per mililitre
µl	micro-litre
µM	micro-molar
µmol/l	micro molar per litre
VCAM-1	vascular cell adhesion molecule-1
VLDL	very low-density lipoprotein
VSMC	vascular SMC
vWF	von Willebrand factor

WHO	world health organization
$\Delta\Psi_m$	mitochondrial membrane potential
°C	degree celcius

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1. Literature Review

1.1 Introduction: Coronary artery disease

Coronary artery disease (CAD) is the leading cause of mortality worldwide, with approximately 4.5 million deaths occurring in the developing world. It has been predicted that CAD will be the major cause of death in developed countries by the year 2020 (Okrainec et al., 2004). CAD is caused by atherosclerosis, which is characterized by the accumulation of cholesterol deposits (plaques) in macrophages on the inner lining of coronary arteries. Plaques usually do not completely obstruct arterial lumen. Rupture of plaques and thrombosis at the lesion may result in blockage of arterial blood flow. This process compromises the supply of oxygen and nutrients to target organs such as heart. Severe ischemia in heart leads to heart attack. Classical risk factors of CAD include smoking, hypertension, dyslipidemia, diabetes, obesity, physical inactivity and aging. The deposition of low-density lipoprotein (LDL) cholesterol in the arterial wall is one of the major sources of mortality in atherosclerosis and CAD patients (Stocker and Keaney, 2004). Oxidation or glycation may increase atherogenecity of LDL. Diabetes is associated with an increased incidence of macrovascular complications including CAD. The risk of CAD is three- to fivefold greater in patients with diabetes compared to non-diabetics (Bierman, 1992). Elevated levels of modified lipoproteins such as oxidized LDL (oxLDL) or glycated LDL (glyLDL) have been implicated in the pathogenesis of atherosclerosis and thrombosis (Holvoet et al., 2001; Lyons et al., 1986). Mechanism involved in atherogenic lipoproteins-induced atherothrombosis remains unclear.

1.1.1 Atherosclerosis

Atherosclerosis is an inflammatory disease and considered as a common cause of mortality around the world. Due to global economic prosperity, atherosclerosis may lead to an epidemic, and claim more lives than all types of cancer combined (Stocker and Keaney, 2004). Endothelial dysfunction and vascular inflammation are crucial for the initiation of atherosclerosis. High plasma concentrations of LDL cholesterol constitute one of the principal risk factors for atherosclerosis (Devaraj and Jialal, 1996; Ross, 1999). Oxidation may increase thrombogenicity as well as atherogenicity of LDL. However, native LDL fails to convert macrophages into foam cells. OxLDL may be incorporated into vascular wall through scavenger receptors (SR). The endothelium locates between vascular wall and circulating blood. Endothelial dysfunction is a key early feature in atherogenesis. OxLDL may trigger inflammation at EC surface. Inflammation increases the release of mitogens and vasoactive factors (cytokines and growth factors), which in turn leads to migration of monocytes/macrophages, neutrophils and T lymphocytes into vascular wall. Modified LDL may be incorporated to macrophages and leads to formation of fatty streak, the initial characteristic lesion of atherosclerosis. Due to vascular injury and lipid accumulation, the surface of atherosclerotic lesion in vascular lumen is more thrombogenic to the intact endothelium. The activated platelets, monocytes, endothelial cells (EC), macrophages, and various growth factors lead to the proliferation of smooth muscle cells (SMC) and fibroblasts. This culminates into the formation of fibrous plaque, an advanced lesion of atherosclerosis. The accumulation of oxLDL in the subendothelial space of the arterial wall is a key step in atherogenesis because it contributes to foam cell

generation, endothelial dysfunction and inflammation (Ross, 1999; Ross and Glomset, 1973; Schachter, 1997) (Fig. 1).

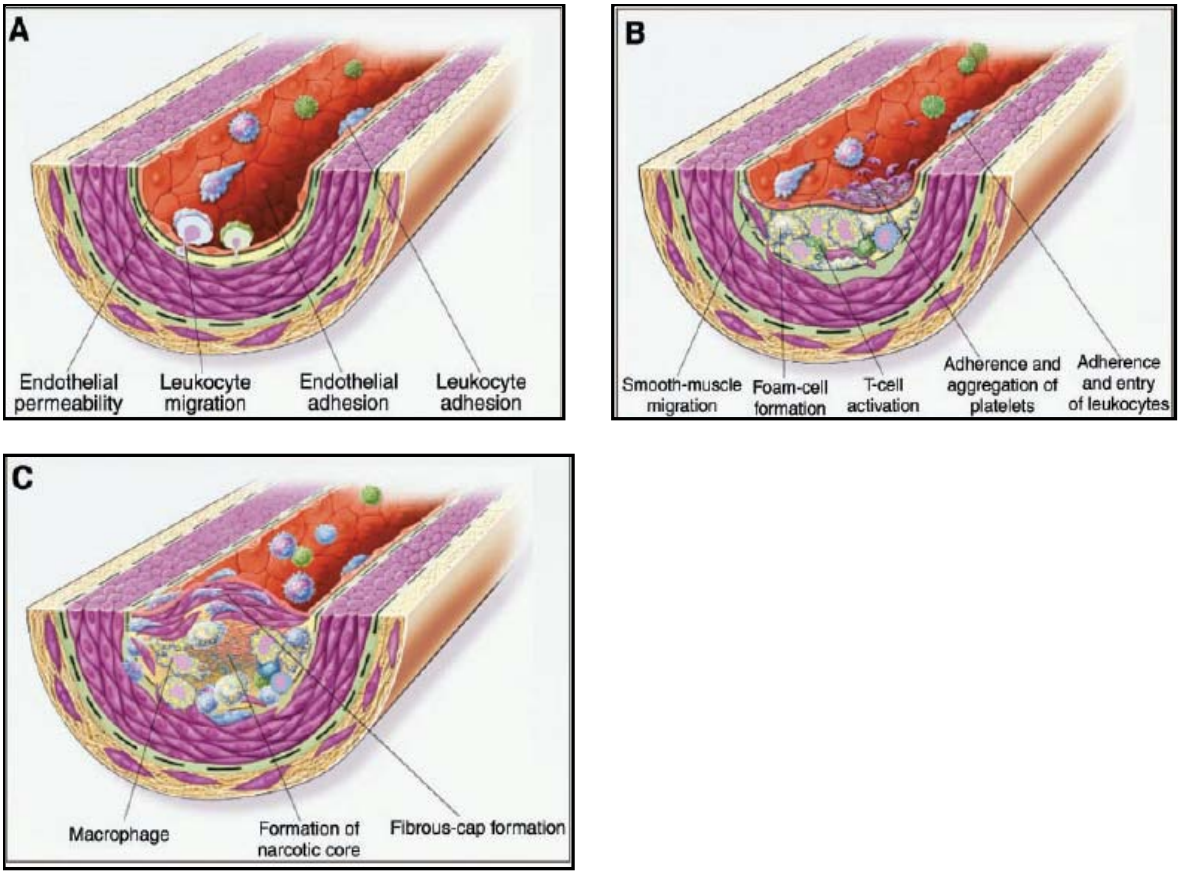


Figure 1. Response-to-injury hypothesis of atherosclerosis. **Panel A**: Explains the process of endothelial dysfunction in atherosclerosis. At this stage, deposition of LDL in the subendothelial space is a critical factor. **Panel B**: Atherosclerosis is characterized by formation of fatty streaks or foam cells. This is an inflammatory stage, where T-cell activation, the adherence and aggregation of platelets, leukocytes and migration of smooth muscle cells into the intima contributes to foam cell formation. **Panel C**: Advanced atherosclerosis is characterized by fibrous cap formation, and necrosis in the core of the lesion. *© 1999 Ross. Mechanisms of disease: Atherosclerosis-an inflammatory disease. Image used with permission from Massachusetts Medical Society on May 5, 2010* (Ross, 1999).

Oxidative modification hypothesis of atherosclerosis is proposed by Steinberg et al which explains a critical role of LDL in atherogenesis (Steinberg et al., 1989). Oxidative stress has been implicated in the oxidation of LDL, vascular SMC (VSMC) proliferation, EC apoptosis and upregulation of matrix metalloproteinases. Increased oxidative stress may act as a pathogenic link between atherosclerosis and endothelial dysfunction (Pennathur and Heinecke, 2007; Stocker and Keaney, 2004). Endothelium produces a number of components of extracellular matrix such as collagen and a variety of regulatory mediators, including nitric oxide (NO), plasminogen activator inhibitor-1 (PAI-1), von Willebrand factor (vWF), and adhesion molecules, and cytokines (Quyyumi, 1998). Reactive oxygen species (ROS) are considered as a critical factor for endothelial dysfunction. ROS is involved in several signaling pathways that regulate vascular inflammation in atherogenesis as well as the development of atherosclerotic lesions (Singh and Jialal, 2006). The sources for excess ROS production include nicotinamide adenine dinucleotide (phosphate) oxidase (NADPH oxidase), xanthine oxidase, lipoxygenase, mitochondria, or the uncoupling of NO synthase in vascular cells (Madamanchi et al., 2005). A reduced level of NO is one of the main alterations responsible for endothelial dysfunction through generation of peroxynitrite, a powerful oxidant in EC. The functions of EC include transport of nutrient substances, diverse biologically active molecules, solutes and blood cells. EC regulate coagulation, thrombosis and fibrinolysis, but high levels of plasma lipids such as LDL, very low-density lipoprotein (VLDL), oxLDL and glyLDL are among the pathophysiologic stimuli that induce endothelial dysfunction (Badimon et al., 2006; Ma et al., 2006). Increased ROS is implicated in the oxidation of LDL, inflammation and thrombogenic conditions

leading to the progression and complications of atherosclerosis. Accumulating lines of evidence suggest that ROS plays a key role in atherosclerosis (Diaz et al., 1997) (Fig. 2).

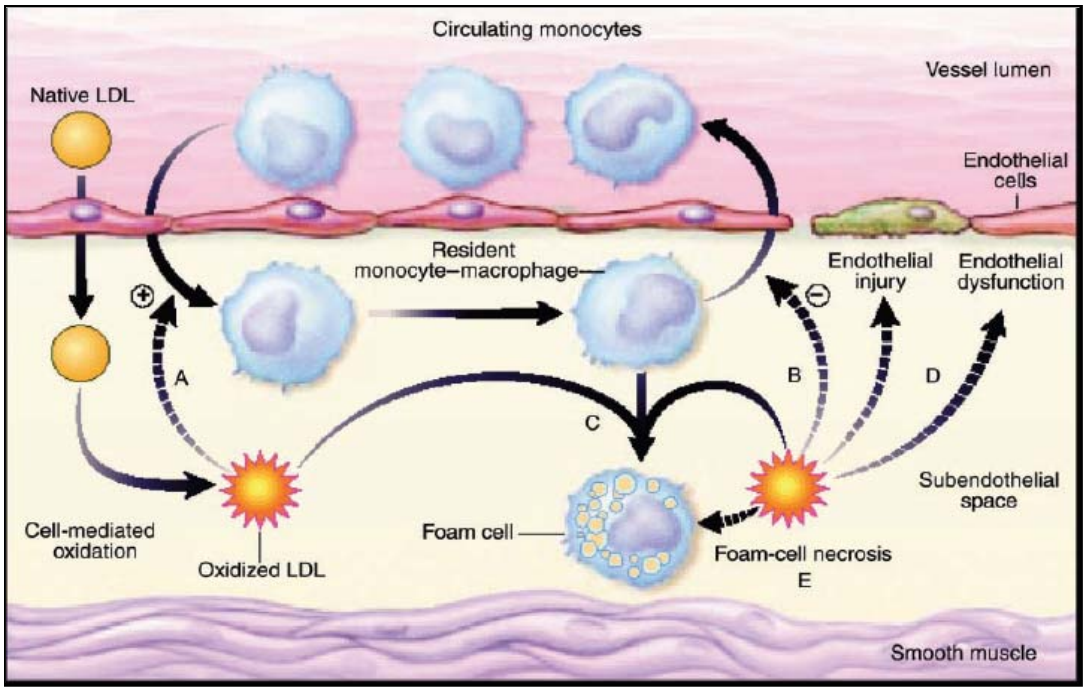


Figure 2. Oxidative modification and atherosclerosis (Steinberg et al., 1989; Stocker and Keaney, 2004). LDL becomes accumulated in the subendothelial space where it undergoes oxidative modification by resident vascular cells such as smooth muscle cells, endothelial cells, and macrophages. OxLDL stimulates monocyte chemotaxis (**A**), prevents monocyte egress (**B**), and supports foam cell formation (**C**). Once formed, oxLDL also results in endothelial dysfunction and injury (**D**), and foam cells become necrotic due to the accumulation of oxLDL (E). *© 1997 Diaz M, Frei B, Vita JA, and Keaney JF Jr. Antioxidants and atherosclerotic heart disease. N Engl J Med. Image used with permission from Massachusetts Medical Society on May 5, 2010.*

1.1.2 Diabetes

According to study by World Health Organization (WHO), the prevalence of diabetes mellitus (DM) in 2002 was about 150 million people worldwide, and the number is predicted to be doubled in the next 2 decades (Wild et al., 2004). Based on these estimates, one can imagine global and societal implications due to the growing epidemic of DM. The increase in the incidence of diabetes is the direct result of altered genes, human behavior and lavish lifestyle over the last several decades (Zimmet et al., 2001). Diabetes is a syndrome of impaired metabolism and increased blood glucose levels (hyperglycemia). DM exists in type 1 DM (impaired insulin production), type 2 DM (T2DM, insulin resistance and β-cell dysfunction) and gestational diabetes. T2DM comprises about 90-95% of all cases of diabetes in North America. About 5-10% of the total health care budget has been spent on diagnosis and treatment of diabetes (Lin and Sun, 2010; Zimmet et al., 2001). The pathogenesis of T2DM is not yet clear. T2DM is a complex heterogeneous group of metabolic conditions characterized by one or more of following key defects: increased hepatic glucose production, reduced insulin secretion, and impaired insulin action or insulin resistance (DeFronzo et al., 1992). Large number of population affected with T2DM has evidence of insulin resistance. Insulin resistance is described by reduced sensitivity of insulin in tissues involved in glucose metabolism, such as the liver, skeletal muscle and the adipose (Reaven et al., 1988). With an alarming increase in incidence, diabetes has already placed a huge financial burden on diabetic population. Early detection, treatment and prevention of DM will be few challenges for scientific community in this century.

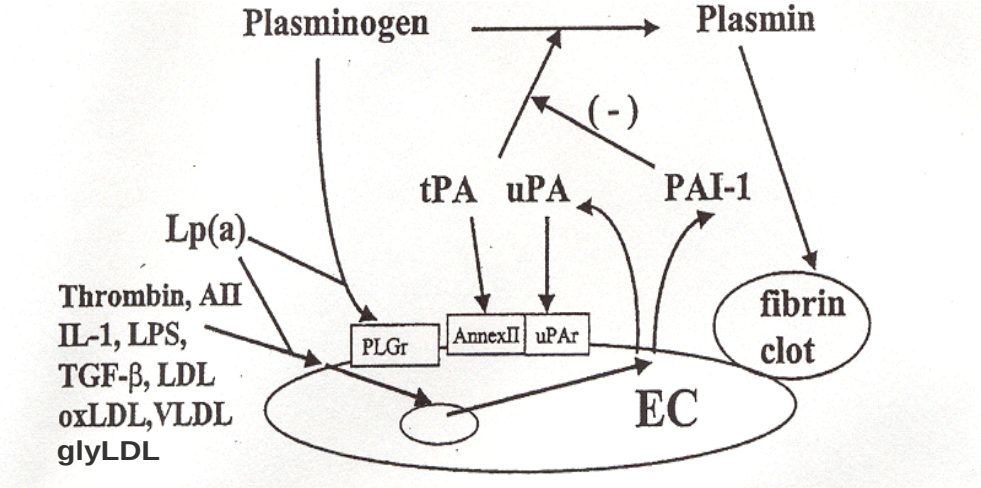
T2DM is an important risk factor for cardiovascular complications. CAD is the most common cause of deaths in diabetic patients (Fox et al., 2004). Diabetes is associated with an increased macrovascular complications such as CAD, cerebrovascular and peripheral vascular disease, and also growing well-recognized microvascular complications including nephropathy, retinopathy and neuropathy. Complications of atherosclerosis contribute to the majority of the deaths in diabetic patients (Beckman et al., 2002). DM in all forms is biochemically characterized by hyperglycemia and dyslipidemia. These abnormalities affect arteries and contribute to atherosclerosis. Diabetes impairs function of several cell types, such as EC, SMC and platelets. Impaired endothelial function has been detected in diabetes, including reduced NO-mediated vasodilation. Hyperglycemia increases oxidative stress, advanced glycation end products (AGE), and further contributes to the activation of nuclear factor κB (NF-κB) transcription factor (Wautier et al., 2001). These factors promote the releases of a number of mediators involved in atherogenesis (Beckman et al., 2002; Schmidt and Stern, 2000). Hyperglycemia-induced oxidative stress is a potential risk factor for accelerated atherosclerosis. Elevated levels of ROS were detected in the circulation of diabetic patients (Baynes, 1991; Giugliano et al., 1996). Evidence shows that modification of LDL under hyperglycemic condition contributes to atherosclerotic lesions via increased migration and apoptosis of VSMC (Taguchi et al., 2000). Pro-inflammatory cytokines, free fatty acids, modified lipoproteins (oxLDL or glyLDL), AGE, adhesion molecules and mitogenic factors seems to play important roles in the pathophysiology of atherosclerosis and diabetic vascular complications (Natarajan and Nadler, 2004). Impaired fibrinolytic activity is detected in diabetic patients due to increased levels of

PAI-1 in atherosclerotic lesions (Carr, 2001). Thus, diabetes increases tendency toward coagulation, impaired fibrinolysis and thrombus formation. These mechanisms explain the acceleration of the development of cardiovascular diseases in diabetic patients.

1.1.3 Fibrinolytic system

Fibrinolysis is important for maintaining blood fluency. Plasmin is the biologically active product of the fibrinolytic system. Plasmin catalyzes fibrin clot degradation and maintains efficient flow of blood through vasculature. The precursor of plasmin, plasminogen, is abundant in most body fluids and generally serves as a limitless supply. The generation of plasmin is regulated by tissue and urokinase plasminogen activators (tPA and uPA). PAI-1, a single chain glycoprotein serine protease inhibitor (serpin), is the major physiological inhibitor of tPA and uPA (Aso, 2007) (Fig. 3). Imbalance between coagulation and fibrinolysis may lead to thrombosis (Libby and Theroux, 2005).

The levels of tPA are higher in circulation while that of uPA is abundant in tissue. The activities of tPA and uPA are primarily regulated by PAI-1, a major physiological inhibitor of fibrinolysis (Shen, 1998). PAI-1 has been synthesized by hepatocytes, fibroblasts, adipocytes, endothelial and mononuclear cells (Kruithof, 1988). Vascular EC and SMC synthesize tPA, uPA and PAI-1 (Shen, 1998). PAI-1 usually forms an active complex with the glycoprotein vitronectin in the circulation. This complex increases PAI-1 biological half-life in the circulation (Aso, 2007). Increased plasma levels of PAI-1 have been considered as a non-traditional risk factor for CAD (Thogersen et al., 1998).



Adapted from Shen GX, Atherosclerosis, Hypertension and Diabetes, 2003

Figure 3. **Scheme for fibrinolytic system.** PAI-1: plasminogen activator inhibitor-1, tPA: tissue plasminogen activator, uPA: urokinase plasminogen activator, EC: endothelial cells, Lp(a): lipoprotein (a) AII: angionetsin II, IL-1: interleukin-1, LPS: lipopolysaccharide, TGF-β: transforming growth factor-beta, LDL: low density lipoprotein, oxLDL: oxidized LDL, VLDL: very LDL, glyLDL: glycated LDL, PLGr: plasminogen receptor, uPAr: urokinase receptor, AnnexII: receptor for tPA.

Increased levels of PAI-1 protein and mRNA were detected in atherosclerotic or thrombotic lesions in vasculature (Arnman et al., 1994; Yorimitsu et al., 1993). PAI-1 levels are elevated in plasma of patients with CAD (Paramo et al., 1985), and are a key risk factor for coronary events in patients with angina pectoris (Juhan-Vague et al., 1996). PAI-1 levels are strongly correlated with circulating triglycerides, apolipoprotein B-100 (apoB) and LDL in CAD or diabetes patients (Juhan-Vague and Vague, 1990; Juhan-Vague et al., 1987). Elevated levels of small dense LDL were positively correlated with the levels of PAI-1 in plasma of general population (Festa et al., 1999). T2DM is usually associated with attenuated fibrinolysis, due to elevated levels of PAI-1. It has been demonstrated that insulin-resistant adipocytes release PAI-1, which confirms the link between diabetes and fibrinolysis (Juhan-Vague et al., 1989). Increased levels of PAI-1 were detected in obesity or diabetes, two classical risk factors for CAD (Carmassi et al., 1992). These findings suggest that dyslipoproteinemia and hyperglycemia may play important roles in the upregulation of PAI-1, which subsequently contributes to atherothrombogenesis.

1.1.4 LDL and its modified forms

Plasma levels of LDL-cholesterol positively correlate with the incidence of CAD. LDL is the major transporter for cholesterol in the blood. The core of the LDL contains cholesterol, triglyceride, phospholipids and apoB. LDL may be divided into subclasses based on their particle sizes, density and lipid composition. Small dense LDL is susceptible to oxidation, and is associated with CAD and insulin resistance (Festa et al., 1999; Stocker and Keaney, 2004). The presence of apoB on the surface of LDL

determines their interactions with cell-membrane receptors. ApoB100 and lipids in LDL particle may be oxidized, glycated and glyco-oxidized (Lyons, 1993). The retention of lipoproteins in plasma is an important etiological factor for the development of atherosclerosis. Modification reduces the uptake of LDL by the LDL receptor (LDLR). Increased LDL residential time in plasma contributes to its modification and lipid deposition in the arterial wall and atherosclerotic lesions (Tozer and Carew, 1997). This modification of LDL by ROS, glucose or EC exposure contributes to the synthesis of modified lipoproteins, including oxLDL or glyLDL (Shen, 2003; Tabas, 1999). Glycation enhances the susceptibility of LDL to oxidation and promotes the formation of glyco-oxidized LDL. Elevated levels of oxLDL or glyLDL have been considered as a key risk factor for cardiovascular complications (Johnston et al., 2006; Shen, 2003; Steinberg et al., 1989). OxLDL and glyLDL-induced oxidative stress may regulate the synthesis of various activators promoting atherosclerosis and/or thrombosis.

Oxidized LDL: It has been very well established that small dense LDL particles represents increased cardiovascular risk. Furthermore, LDL particles have atherogenic properties and are more susceptible to oxidation. LDL particles undergo oxidation to more actively atherogenic forms in the vessel wall, and the macrophage is a key element in this process. Generally, small dense LDL particles show lesser affinity for the LDLR and are preferentially taken up by macrophage SR, leading to foam cell formation (Chapman et al., 1998). OxLDL has been considered as a key risk factor in foam cell formation, endothelial dysfunction and inflammation. Oxidation of LDL can be achieved using metal ions such as Cu^{2+} (extensively oxLDL), Fe^{2+} (one type of minimally oxLDL),

biological products (e.x. H_2O_2), peroxynitrite, 15-lipoxygenase, ultraviolet, radiation or prolonged incubation with cells (EC, SMC and monocyte-macrophages) (Morel et al., 1983; Shen, 2003; Steinbrecher et al., 1984; Watson et al., 1995). Minimally oxLDL and extensively oxidized LDL both are implicated in CAD, and detected in atherosclerotic plaques (Steinberg, 1997). EC-mediated oxidation may be an important source of oxLDL in blood circulation. OxLDL is implicated in atherosclerosis and is cytotoxic to a variety of vascular cells including EC, SMC and fibroblasts (Morel et al., 1983). OxLDL constitutes various lipid peroxidation products, including thiobarbituric reactive substances, conjugated dienes, lipid hydroperoxides, and aldehydes (Steinberg, 1997). Initially macrophages oxidize minimally oxLDL. This form of oxLDL is no longer recognized by the LDLR and taken up by SR on the surface of macrophages. Previous studies demonstrated that exposure to oxLDL for a prolonged period may cause cytotoxic effects in vascular cells (Cathcart et al., 1991; Steinberg, 1997).

OxLDL induces a variety of cellular activities in EC, including the upregulation of monocyte chemoattractant protein-1, tissue factor, PAI-1, pro-inflammatory cytokines, apoptosis, the downregulation of NO generation and increased expression of vascular cell adhesion molecule-1 (VCAM-1) (Steinberg, 2009). Multiple lines of *in-vivo* evidence confirm a strong association between oxLDL and CAD. Increased levels of oxLDL have been detected in the plasma of CAD patients (Holvoet et al., 2001). OxLDL has also been found in atherosclerotic lesions of experimental animals and humans (Yla-Herttuala et al., 1989). Multiple clinical studies have been done for the measurement of oxLDL in the blood. According to Holvoet et al (2001), the typical blood levels of oxLDL in control

subject were 1.30±0.88 mg/dl compared to that of CAD patients: 3.11±1.19 mg/dl. Small dense LDL contains less antioxidant, and is more prone to oxidation (Holvoet et al., 2008). Autoantibody against oxLDL has been detected in human plasma and atherosclerotic lesions (Erkkila et al., 2000). Previous studies suggest that oxLDL may enhance thrombogenecity through increased expression of PAI-1 in cultured EC (Drake et al., 1991). OxLDL contributes to endothelial dysfunction and inflammation in vasculature by upregulating the expression of inflammatory mediators (Maziere and Maziere, 2009). An elevated level of circulating oxLDL is associated with metabolic syndrome and may contribute to atherogenesis (Holvoet et al., 2008).

Glycated LDL: Hyperglycemia promotes nonenzymatic glycation of proteins and lipids, further leading to the formation of AGE. AGE result from a chain of chemical reactions after prolonged glycation (Ramasamy et al., 2005). High levels of glucose increases glycation of the apoB or phospholipids in LDL. Chronic hyperglycemia enhances LDL oxidation and/or glycation, which is considered as pro-atherogenic. Small dense LDL is one type of LDL particles favors atherogenesis, which is considered as more susceptible to glycation than more buoyant LDL (Younis et al., 2008). Glycation is the nonenzymatic reaction between glucose and lysine residues of proteins leading to the formation of stable products. These irreversible reactions are also called as "Browning" or the "Maillard" reaction. Increased *in-vivo* glycation of apoB moiety in LDL contribute to the accelerated atherosclerosis. Combined glycation and oxidation of proteins, termed as "glycoxidation", promotes the generation of atherogenic products (Baynes, 1991; Lyons et al., 1986; Zhao and Shen, 2007). Elevated levels of glyLDL were detected in diabetic

patients (Lyons, 1993; Tames et al., 1992). Glycation increases the susceptibility of LDL to oxidation. This prevents the uptake of glyLDL via LDLR and promotes the uptake through macrophage SR (Kobayashi et al., 1995; Younis et al., 2008). Previous reports demonstrated impaired catabolism of LDL in T2DM due to reduced number of LDLR on cell surface (Duvillard et al., 2003). The modification of lipoproteins induced by AGE is implicated in dyslipidemia. Previous studies demonstrated that LDLR and plasma lipoproteins undergo glycation, which further affects cholesterol metabolism (Morigi et al., 1998). Several studies found the implication of glycation of LDL and collagen in the pathogenesis of atherosclerosis (Berliner et al., 1992; Lyons, 1992). Recent *in-vivo* studies demonstrated that LDL apoB from diabetic patients contains a higher proportion of lysine-bound glucose compared with LDL isolated from nondiabetic individuals (1.9 versus 0.5 mol glucose/mol protein). The levels of serum glycated apoB were significantly higher in patients with diabetes than in controls without diabetes (Schleicher et al., 1981; Tames et al., 1992). Elevated levels of glyLDL were not only detected in diabetes, but also under hypercholesterolemia, due to more LDL available for glycation (Tames et al., 1992).

Several factors are involved in the formation of AGE, such as oxidation of proteins and lipids, hyperglycemia, oxidant stress in the environment and cross linking reactions (Brownlee, 1995; Schmidt et al., 1999). GlyLDL-induced oxidative stress is a key factor in the synthesis of several cardiovascular markers involved in the pathogenesis of diabetic complications. Our previous studies demonstrated that glyLDL increased the levels of mRNA and the release of PAI-1 from EC (Ma et al., 2006; Zhang et al., 1998;

Zhao and Shen, 2007). GlyLDL attenuates NO production and impairs LDL clearance through its receptor on EC (Posch et al., 1999). Previous studies including ours demonstrated increased levels of superoxide in EC induced by glyLDL (Posch et al., 1999; Zhao and Shen, 2005). Previous reports demonstrated multiple deleterious effects of glyLDL on endothelial function (Lyons et al., 1994), platelet (Watanabe et al., 1988) and macrophages (Lopes-Virella et al., 1988). Moreover, AGE-LDL increased the production of proinflammatory cytokines in EC and macrophages via toll-like 4 receptor pathway (Hodgkinson et al., 2008). After glycation of LDL, it is poorly recognized by lipoprotein receptors such as the LDLR and SR. Therefore, glyLDL is a target of oxidative stress (Zimmermann et al., 2001). These studies suggest that the glycation of lipoproteins may play a significant role in the development of diabetic cardiovascular complications.

1.1.5 Regulation of PAI-1 production in vascular EC

The endothelium is a single layer of cells that covers entire vasculature and separates blood components from other tissues in the body. EC dysfunction is the central feature of atherosclerosis. The first report on the effect of lipoproteins on the production of PAI-1 in EC came in 1990. VLDL isolated from hypertriglyceridemic patients increases PAI-1 release from EC (Stiko-Rahm et al., 1990). The generation of fibrinolytic regulators in EC is regulated by a variety of biological activators, including thrombin, insulin, fatty acids, glucose and lipoproteins (Plow et al., 1995). The release of PAI-1 in plasma is mediated by multiple sources, including the vascular EC, adipose tissue, and liver (Vaughan, 2005). The binding of PAI-1 to vitronectin gives stability to stay in the active

conformation (Declerck et al., 1988). LDL and its structural homologue, lipoprotein(a) [Lp(a)], increased the transcription and secretion of PAI-1 in EC (Ren et al., 1997; Tremoli et al., 1993). OxLDL modified by ultraviolet or copper significantly increased the antigen and activity of PAI-1 in cultured vascular EC. The effects of oxidatively modified LDLs on PAI-1 production were significantly greater compared to LDL without modification (Latron et al., 1991; Ren et al., 1997; 2000). Several inflammatory cytokines, including interleukin-1 (IL-1) and tumor necrosis factor α (TNF-α), stimulated the production of PAI-1 in EC (Emeis and Kooistra, 1986; Sawdey et al., 1989). Angiotensin II (Ang-II) induced dose- and time-dependent increase in PAI-1 expression in vascular EC (Feener et al., 1995). Previous studies demonstrated that prolonged incubation of LDL with EC, SMC or monocytes undergoes modification. Increased formation of lipid peroxidation products was detected in LDL incubated with EC, and antioxidants inhibited LDL-induced PAI-1 generation or lipid peroxidation. LDL-induced PAI-1 expression in EC may result from EC-modified oxidation of LDL. High density lipoprotein (HDL) did not affect the generation of PAI-1 from EC. HDL protects LDL from oxidative modification and LDL-induced PAI-1 production from EC, which suggests antioxidant properties of HDL (Ren and Shen, 2000).

Impaired fibrinolytic activity in diabetic subjects is associated with cardiovascular complications. Elevated PAI-1 production could be a link between insulin resistance/metabolic syndrome and atherothrombosis (Dandona et al., 2005). Glycation increases the oxidative stress of lipoproteins. Previous studies from our lab first time demonstrated that glycation enhanced LDL-induced overproduction of PAI-1 in vascular

EC. GlyLDL increased the levels of PAI-1 mRNA but not significantly alter tPA mRNA in EC (Zhang et al., 1998). Moreover, glycation enhanced the effect of Lp(a) on the generation of PAI-1 from EC (Zhang et al., 2000). Recent studies suggest that stimulation with high-glucose increased PAI-1 expression as well as gene transcription in aortic EC (Iwasaki et al., 2008; Rikitake and Liao, 2005). The production of fibrinolytic regulators in EC induced by glycated HDL was significantly less compared to glyLDL. Co-stimulation with glycated HDL reduced PAI-1 production and increased the generation of tPA induced by glyLDL (Ren and Shen, 2000). Our previous study demonstrated that LDL isolated from diabetic patients increased the generation of PAI-1 from EC compared to LDL isolated from healthy subjects (Ren et al., 2002). These studies suggests that oxidized and glycated forms of lipoproteins affect the generation of PAI-1 from vascular EC, which may contribute to the development of CAD in diabetic patients.

1.1.6 Stress response and atherothrombosis

Stress responses are considered as cellular self-defense reactions against severe environmental stresses. Cells respond to environmental stresses, such as heat, ROS, and shearing force, radiation and hypoxia via a variety of post-translational modifications of proteins. Stress responses in cells are mediated by heat shock proteins (Hsp). Increased expression of Hsp has been implicated in the pathogenesis of atherosclerosis (Metzler et al., 2003). Hsps are molecular chaperones which protect cellular proteins from stresses, and help proteins to repair if they become damaged. The transcription of Hsp genes is regulated by heat shock factor (HSF). HSF undergoes activation and translocation from the cytoplasm to the nucleus in cultured cells induced by stress stimuli. In the trimetric

state, HSF has a high affinity for cis-acting DNA sequence elements called heat shock elements (HSEs) in the promoter region of heat shock protein (Craig et al., 1994; Snoeckx et al., 2001). Interaction between HSF and HSE in promoters of targeted proteins triggers the transcription of Hsp genes (Pirkkala et al., 2001).

HSF family consists of four members. HSF1, HSF2 and HSF4 are expressed in humans. HSF2 and HSF4 are tissue-specific. HSF1 is the most widely distributed HSF in human tissues (Snoeckx et al., 2001). Increased expression of HSF1 and various subtypes of Hsp, such as Hsp70, Hsp60, Hsp90 and Hsp27, are detected in atherosclerotic lesions (Berberian et al., 1990; Chan et al., 2003; Roma and Catapano, 1996). Increased expression of HSF1 or Hsp70 was detected in spontaneous hypertensive rats compared to control animals (Chan et al., 2003). Increased expression of Hsp70 along with PAI-1 was detected in wounded renal cells (Pawar et al., 1995). Oxidative stress and redox system have profound effects on HSF activation and Hsp production. Recent studies in our laboratory demonstrated that glyLDL, oxLDL or oxidized VLDL enhanced the generation of ROS, and the expression of HSF1, prior to the increase of PAI-1 expression in EC or fibroblasts (Zhao et al., 2008; 2009; Zhao and Shen, 2007). These findings suggest that stress responses may play an important role in the upregulation of PAI-1 in vascular EC, which may contribute to atherothrombosis.

1.2 Signaling mechanisms for lipoprotein-induced oxidative stress or PAI-1 in EC

Signal transduction mechanisms involved in lipoprotein-induced PAI-1 production remains uncharacterized. Multiple biochemical mechanisms have been described for

lipoprotein-induced oxidative stress. Signaling activates various downstream pathways that affect fibrinolysis, leading to cardiovascular complications (Shen, 2003; Way et al., 2001). Small G-proteins act as a biological switch for various cellular processes. Several studies suggested the involvement of small G-proteins in cell proliferation and differentiation (Kerkhoff and Rapp, 1998; Takai et al., 2001). Small G proteins are involved in modulation of signaling between multiple membrane receptors and downstream signal transduction systems (Takai et al., 2001). The mitogen activated protein kinase (MAPK) family is comprised of key regulatory proteins that control cellular response to both proliferation and extracellular stimuli (Guyton et al., 1996). The activation of extracellular signal-regulated kinase-1/2 (ERK-1/2) is required for oxLDL-induced TGF-β/Smad3 activation and PAI-1 expression in mesangial cells (Hong et al., 2006). Results from our earlier studies suggest that the activation of protein kinase c-beta (PKC-β) is required for oxLDL-induced PAI-1 production in EC (Ren et al., 2000).

In 1997, Sawamura et al (1997) discovered LOX-1 in bovine aortic EC. Recently the importance of LOX-1 in the pathogenesis of cardiovascular disease, including atherosclerosis has been reviewed (Chen et al., 2007b; Mehta et al., 2006). LOX-1 mediates oxLDL-induced cellular events in EC (Cominacini et al., 2000; Li et al., 2003a; Li and Mehta, 2000). LOX-1 is also involved in oxLDL-induced endothelial dysfunction including NO regulation, ROS generation and inflammatory activation of EC (Mehta et al., 2007). LOX-1 mediates oxLDL-induced intracellular signaling pathways including p38 MAPK (Mehta et al., 2004), p44/42 MAPK (Li et al., 2003b), PKC (Li et al., 2003a), NOX (Zhao et al., 2009) and protein kinase B (Li et al., 2001). OxLDL is implicated in

the activation of various transcription factors, including HSF1 (Zhao et al., 2009), activator protein 1 (Maziere et al., 1997) and NF-κB (Cominacini et al., 2000). The activation of these transcription factors plays important roles in oxLDL-induced oxidative stress and inflammation (Maziere and Maziere, 2009).

A recent study demonstrated that high glucose-induced PAI-1 expression in EC is mediated via Rho/Rho-kinase and NF-κB pathway (Iwasaki et al., 2008). Small G-proteins are implicated in diabetes induced oxidative stress, ROS production and vascular disease (Kowluru et al., 2004; Vecchione et al., 2006). Increased expression of receptor for AGE (RAGE) has been associated with diabetic vascular complications (Yamamoto et al., 2000). Previous studies from our group demonstrated that glyLDL significantly increased ROS production in EC. Oxidative stress activates HSF-1. HSF1 regulates glyLDL-induced PAI-1 transcription in vascular EC (Zhao and Shen, 2005; 2007). Previous studies demonstrated that AGE/RAGE-induced oxidative stress activates H-Ras, and a cascade of MAP kinase, resulting in the activation of NF-κB in SMC (Lander et al., 1997). The engagement of RAGE by AGE triggers the production of ROS via the activation of NOX (Wautier et al., 2001). Increased AGE-LDL in diabetes induces oxidative stress and pro-inflammatory state in human EC. Increased formation of AGE-LDL may directly affect endothelial function by activating RAGE (Toma et al., 2009). Studies on signaling mechanisms for modified LDL-induced atherothrombosis related cellular effect will help us to understand the pathogenesis of diabetic vascular complications and identify new drug target for the management of this harmful disease.

1.2.1 LOX-1

LOX-1 is considered as a major receptor for oxLDL in EC. LOX-1 mediates the binding, internalization and degradation of oxLDL in EC (Sawamura et al., 1997). Based on sequence and structural analysis of LOX-1, this receptor is unique and has no resemblance to any known SR. However, LOX-1 shares significant similarity to natural killer (NK) cell receptors family (CD94 and NKR-P1), which are essential in target cell recognition and NK cell activation (Aoyama et al., 1999; Yamanaka et al., 1998). Human LOX-1 (OLR1; low density lipoprotein, oxidized, receptor 1; OMIM#602601) gene consists of more than 7000 base pairs (bp), and 6 exons interrupted by 5 introns. All the NK cell receptors are encoded in the NKC. In case of LOX-1, it is a type II membrane protein (~50 kDa). Structurally LOX-1 belongs to the C-type lectin family. These family proteins comprised of four domains: a short N-terminal cytoplasmic domain, a single transmembrane domain, a connecting neck domain and a lectin-like domain at the C-terminus, all binds carbohydrates in a Ca2+-dependent manner (Mehta et al., 2006). Human LOX-1 protein contains 273 amino acids, and has been shown to be 72% identical for its bovine counterpart (Sawamura et al., 1997). Previous studies demonstrated that the C-terminal lectin-like domain is essential for the binding of oxLDL (Xie et al., 2003).

Previous studies demonstrated that LDLR blocking antibody had no effect on LDL or oxLDL-induced PAI-1 release from EC (Tremoli et al., 1993). The expression of LOX-1 was also found in macrophages (Yoshida et al., 1998), platelets (Chen et al., 2001a), monocytes and SMC (Draude et al., 1999). Several other cell-surface receptors

(Dhaliwal and Steinbrecher, 1999; Nagase et al., 2000) may be involved in oxLDL uptake, including SR-AI/II, CD36, and SR-BI (Steinbrecher, 1999). However, Mehta et al (2004) indicated very low levels of other SR in EC. LOX-1 mediates oxLDL-induced secretion of matrix metalloproteases (Li et al., 2003a). LOX-1 expression usually elevated under pro-inflammatory states and also detected in human atherosclerotic lesions (Kataoka et al., 1999). LOX-1 levels are usually higher after oxLDL binding, which may further enhance vascular dysfunction. Activation of LOX-1 leads to increased expression of endothelin-1 (ET-1), angiotensin type 1 receptor, and several inflammatory mediators including E-selectin, P-selectin, VCAM-1 and intercellular adhesion molecule (ICAM)-1 in EC, which further contribute to endothelial dysfunction (Chen et al., 2007b; Mehta et al., 2006). These findings suggest that LOX-1 regulate an initiation and formation of atherosclerotic plaques induced by oxLDL (Chen et al., 2000). Previous study suggests that AGE or glucose also serve as a ligand for LOX-1. The expression of LOX-1 is increased in the vascular endothelium of diabetic rats, which may indirectly suggest the increase of oxLDL in diabetic condition (Chen et al., 2001b).

LOX-1 mediates oxLDL-induced increase of ROS in EC (Cominacini et al., 2000). Previous studies from our laboratory showed increased generations of ROS as well as functional activities of multiple antioxidant enzymes in EC induced by oxLDL or glyLDL (Zhao and Shen, 2005). Both, superoxide and H_2O_2 levels were increased after LOX-1 activation (Nagase et al., 2001). Pioglitazone, a PPAR-γ agonist inhibited oxLDL-induced oxidative stress and LOX-1 upregulation in coronary artery EC (Mehta et al., 2003). Our laboratory previously demonstrated that oxLDL is a potent agonist for

the production of PAI-1 from cultured EC (Ren et al., 1997; 2000). The expressions of both LOX-1 and PAI-1 are increased in atherosclerotic tissues (Arnman et al., 1994; Chen et al., 2000; Kataoka et al., 1999). LOX-1 antibody reduced arterial thrombus formation in rats (Kakatani et al., 2000). Cominacini et al (2000) reported that LOX-1 blocking antibody prevented ROS production induced by oxLDL. These studies prove that LOX-1 plays an important role in oxLDL-induced oxidative stress and thrombosis-related process. LOX-1 activation may be an initial event for many oxLDL-induced effects in EC, which mediates the activation of downstream signaling pathway. Membrane receptor for oxLDL-induced PAI-1 production in EC remains unclear.

1.2.2 RAGE

High levels of glucose increase nonenzymatic glycation at amino groups on proteins, lipids or nucleic acids. This process is known as the "Maillard reaction", and participates in the formation of AGE. Proteins with abundant lysine and arginine residues are most vulnerable to AGE modification. ApoB is rich in lysine residues (Rabbani et al., 2010; Ramasamy et al., 2005). Elevated levels of glyLDL, a type of AGE, were detected in diabetic patients (Lyons, 1993; Tames et al., 1992). Increased expression of RAGE was detected on surface of vascular cells.

RAGE is as multi-ligand receptor of the immunoglobulin superfamily of cell surface molecules that can act as a pattern recognition receptor. This receptor consists of one "V"-type and two "C"-type immunoglobulin domains in extracellular region, with AGE binding occurring with the V domain (Neeper et al., 1992). RAGE is a signal

transduction receptor, which regulates the generation of ROS, pro-inflammatory and pro-thrombotic molecules contributing to cellular damage (Ramasamy et al., 2005). Other than AGE, RAGE also has affinity for non-AGE ligands such as β-amyloid protein, S100/calgranulins, high-mobility group box-1, β-sheet fibrils, Mac-1 and a neuroregulatory protein, amphoterin (Hofmann et al., 1999; Ramasamy et al., 2009). Recent study demonstrated that RAGE cytoplasmic domain interacts with Diaphanous-1, which is required for activation of cell signaling via RAGE (Hudson et al., 2008).

The exact role of RAGE in normal physiology has not been fully understood. However, this receptor may be involved in excretion or catabolism of damaged or senescent tissue elements as well as in the regulation of signal transduction pathways on the binding of AGE. The upregulation of RAGE was detected in diabetic versus nondiabetic lesions. The increased expression of RAGE was associated with oxidative and inflammatory stress, such as elevated expression of matrix metalloproteinases (Cipollone et al., 2003). These findings suggest the involvement of AGE in inflammation and signal transduction. The activation of RAGE is associated with multiple cascades of intracellular signaling pathways. Further, RAGE is involved in modulation of cellular responses to various stress conditions. Multiple pro-inflammatory and pro-atherogenic mediators are implicated in RAGE-mediated signaling pathways, including NF-κB-dependent mediators, VCAM-1, ICAM-1, interleukin-6 (IL-6), IL-1α, TNF-α, E-selectin, tissue factor, ET-1 and RAGE itself (Basta et al., 2002; Huttunen et al., 1999; Yan et al., 1994). RAGE is also involved in activation of PI3K/Akt and MAP kinases, such as c-Jun N-terminal kinases (JNK), p38, and ERK-1/2 kinase (Stern et al., 2002). AGE binding to

RAGE triggers ROS production via NOX activation (Wautier et al., 2001). AGE/RAGE interaction is also involved in the activation of coagulation. Previous study demonstrated that reduced thrombomodulin activity as well as increased expression of tissue factor in EC is induced by AGE/RAGE system activation (Esposito et al., 1989). RAGE is considered as a potential target for the prevention of CAD in animal model of diabetes. Recent study demonstrated that RAGE deficiency significantly inhibited plaque accumulation, vascular inflammation and oxidative stress in diabetic animal model (Soro-Paavonen et al., 2008). It has been demonstrated that oxLDL is prone to glycoxidation (Gugliucci Creriche and Stahl, 1993). Recent study reported that incubation of RAGE-deficient murine aortic EC with oxLDL failed to upregulate inflammatory molecules (Harja et al., 2008). These findings suggest that oxLDL may have some effects via RAGE under hyperglycemia. The role of RAGE in glyLDL-induced PAI-1 expression in EC remains unclear.

1.2.3 Small G-proteins

Small G-proteins (small GTPases) are an important class of signaling molecule and act as biological switch for various cellular processes. They have been identified in eukaryotes from yeast to human, and comprise a superfamily of more than 100 members (Takai et al., 2001). A variety of membrane receptors and upstream regulators are involved in the regulation of small G-protein activation (Bar-Sagi and Hall, 2000; Bishop and Hall, 2000). The superfamily of small G-proteins can be divided into five families according to their structures and functional characteristics: Ras, Rho, Rab, Sar1/Arf, and Ran families

(Takai et al., 2001). The Ras family is of special interest as its members couple upstream signaling pathways to changes in the external environment.

In mammals, the Ras family includes three isoforms, namely H-, N- and K-Ras. H-Ras is the most widely distributed form of Ras in tissues, which modulates signaling between multiple membrane receptors and downstream signal transduction systems. H-Ras proteins usually cycles between two interconvertible forms, guanosine diphosphate (GDP)-bound inactive cytosolic form and guanosine-5'-triphosphate (GTP)-bound active membrane form. Due to cycling between two forms, H-Ras mediates multiple signaling pathways that originate from the activation of membrane receptors. Guanine-nucleotide-exchange factors (GEFs) regulate the conversion of Ras from its inactive to active form by promoting the binding of GTP. Full activation also requires post-translational lipidation of Ras proteins (farnesylation). The process of farnesylation of Ras is catalyzed by farnesyltransferase (Stephens et al., 2001; Takai et al., 2001). Farnesylation is essential for the association of cytosolic Ras in to the plasma membrane (Zhang and Casey, 1996). Previous studies showed that H-Ras activates, and K-Ras reduces, intracellular ROS production in EC. Activation of K-Ras is linked to low levels of ROS. Under oxidative stress condition K-Ras stimulated ROS scavenging by activating the mitochondrial superoxide dismutase (SOD) decreasing H_2O_2-induced cell apoptosis. Several studies demonstrated the protective effect of K-Ras under oxidative stress condition (Cuda et al., 2002; Santillo et al., 2001). H-Ras has been suggested as novel therapeutic target in cardiovascular medicine (Ohtsu et al., 2006).

Previous studies demonstrated close interactions between oxidative stress and H-Ras (Cuda et al., 2002; Lander et al., 1996). Cuda et al (2002) showed potential regulatory role of H-Ras in H_2O_2-induced oxidative stress in EC. The oxidizing agents act as an upstream signal and affect the interactions between Ras and several of its downstream effectors (Accorsi et al., 2001; Cheng et al., 2001). In EC, oxidative stress-induced ROS generation acts as a trigger for H-Ras activation, and further activates NOX complex (Cuda et al., 2002; Irani et al., 1997). Recent studies suggest that ROS is involved in strain-induced ET-1 gene expression via Ras/Raf/ERK signaling pathway in EC, which may be relevant to cardiovascular disease (Cheng et al., 2001). Membrane-associated small G-proteins, such as Ras and Rho, often mediate the activation of membrane receptors (Puddu et al., 2005; Ruiz-Velasco et al., 2004). Iwasaki et al (2008) demonstrated the involvement of Rho proteins in high glucose-induced PAI-1 production in bovine aortic EC. Kowluru et al (2004) demonstrated involvement of H-Ras in glucose-induced oxidative stress in EC. Previous studies demonstrated that LDL increased the membrane translocation and activation of H-Ras in human EC (Zhu et al., 2001). The interaction between oxidative stress and H-Ras stimulates cascade of kinases including MAP kinase, ERK, JNK or SAPK, and p38 kinase (Halfon et al., 2000; Xia et al., 1995). Ras is a common signaling target of ROS and cellular redox stress, which eventually transmit the signal to the nucleus (Lander et al., 1995). Further, H-Ras activation regulates downstream signal transduction cascades that include the activation of the transcription factor, NF-κB. Atherogenic stimuli mediated by Ras can induce cell senescence and inflammation in VSMC, eventually contributing to the development of atherogenesis (Minamino et al., 2003). These findings suggest that oxLDL or glyLDL-

induced oxidative stress may activate H-Ras, and downstream signaling pathways. The role of H-Ras and associated signaling involved in oxLDL or glyLDL-induced upregulation of PAI-1 is not clear.

1.2.4 Raf-1 and ERK-1/2 kinase

It is well established that phosphorylation of signaling protein kinases plays critical role in the regulation of cellular functions including cell growth, migration, and differentiation (Yang et al., 2001). Raf-1 is a serine-threonine kinase which undergoes phosphorylation in response to mitogenic stimulation. Raf-1 plays a critical role in the transmission and amplification of mitogenic signals from the cell surface to the nucleus (Morrison, 1990). Raf-1 is activated when it binds to Ras, and plays a key role in the downstream MAPK/ERK pathway. It is well known that phosphorylation of Raf-1 upon binding to Ras locks it into an activated conformation (Avruch et al., 2001). Small G-proteins mediate their downstream effects on cell growth, proliferation and differentiation by the activation of a cascade of protein kinases: including Raf-1. Raf-1 is one of the best characterized downstream effectors of Ras. Ras activates Raf-1 by recruiting Raf-1 from cytosol to cell membrane (Van Aelst et al., 1993; Warne et al., 1993). Raf-1 phosphorylates MEK, which in turn activates ERK-1/2 (Kyriakis et al., 1992).

The MAPK family is comprised of key regulatory proteins that control the cellular responses to extracellular stimuli. MAPK forms a group of serine/threonine specific kinases which are activated through phosphorylation at conserved threnonine and tyrosine residues. These kinases are classified in three groups; ERK, JNK and the p38

kinases (Guyton et al., 1996; Tomlinson, 1999). Many growth factor receptors have been shown to activate MAP kinases (Lange-Carter et al., 1993). The involvement of Ras, Raf-1, MEK and ERK-1/2 in cell proliferation has been documented for several cell types (Blenis, 1993; Marrero et al., 1997). Previous studies demonstrated the involvement of various signaling mechanisms in hyperglycemia-induced oxidative stress, such as MAP kinase, PI3 kinase, Jak/STAT, p21Ras, cdc42 and Rac1 (Lander et al., 1997; Schiekofer et al., 2003; Simm et al., 1997; Wautier et al., 2001; Yan et al., 1994). ROS mediates strain-induced ET-1 gene expression via Ras/Raf/ERK signaling pathway in EC, which may be relevant to pathological states of the cardiovascular system, such as atherogenesis (Cheng et al., 2001). Diabetes-associated AGE induced ROS generation is implicated in the activation of the Ras/Raf/MEK signaling cascade (Urata et al., 2002; Xu and Kyriakis, 2003). Recent study has demonstrated that native, mildly and highly modified LDL preparations stimulate PKC and MAPK pathways in VSMC. The findings further suggest potential links between modified LDL, vascular function, and the development of atherosclerosis in diabetes (Velarde et al., 2001). LDL activates both p38 and JNK signaling pathways through Ras activation, and further plays an important role in LDL-induced endothelial activation (Zhu et al., 2001). OxLDL mediates its mitogenic effects through the activation of Ras/Raf/MEK/MAPK pathway in rat cultured VSMC (Yang et al., 2001). Recent study demonstrated that Raf-1/ERK-1/2 signaling links to cytoskeletal remodeling to facilitate glucose-induced insulin secretion in pancreatic beta-cells (Kowluru et al., 2010). The involvement of Raf-1 and ERK-1/2 in lipoprotein-induced PAI-1 production is unknown.

1.2.5 Lipoproteins and Stress response

Stress responses have been implicated in the pathogenesis of atherosclerosis (Metzler et al., 2003; Sangle and Shen, 2010). Environmental stresses enhance the expression of Hsps, which is a family of conserved proteins or stress proteins that are identified in most of the mammalian cells (Morimoto, 1998). HSF is transcription factor of Hsps. Recent study demonstrated that both heat stress and H_2O_2 activate HSF1, *in-vitro* and *in-vivo* (Ahn and Thiele, 2003). Several studies demonstrated that risk factors of atherosclerosis, such as biomechanical stress and cytokines under hypercholesterolemic condition, stimulate the expression of HSF1 in cells of arterial wall, which increases the expression of Hsps (Metzler et al., 2003).

Various stress stimuli, such as oxLDL, heat shock, oxidants, and cytokines, stimulate the expression of Hsps by preventing damage from these stresses (Benjamin and McMillan, 1998; Xu and Wick, 1996). Previous studies demonstrated that glyLDL or oxLDL increased expression of Hsp70 in vascular EC (Zhao et al., 2009; Zhao and Shen, 2007), which is supported by other studies (Zhu et al., 1996). OxLDL has been shown to increase the expression of other Hsp isoforms, including Hsp23/32 in mouse macrophages (Yamaguchi et al., 1993), Hsp60 in monocytes/macrophages (Frostegard et al., 1996), and Hsp70 in SMC (Zhu et al., 1995). Increased production of Hsps was associated with the protection of cells against apoptosis induced by oxidative stress or heat shock (Buzzard et al., 1998). Previous studies demonstrated increased HSF1 activation in atherosclerotic lesions *in-vivo*. Furthermore, TNF-α, an inflammatory cytokine, can activate HSF1 in SMC, supporting the role of cytokines in HSF1 activation

in atherosclerotic lesions (Metzler et al., 2003). Our group originally demonstrated the involvement of HSF1 in glyLDL, oxLDL or oxidized VLDL-induced upregulation of PAI-1 gene transcription in vascular EC through the binding of HSF1 to PAI-1 promoter. Modification of LDL and EC-derived ROS is crucial in the upregulation of HSF1 and PAI-1 induced by these diabetes or atherosclerosis-associated lipoproteins. Oxidation contributes to glyLDL-induced HSF1 or PAI-1 expression in EC (Zhao et al., 2008; 2009; Zhao and Shen, 2007). Our group demonstrated that treatment with glyLDL or oxLDL dose and time-dependently increased the abundance of HSF1 and the levels of HSF1 mRNA in venous or arterial EC. HSF1 is involved in glyLDL or oxLDL-induced activation of PAI-1 promoter. SiRNA against HSF1 inhibited the levels of HSF1 and PAI-1 protein in EC induced by glyLDL or oxLDL. In addition, recent results from our laboratory demonstrated that antioxidant inhibited glyLDL or oxLDL-induced expression of HSF1 and PAI-1 in EC, which was associated with a reduction in the release of ROS from EC (Zhao et al., 2009; Zhao and Shen, 2007). HSF1 mediated stress response is an essential chain reaction for self-defense mechanisms against environmental stressor in the body. The upregulation of PAI-1 mediated by HSF1 in EC may be a part of defensive mechanism against oxidative stress and diabetes-associated metabolic stress. These findings demonstrated close interactions between metabolic stress and HSF1.

1.3 Oxidative stress

Oxidative stress in general referred as imbalance between ROS and antioxidant defense in cell metabolism, which occurs when the generation of ROS exceeds the antioxidant capacity of cells. Oxidative stress is thought to play an important role in the pathogenesis

of a variety of human diseases, including hypertension, heart failure, stroke, Alzheimer's disease, kidney disease, cancer, atherosclerosis and diabetes (Madamanchi et al., 2005; Roberts and Sindhu, 2009). Based on experimental and clinical studies, oxidative stress may be induced by multiple atherosclerosis-associated risk factors and contributes to atherosclerotic vascular damage. Hypercholesterolemia and diabetes are implicated in vascular ROS generation and ROS-induced endothelial dysfunction. The generation of ROS in EC, SMC, and macrophages is increased by oxLDL. ROS as well as reactive nitrogen species (RNS) are products of normal cellular metabolism. In the condition of increased production of ROS/RNS or decreased activities of anti-oxidant enzymes, biological damages on lipids, proteins and DNA may occur (Puddu et al., 2009). Cells naturally have enzymatic and nonenzymatic mechanisms to protect against pro-oxidants. The enzymatic mechanisms include antioxidant enzymes such as SOD, catalase, and glutathione peroxidase (GPx). The nonenzymatic antioxidant includes glutathione, ascorbate, β-carotene and α-tocopherol (Madamanchi et al., 2005).

ROS are ubiquitous, highly reactive with short half-life and formed as a part of oxygen metabolism in all biological systems. ROS species includes superoxide anion, the hydroxyl radical, H_2O_2, ozone, NO and peroxynitrite, all of them play key role in vascular biology (Pourova et al., 2010; Roberts and Sindhu, 2009). The sources for ROS production in cellular physiology includes NOX, xanthine oxidase, uncoupled endothelial NO synthase (eNOS), lipoxygenases and cycloxygenases. Superoxide is the primary ROS, formed in the cytosol by NOX and xanthine oxidase by reduction of molecular oxygen. The mitochondrial respiratory chain is another important source of ROS in cells

(Ballinger, 2005; Esposito et al., 1999; Zhang and Gutterman, 2007). NO is a reactive radical and considered as a signaling molecule in physiology. NO can react with superoxide to produce peroxynitrite anion, which is a potent oxidizing agent (Madamanchi and Runge, 2007). Increased ROS promotes oxidation of lipids which further decreases biological activity, leading to alterations in cell signaling and cellular function (Chopra and Wallace, 1998). Multiple epidemiological and experimental studies support correlations between oxidative stress and vascular disease. However, due to disappointing results of antioxidant therapy in humans in large clinical trials, in the prevention and control of CAD, the importance of antioxidants for the prevention of CAD is controversial.

Increasing lines of evidence indicate that oxidative stress mediates oxidation of apoB-containing lipoproteins, which might play an important role in lipoprotein atherogenecity (Lusis, 2000). Oxidative modification of LDL in arterial wall induced by ROS is thought to be a key factor for atherosclerosis. Clinical studies have proved a strong association between oxidative stress and atherogenic lipoproteins including oxLDL, in patients with cardiovascular disease (Kotur-Stevuljevic et al., 2007). Previous study in animal models demonstrated that aortae from hypercholesterolemic rabbits produced significantly more superoxide than control aortae, which supports a role of LDL in the induction of oxidative stress *in-vivo* (Mugge et al., 1994). Incubation of cultured vascular EC with oxLDL stimulated ROS production (Cominacini et al., 2000; Zhao and Shen, 2005). Previous studies including our own, demonstrated that NOX is activated by oxLDL, and mediates the generation of ROS in EC (Honjo et al., 2008; Zhao

et al., 2009). OxLDL-induced ROS production can also be mediated by mitochondrial Complex II (Cheng et al., 2007) and uncoupled eNOS (Fleming et al., 2005). Both cholesterol and oxLDL are associated with mitochondrial damage (Ballinger, 2005). LOX-1 mediates oxLDL-induced ROS formation in EC (Cominacini et al., 2000). These observations suggest that oxLDL contributes to oxidative stress in vasculature through multiple pathways.

Increased oxidative stress is considered as a major factor in the onset and development of diabetic complications including atherogenesis. Hyperglycemia increases ROS production in EC, and most of this ROS comes from mitochondria (Nishikawa et al., 2000). Nonenzymatic glycation of proteins and autoxidation of glucose generates ROS (Baynes, 1991). Elevated levels of glyLDL and ROS were detected in diabetic patients (Lyons, 1993). Consistent with this, an increased production of AGE is associated with CAD in patients with diabetes. Previous studies from our laboratory demonstrated that glyLDL increased the production of ROS in EC, which contributes to oxidative stress and stress response (Zhao and Shen, 2005; 2007). Incubation of EC with increasing concentrations of glucose induces oxidative stress (Ceriello et al., 1996). AGE are considered as an important source of vascular oxidative stress in diabetes. Accumulation of AGE stimulates the expression and activity of NOX in EC. Oxidative stress plays an important role in RAGE-mediated signaling (Wautier et al., 2001). The role of oxidative stress in lipoprotein-induced upregulation of PAI-1 in EC remains uncharacterized.

1.3.1 NADPH oxidase (NOX)

NOX plays an important role in the dysfunction of vascular cells (Petry et al., 2006). Clinical studies have shown an increased plasma level of oxidative stress markers in CAD patients (Kotur-Stevuljevic et al., 2007). Recent evidence indicates that NOX is the major source of ROS generation, which transfers electrons from NADPH across intracellular organelle membranes (Jones et al., 1996). Increased ROS production by NOX is associated with endothelial dysfunction and clinical risk factors of atherosclerosis (Guzik et al., 2000). The NOX complex consists of different catalytic subunits including one NOX family member, such as NOX1, NOX2/gp91^{phox}, NOX3, NOX4, NOX5, DUOX1, DUOX2, NOXO1 (NOX organizer 1) or NOXA1 (NOX activator 1), multiple other subunits including p22^{phox}, p40^{phox}, p47^{phox} and p67^{phox} and a small G-protein, Rac1. NOX isoforms are expressed and regulated in various cell types under pathological conditions (Bedard and Krause, 2007). NOX isoforms are involved in oxygen sensing. NOX1, NOX4 and their regulatory molecule p22^{phox} are involved in the cellular responses to changes in oxygen levels (Block et al., 2007). A gp91^{phox}/NOX2 complex is an essential catalytic subunit of NOX. Activation of gp91phox/NOX2 requires translocation of cytosolic factors to the NOX2/p22^{phox} complex (Lambeth et al., 2000). Interactions between subunits of NOX, including p22^{phox}, p47^{phox} p67^{phox} and p40^{phox}, are required for the activation of NOX complex (Babior, 1999).

OxLDL demonstrated 2-fold increase in gp91^{phox}/NOX2 mRNA as well as NOX activity in EC. The elevated expression of gp91^{phox}/NOX2 mRNA was accompanied by increased superoxide formation induced by oxLDL suggesting a correlation with

formation of an active NOX protein complex (Rueckschloss et al., 2001; 2003). The elevated ROS generation of oxLDL-treated EC is associated with NOX activity (Heinloth et al., 2000). OxLDL contributes to increased oxidative stress in EC, as well as in aortic segments of rabbits (Galle et al., 1999). Previous study from our laboratory demonstrated that oxLDL and glyLDL stimulated the generation of superoxide from EC (Zhao and Shen, 2005). The expression of NOX and ROS generation is increased in vascular cells by a number of stimulators, including Ang-II, thrombin, and TNF-α (De Keulenaer et al., 1998; Patterson et al., 1999; Ushio-Fukai et al., 1996). Increased accumulation of ROS and oxLDL was detected in atherosclerotic lesions in humans, which was associated with an increased level of $p22^{phox}$ (Azumi et al., 2002). $P22^{phox}$ is an essential component of the superoxide-generating NOX system and involved in Ang-II-mediated generation of ROS in VSMC (Ushio-Fukai et al., 1996). The expression of $p22^{phox}$ has been detected in advanced atherosclerosis plaques (Azumi et al., 1999). Previous studies suggest that siRNA against $p22^{phox}$ mRNA suppressed the expression of multiple NOX subunits and the activity of NOX complex (Modlinger et al., 2006). AGE-LDL increased the gene expression of NOX subunits, including NOX4, $p22^{phox}$ and $p67^{phox}$ in VSMC or EC. Further, AGE-LDL increased NOX activity and ROS production in VSMC or EC (Sima et al., 2009; Toma et al., 2009). AGE triggers the generation of ROS via the activation of NOX (Wautier et al., 2001). Previous studies demonstrated that NOX inhibitor, DPI, blocked oxLDL or AGE-induced ROS production (Nitti et al., 2007; Stielow et al., 2006)). Activation of NOX and increased ROS production were detected in the aortae of STZ-induced diabetic rats, which further implicates a direct association with

atherosclerosis pathogenesis (Hink et al., 2001). Nonetheless, precise role of NOX in lipoprotein-induced HSF1 or PAI-1 in EC remains unclear.

1.3.2 Mitochondrial electron transport chain (ETC)

Adenosine triphosphate (ATP) is the major supplier of energy in living cells. Mitochondria are the major source of energy (ATP) via oxidative phosphorylation. Mitochondria have two layers of membranes, outer membrane and inner membrane. The process of oxidative phosphorylation is based on chemiosmotic hypothesis proposed by Mitchell, which takes place in the inner membrane (Mitchell, 1984; Madamanchi et al., 2005). The electron transport chain (ETC) is composed of 5 multiple subunit complexes located in the inner mitochondrial membrane: Complex I (NADH-ubiquinone dehydrogenase), Complex II (succinate cytochrome *c* reductase), Complex III (ubiquinone cytochrome *c* reductase), Complex IV (cytochrome *c* oxidase), and Complex V (ATP synthase). The citric acid cycle generates NADH and $FADH_2$ as the final product from multiple chains of reactions. Electrons are transferred from NADH to Complex I or $FADH_2$ to Complex II and passed on to ubiquinol via coenzyme Q. Then electrons are transferred to Complex III via ubiquinol. Cytochrome *c* transfers electrons from Complex III to Complex IV and in this process molecular oxygen reduces to form water. The transfer of electrons in ETC creates transmembrane electrochemical gradient, through constant pumping of electrons across inner mitochondrial membrane at Complexes I, III and IV. The proton-motive force initiates reentry of protons in to matrix, which is used by Complex V to synthesize ATP from inorganic phosphate and ADP. The proton-motive force also mediates ATP-ADP exchange by the adenine nucleotide translocase (ANT). In

mitochondria, 0.2 – 2.0% of the molecular oxygen leaks through ETC to form ROS (Chance et al., 1979; Madamanchi and Runge, 2007) (Fig. 4).

Mitochondria play an important role in the development of atherogenesis. In fact, the mitochondrion is one of the major source of and primary target of ROS (Esposito et al., 1999; Wallace, 1992). In mitochondria, Complex I and III are considered as primary sites for ROS production (Turrens, 2003). In the absence of ATP, electrons derived from $FADH_2$ via Complex II can undergo "reverse electron transport" into Complex I generating more ROS production (Han et al., 2003; Liu et al., 2002). Based on these evidences, Complex I is considered as a pathophysiologically relevant source of ROS production in ETC. Many factors are involved in the regulation of ROS in mitochondria such as ETC, oxygen concentration, availability of NADH and $FADH_2$, uncoupling proteins (UCPs), antioxidant defenses and the modulation of nuclear factors (Ballinger, 2005; Droge, 2002). ROS formed in mitochondrial matrix is quickly converted to H_2O_2 by an antioxidant enzyme, SOD2. Another set of antioxidant enzymes such as GPx or catalase, converts H_2O_2 to water. However, transition metals may reduce H_2O_2 to a highly reactive hydroxyl radical form of ROS (Ide et al., 2000; Madamanchi and Runge, 2007). The reaction between NO and superoxide generates peroxynitrite, which is capable for the inactivation of enzymes, DNA damage and mitochondrial dysfunction (Ballinger et al., 2000; Cassina and Radi, 1996; Radi et al., 1991). Several lines of evidence indicate that ROS produced in cell by NOX act as a positive feedback, leading to increased production of ROS from mitochondria. This process is termed as ROS-induced ROS release (Brandes, 2005).

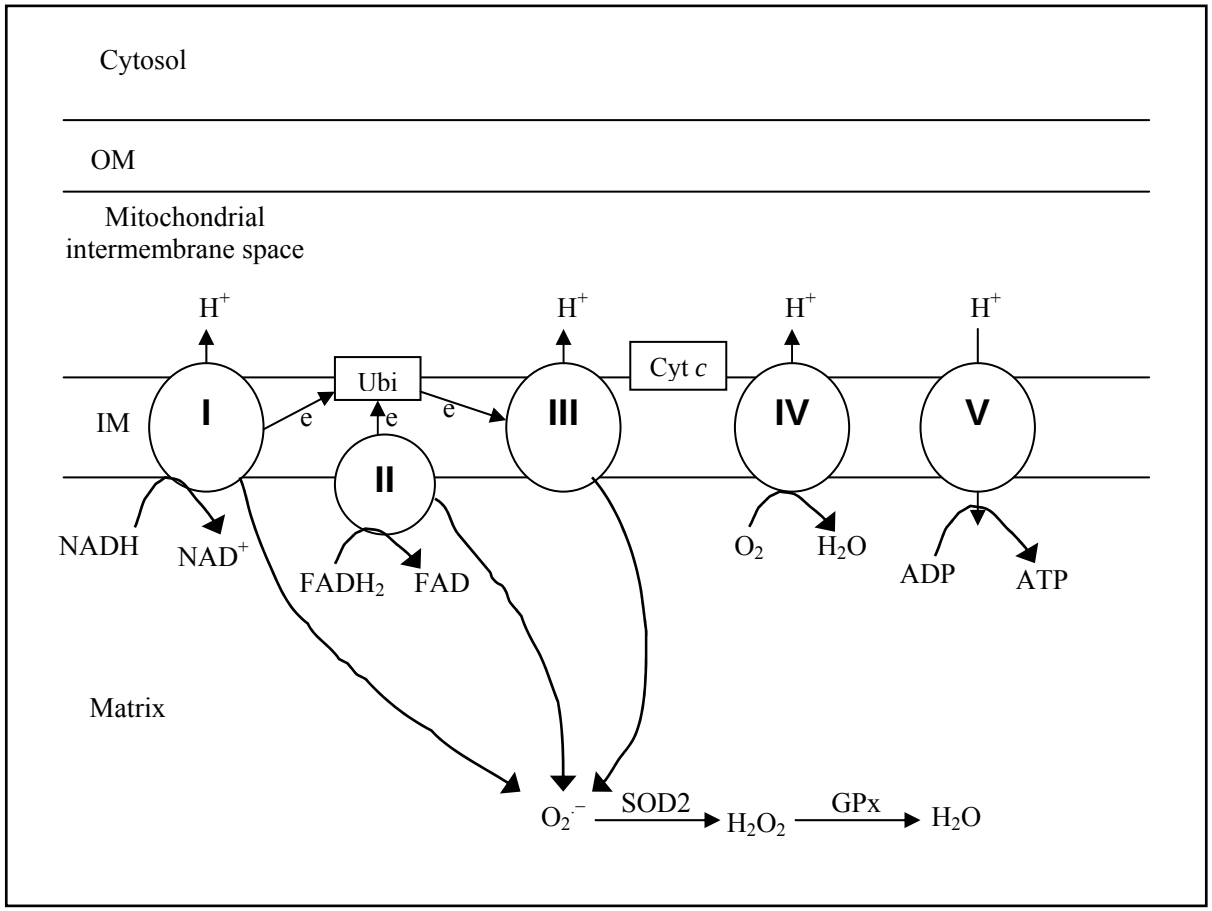


Figure 4. Scheme for mitochondrial electron transport chain. OM: outer membrane in mitochondria, IM: inner membrane, H^+: proton, e: electron, ubiq: ubiquinone, cyt *c*: cytochrome *c*, SOD2: superoxide dismutase, GPx: glutathione peroxidase, NADH/$FADH_2$: substrates from citric acid cycle.

Mitochondria-derived ROS causes multiple deleterious effects which may be neutralized by various antioxidants systems under normal physiology. However, when the balance between ROS and antioxidants is impaired, the conversion of ROS to H_2O_2 disrupts and contributes to oxidative damage in the cell (James and Murphy, 2002). Mitochondria-derived ROS is implicated in endothelial dysfunction, VSMC proliferation, and apoptosis of VSMC and macrophages, which can contribute to atherogenesis (Madamanchi and Runge, 2007). However, generation of ROS in mitochondria can cause damage to lipids, carbohydrates, proteins, and mitochondrial DNA (mtDNA) (Puddu et al., 2009). Damage of mtDNA is associated with atherogenesis. Each mammalian cell contains hundreds of mitochondria, and each mitochondrion has 5-10 copies of mtDNA (Wallace, 1999). Previous reports demonstrated that mtDNA damage contributes to increased ROS production and atherogenesis. The mutations at Complex I gene increases mitochondrial ROS production (Chomyn and Attardi, 2003; Pitkanen and Robinson, 1996; Puddu et al., 2005). It has shown that mtDNA is a cellular target of ROS due to its proximity to the source of ROS in inner mitochondrial membrane and the lack of protective histone-like proteins (Ballinger et al., 2002; Clayton, 1984). These evidences suggest that mtDNA damage, mutations and impaired mitochondrial function are associated with cardiovascular disease. The role of mitochondria in lipoprotein-induced oxidative stress remains unclear.

1.3.3 Mitochondrial ETC enzymes

Mitochondrial ETC enzymes play an important role in the regulation of oxidative phosphorylation. In the 1960s, Hatefi first purified four respiratory chain enzymes in

mitochondria (Hatefi, 1976). The respiratory chain consists of four inner-membrane bound enzymes: NADH-ubiquinone dehydrogenase, succinate cytochrome *c* reductase, ubiquinone cytochrome *c* reductase and cytochrome *c* oxidase. The molecular weights of the mitochondrial complexes in which they reside are: 700,000 for Complex I, 200,000 for Complex II, 300,000 for Complex III and 160,000 for Complex IV (Gautheron, 1984). The enzymes in mitochondrial complexes are of greatest interest due to their roles in the ETC, which places them at the heart of cellular physiology (Crofts, 2004).

NADH-ubiquinone dehydrogenase (ND) or Complex I: is composed of more than 40 protein subunits. ND is located in the inner mitochondrial membrane, and is the point of entry for electrons through the ETC. Complex I regulates an initial stage of oxidative phosphorylation in mitochondria by oxidizing NADH in the matrix and transferring electrons from NADH to a lipid-soluble carrier, ubiquinone (Q) at the inner membrane (Schultz and Chan, 2001; Walker, 1992). At the same time, the passage of two electrons from NADH to Q is coupled to the translocation of four protons from mitochondrial matrix to intermembrane space. ND catalyzes the reaction (Grivennikova et al., 2007; http://en.wikipedia.org/wiki/NADH_dehydrogenase):

$$NADH + H^+ + CoQ + 4H^+_{in} \rightarrow NAD^+ + CoQH_2 + 4H^+_{out}$$

Seven ND subunits are encoded by mitochondrial DNA (Hirst et al., 2003; Walker, 1992). Mutations of Complex I have been detected in humans and associated with neurological and neuromuscular diseases (Smeitink et al., 2001). Glutamate and

malate acts as a substrate for Complex I (Chowdhury et al., 2000). Rotenone is the best-known inhibitor of Complex I (Carroll et al., 2006). Complex I-dependent superoxide release exclusively occurs in matrix and no detectable levels escape from intact mitochondria. The most likely site of electron leakage in Complex I is the iron-sulfur clusters (Barja, 1999). Previous studies demonstrated reduced activities of Complex I in aging rats and mice (Navarro and Boveris, 2004; Navarro et al., 2002).

Succinate cytochrome *c* reductase (SCCR): Membrane bound SCCR embedded in the inner mitochondrial membrane in all aerobic cells. This enzyme is also called as Complex II. SCCR is important due to its tendency to contribute to both the citric acid cycle and the ETC. SCCR catalyzes the oxidation of succinate to fumarate and transfers its reducing equivalent to the respiratory chain (Lancaster, 2002). This enzyme is water soluble and contains covalently bound flavoprotein, non-heme iron, and acid-labile sulfur. SCCR is composed of 4 polypeptides with molecular masses of 70, 30, 15, and 13 kDa. It has five prosthetic groups, i.e. one covalently linked flavin adenine dinucleotide (FAD), three iron sulfur clusters (2Fe-2S, 4Fe-4S, and 3Fe-4S), and heme b. Electrons are sequentially transferred to Q via an ETC consisting of a covalently bound FAD and three iron-sulfur centers (Hederstedt and Rutberg, 1981; Tomitsuka et al., 2003). Succinate acts as a substrate for Complex II (Chowdhury et al., 2000). The human Complex II has mutations in the four subunits, which are associated with a wide spectrum of clinical pathologies (Rustin et al., 2002). Defects in human Complex II are associated with cancers as well as mitochondrial diseases (Tomitsuka et al., 2003).

Ubiquinone cytochrome *c* reductase (UCCR): is also called the cytochrome *bc1* complex or Complex III. It is a multifunctional membrane protein complex, which catalyzes electron transfer, proton translocation, peptide processing, and superoxide generation. The UCCR complex is an intrinsic membrane protein that catalyzes electron transfer from ubiquinol to cytochrome *c*. The reaction mechanism of this complex operates through a Q-cycle that couples electron transfer to the generation of a proton gradient that drives ATP synthesis. There is constant pumping of 4 protons from the mitochondrial matrix to intermembrane space. The reaction in Complex III reduces quinone (Q) to quinol (QH_2):

$$QH_2 + 2 \text{ cytochrome } c\ (Fe^{III}) + 2\ H^+_{in} \rightarrow Q + 2 \text{ cytochrome } c\ (Fe^{II}) + 4\ H^+_{out}$$

Q-cycle coupled electron transfer operates with the consumption of two protons into the matrix, four protons are released into the inter membrane space and two electrons are passed to cytochrome *c* (Crofts, 2004; Kramer et al., 2004; http://en.wikipedia.org/wiki/Cytochrome_bc1_complex). This complex consists of four redox prosthetic groups including cytochromes b566 (bL) and b562 (bH) cytochrome c1, and a high-potential iron–sulfur cluster [(2Fe–2S)]. It contains eleven proteins including subunits III, IV, and V, house *b*-type cytochromes, cytochrome *c1*, and the iron–sulfur cluster, respectively, and the rest eight protein (subunits I, II, VI–XI) contains no redox prosthetic groups are termed supernumerary subunits (Yue et al., 1991). Complex III releases ROS to both sides of the inner mitochondrial membrane. The locus of superoxide production in Complex III, the ubiquinol oxidation site, is situated immediately next to

the intermembrane space (Muller et al., 2004). Antimycin A, myxothiazol and stigmatellin are the inhibitors for Complex III. The mutations in genes encoding the proteins of the Complex III have been associated to a wide range of mitochondrial myopathies (Crofts, 2004).

Cytochrome *c* oxidase: is the terminal enzyme of the ETC, which plays an important role in the regulation of aerobic energy production. Located in the inner mitochondrial membrane, it catalyzes the oxidation of cytochrome *c* and the reduction of oxygen to water. In this reaction four protons are transferred to oxygen to form water in the matrix, and four protons are transported across the inner mitochondrial membrane. Cytochrome *c* oxidase is considered as the rate-limiting step of respiration (Babcock and Wikstrom, 1992), thus highlighting its key role in regulating the rate of respiration and ATP synthesis. This enzyme consists of a dozen or more subunit polypeptides, of which three (Cytochrome *c* oxidase I, II and III) are encoded by mtDNA; and the remaining ten subunits by the nuclear genome (Nijtmans et al., 1998; Yoshikawa et al., 1998). Mutations corresponding to nuclear genes encoding Cytochrome *c* oxidase results in clinical encaphalopathies such as Leigh syndrome, fatal cardiomyopathies, hepatic failure and leukodystrophy (Shoubridge, 2001). The involvement of respiratory chain enzyme complexes (Complex I-IV) in lipoprotein-induced mitochondrial dysfunction not documented.

2. Hypothesis

Based on literature, several studies demonstrated signal transduction mechanisms for oxLDL or glyLDL and their involvement in diabetic cardiovascular complications. However, these studies have not discussed the role of transmembrane receptors in oxLDL or glyLDL-induced signaling for PAI-1 production in EC. Elevated levels of oxLDL or glyLDL were detected in diabetic patients. PAI-1 is considered as a non-traditional risk factor for diabetes-associated cardiovascular disease. Membrane receptors incuding LOX-1, RAGE, small G-proteins and downstream signal transduction pathways such as Raf-1/ERK-1/2 have been linked to atherosclerosis and inflammation. However, the link between these signaling mediators and any role in oxLDL or glyLDL-induced PAI-1 production is not known. Atherogenic lipoproteins play an important role in oxidative stress and stress response in cells. Mitochondria and NOX are the major sources for ROS production. The effects of oxLDL or glyLDL on NOX and mitochondrial function in EC not yet clear. Previous studies in our laboratory demonstrated that oxLDL or glyLDL increased the production of PAI-1, HSF-1 and ROS in vascular EC. The current project is designed to address above discussed questions with following hypotheses:

1. OxLDL or glyLDL stimulates the generation of PAI-1 in EC via a membrane receptor, and transmembrane signal transduction pathways.
2. Elevated ROS may be implicated in oxLDL or glyLDL-induced oxidative stress in EC.

3. Objectives

1. To identify the involvement of membrane receptor, LOX-1, small G-protein, H-Ras and Raf-1/ERK-1/2 pathway in the upregulation of PAI-1 in cultured EC induced by oxLDL.
2. To examine the involvement of RAGE (a receptor for AGE), NOX, H-Ras/Raf- pathway in glyLDL-induced HSF1 and PAI-1 upregulation in EC.
3. To determine the impact of oxLDL or glyLDL on oxygen consumption, the activities of key enzymes in the mitochondrial respiratory chain and mitochondria-derived ROS production in EC.

4. Materials and Methods

4.1 Reagents: All chemicals were purchased from either Sigma-Aldrich® Inc. (Oakville, ON. Canada) or Calbiochem (San Diego, CA, USA) unless otherwise mentioned. All antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), Sigma-Aldrich Inc. (Oakville, ON, Canada), Cell Signaling Technology® Inc. (Danvers, MA, USA), Abcam® Inc. (Cambridge, MA, USA) or Oxford Biomedical Research (Oxford, UK).

4.2 Isolation and modification of LDL: Plasma was freshly prepared from blood of healthy donors by centrifugation (2,000 xg) for 15 min at 4°C. LDL (density 1.019-1.063) was isolated from plasma using sequential floating ultracentrifugation. Copper-oxLDL was prepared by incubating LDL with 5 μM $CuSO_4$ at 22°C for 24 h (Ren et al., 1997), and that of $FeSO_4$-modified LDL (F-oLDL) was generated through dialysis against 6 μM $FeSO_4$ for 24 h at 22°C (Watson et al., 1995). In a parallel preparation, LDL was oxidized with the presence of 80 μmol/l butylated hydroxytulene (BHT) (BHT-oxLDL). Mildly oxidized LDL (moLDL) was prepared by incubation of LDL with 5 μM $CuSO_4$ at 22°C for 6 h. Free copper or iron ions in oxidized LDLs were removed via dialysis (Zhao et al., 2009). The extent of oxidation of LDL was confirmed using thiobarbituric acid-reactive substance (TBARS) assay (Ohkawa et al., 1979). The levels of malondialdehyde in oxidized LDLs were 10-fold greater than that in comparing batches of LDL. Lipoproteins were excluded from experiments if the level of endotoxin in lipoproteins was >0.05 ng/ml measured using the E-Toxate kit (Sigma, St. Louis, MO).

The oxLDL preparations were stored in sealed tubes at 4°C in the dark under a layer of nitrogen to prevent autooxidation (Ren et al., 2000).

LDL was glycated by incubation with 50 mmol/L glucose and 50 mmol/L sodium cyanoborohydride in the presence of 0.01% EDTA to prevent oxidation for 2 weeks at 37°C as previously described (Zhang et al., 1998). In parallel experiment, LDL was modified with 50 mM sodium cyanoborohydride alone (as control) for 2 weeks at 37°C. GlyLDL preparation was thoroughly dialysed to remove free glucose or chemicals. The extent of glycation in glyLDL was confirmed using trinitrobenzenesulfonic acid assay (Duell et al., 1990). Approximately 60% of lysine residues were glycated in the preparations of glyLDL used in the following experiments. Endotoxin level in lipoproteins was monitored using E-Toxate kit with a threshold of 0.05 ng/ml (Sigma). The glyLDL preparations were stored in sealed tubes at 4°C in dark under a layer of nitrogen to prevent auto-oxidation (Zhao and Shen, 2007).

4.3 Cells and cell culture: Seed human umbilical vein EC (HUVEC), most commonly used model of cultured EC, were obtained from American Type Culture Collection (Manassas, VA, USA). Cells were grown in F12K medium received from Invitrogen (Burlington, ON, Canada) and volume of 500 ml were supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (Invitrogen), 0.1 mg/ml heparin, and 30 µg/ml endothelial cell growth supplements (Sigma) (Ren and Shen, 2000). Human coronary artery EC (HCAEC), a type of EC directly related to CAD, were originally

received from Clonetics (San Diego, CA, USA). The cells were cultured in endothelial growth medium-MV (Clonetics) and used within eight passages from seed cells.

Porcine aortic EC (PAEC) were obtained from Dr. P.E. DiCorleto in Cleveland Clinic Foundation (Cleveland, OH, USA). Primary cultures of porcine aortic EC were isolated from healthy, plaque free porcine aorta. Briefly, thoracic aorta segments were opened longitudinally, rinsed with serum-free media and the exposed intimal surface was digested with collagenase (2 mg/ml in serum-free media) for 15 min at 37°C. The detached EC patches were gently collected with a rounded spatula or cotton swab and placed in primary culture in Dulbecco's modified Eagles media/Ham's F12 media (DME/F12. 1:1) (Irvine Scientific Co., Santa Anna, CA) supplemented with 0.24% sodium bicarbonate, 0.1 mM modified Eagle's media (MEM) non-essential amino acids and penicillin (100 U/ml) and streptomycin (100 ~g/ml) (supplements from Sigma Chemical Co.) containing 5% fetal bovine serum (FBS) (Gibco) and incubated at 37°C in 5% CO2 in a humidified chamber. At confluence the cells were subcultured at 1: 3 or 1: 5 ratios by trypsin/EDTA treatment. EC between the third and tenth passage were seeded into 150 mm tissue culture dishes (Falcon, Lincoln Park, N J) and grown to confluence (Shen et al., 1990). In present study, PAEC were grown in Dulbecco's Modified Eagle Medium, D-MEM (Invitrogen) supplemented with 10% FBS and 1% penicillin/streptomycin (Invitrogen). PAEC were used for mitochondrial study, which generally required large number of cells.

4.4 Cell treatment: EC were treated with physiological concentrations of LDL, F-oLDL, oxLDL or glyLDL. OxLDL (50-150 μg/ml), glyLDL (25-150 μg/ml), F-oLDL or LDL (100 μg/ml) were incubated with EC for various lengths of time as indicated. Equal volume of vehicle was added to control cultures for the matching period. LOX-1 blocking antibody (a gift from Dr. T. Sawamura, National Cardiovascular Center Research Institute, Osaka, Japan) was used at 10 μg/ml and LDLR blocking antibody (R&D Systems, Minneapolis, MN, USA) at 5 μg/ml. Polyclonal RAGE-blocking antibody (a gift from Dr. A.M. Schmidt, Columbia University, New York, USA) and control polyclonal goat IgG (Santa Cruz) were used at 10 μg/ml with a 30 min pre-incubation. Farnesyltransferase inhibitor, FTI-277 (10 or 20 μM; Calbiochem), Raf-1 inhibitor, GW 5074 (1 μM; Calbiochem), or ERK-1/2 inhibitor, PD-98059 (10 μM; Sigma) were used in following experiments. For experiments using blocking antibody or various signaling inhibitors, 30 min of pre-incubation was applied unless otherwise indicated. Diphenyleneiodonium (DPI; Sigma), a NOX inhibitor, was used at 10 μM with 4 h pre-incubation.

Various substrates and inhibitors for mitochondrial respiratory chain Complexes were used: Glutamate (10 mM) + malate (5 mM), succinate (10 mM), or ascorbate (5 mM) + TMPD (0.5 mM) for Complex I, II/III, or IV, respectively. Rotenone (1 μM), antimycin A (1 μg/mL), and potassium cyanide (KCN, 0.25 mM) were used as inhibitors for Complex I, III, or IV, respectively. Oligomycin (1 μM) was used as an ATP synthase inhibitor and carbonylcyanide p-trifluoromethoxyphenylhydrazone (FCCP, 1 μM) was

used as a mitochondrial uncoupler. All above mentioned chemicals were obtained from Sigma.

4.5 Western blotting: The cells were treated after reaching 70-80% confluency in all the experiments. The medium was removed from the cells and the cell plates were kept in the ice. The cells were washed with 1X phosphate buffered saline. After addition of lysis buffer (50mM HEPES, 5mM EDTA, 50mM NaCl, 1% Triton X-100, 5mM Protease inhibitor, pH 7.5 with HCl), cells were incubated at 4°C for 30 min. The lysates were collected in precooled microcentrifuge tubes using scraper. The samples were vertex for 30 sec and spin at 4°C for 10 min at 13,000 rpm. The supernatant was transferred to fresh tubes. The samples were stored at -70°C and used for Western blotting. Protein concentrations in the cell lysate were estimated using Lowry assay.

Total cellular lysate (30-80 µg) were mixed with 5X SDS loading dye and denatured for 10 minutes at 99°C. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes using a transfer apparatus (BioRad® Hercules, CA, USA) for 1 h at constant current of 100V. Membranes were blocked for 1 h with 5% non-fat dry milk powder in Tris-buffered saline with 0.25% v/v Tween-20 (TBS-T) and incubated for 2 h with appropriate primary antibody diluted in TBS-T containing 5% non-fat dry milk powder. The following primary antibodies were used: anti-human LOX-1, anti-human LDLR, anti-human RAGE (polyclonal: R & D Systems), human NOX2 ($gp91^{phox}$), H-Ras, phosphorylated Raf-1 (pRaf-1), H-Ras, NOX2, HSF1 (polyclonal: Santa Cruz), pERK-1/2, ERK-1/2 (polyclonal: Cell Signaling), human PAI-1, HSF1 (monoclonal:

Santa Cruz), β-actin (monoclonal: Sigma), PAI-1 (monoclonal: Oxford Biomedical Research), and β-actin (monoclonal: Abcam). Membranes were washed 2 times for 10 minutes in TBS-T and subsequently incubated with an appropriate secondary antibody conjugated with horseradish peroxidase (HRP) for 1 h at room temperature. After the repeated washing steps (15 minutes and 5 minutes-x3) the specific proteins on the membrane were visualized using enhanced chemiluminence (ECL) reagents (Amersham Biosciences®, Piscataway, NJ, USA). Relative intensities of protein bands were detected using Chemi-Doc system or autoradiography and analyzed using Quantity One software (BioRad).

4.6 Reverse transcription-polymerase chain reaction (RT-PCR): The levels of PAI-1 or H-Ras mRNA were assessed using RT-PCR and justified with the level of β-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA in corresponding samples. Total RNA was isolated from cultured EC or heart tissues of STZ-diabetic mice using TRI Reagent (MRC, Cincinnati, OH) or TRIZOL Reagent (Invitrogen, Burlington, ON) respectively. RT-PCR was performed using access RT-PCR system (Promega, Madison, WI, USA) to synthesize cDNA. The levels of H-Ras or PAI-1 mRNA were examined using RT-PCR. Primers for H-Ras mRNA (sense: 5'-GAATCTCGGCAGGCTCAG-3', antisense: 5'-CACTCTCATCAGGAGGGTTC-3'), PAI-1 mRNA (sense: 5'-CAGACCAAGAGCCTCTCCAC, antisense: 5'ATCACTTGGCCCATGAAAAG) and β-actin gene (sense: 5'-CGTGGGCCGCCCTAGGCACCA, antisense: 5'-TTGGCCTTAGGGTTCAGGGGGG) were prepared according to reported cDNA sequences (Antalis et al., 1988; Ponte et al., 1984; Tsai et al., 2006). PCR for H-Ras was

performed using 35 cycles: denaturation at 95°C for 15 s, annealing and extension at 60°C for 1 min. In case of PAI-1 or β-actin, PCR comprised 35 cycles and performed at 95, 60, and 72°C for 1, 2, and 3 minutes, respectively.

Primers for mouse PAI-1 (sense: GAGTGGCCTGCTAGGAAATCCATTC, antisense: GACCTTGCC AAGGTGATGCTTGGCAAC) and GAPDH gene (sense: ATGTTCCAGTATGACTCCACTCACG, antisense: GAAGACACCAGTAGACTCCACGACA) were generated as previously reported (Krag et al., 2007). PCR for PAI-1 mRNA in mouse hearts was performed at 95°C, 61°C and 72°C for 1, 1, and 2 minutes with 35 cycles. In the case of GAPDH mRNA, PCR was performed at 95°C, 60°C and 72°C for 1, 1, and 2 minutes with 35 cycles. H-Ras (140 bp), PAI-1 (202 bp) and β-actin (300 bp) mRNA fragments were visualized on 1% agarose gel stained with ethidium bromide, and semi-quantified using Chemi-Doc system plus Quantity One software. The abundance of specific mRNA was normalized with the intensity of β-actin or GAPDH mRNA in same samples.

4.7 Detection of translocation of H-Ras: After treatment of EC with lipoproteins, cells were harvested with a rubber policeman and homogenized in a Dounce homogenizer. Cytosolic and membrane fractions from cell lysate were separated using a Beckman TLX-100 (Beckman Coulter Canada Inc., Mississauga, ON, Canada) table top ultracentrifugation at 100,000 g at 4°C for 1 h (Ren et al., 2000; Zhu et al., 2001). Expression of H-Ras in the membrane fraction was detected using Western blotting with a polyclonal antibody against human H-Ras (Santa Cruz).

4.8 Gene silencing: EC were transfected with desired siRNA using Silence siPort Lipid kit (Ambion, Austin, TX, USA). Cells were plated in serum-free medium 24 hours prior to transfection. SiRNA was added to 100µl of medium in a tube at recommended concentrations (typically 2-5µl for a 6 well plate) at the time of transfection. Lipid reagent (2-5µl for a 6 well plate) was diluted in 100µl of medium. SiRNA and lipid reagent were mixed and then incubated for 20 minutes at room temperature to allow the formation of siRNA-lipid complexes. SiRNA for H-Ras or human $p22^{phox}$ (Santa Cruz)-were used to transfect EC in serum free medium as previously described (Zhao and Shen, 2007). SiRNA for β-actin, scramble or negative control siRNA (Ambion) was transfected in parallel cultures to verify the methodology.

4.9 PAI-1 antigen measurement: The levels of PAI-1 antigen in experimental media of cultured EC were measured using human PAI-1 enzyme-linked immunosorbent assay (ELISA) kit (American Diagnostic, Stamford, CT, USA) as previously described (Cockell et al., 1995).

4.10 Measurement of hydrogen peroxide (H_2O_2): The levels of H_2O_2 in post-culture media of EC were measured using PeroxiDetect^{TM} kit from Sigma as previously described (Zhao and Shen, 2005). The measurement was based on the fact that peroxides convert Fe^{2+} to Fe^{3+} at acidic pH. Fe^{3+} +forms a color-adduct with xylenol orange, which was detected at 560 nm.

4.11 Measurement of oxygen consumption in mitochondria using oxygraphy: Mitochondrial oxygen consumption in EC was determined at 37°C using highly sensitive OROBOROS oxygraphy-2K (Oroboros, Innsbruck, Austria) (Chowdhury et al., 2000). PAEC were trypsinized and counted using hemocytometer for each measurement. Cells were harvested and resuspended in KCl medium (80 mM KCl, 10 mM Tris-HCl, 3 mM $MgCl_2$, 1 mM EDTA, 5 mM potassium phosphate, pH 7.4) at a concentration of $1.5x10^6$ cells/ml. Cell suspension (2 ml) was added in each oxygraphy chamber with continuous magnetic stirring. EC were permeabilized using digitonin (25 μg/10^6 cells) to permeabilize plasma membrane of cells, but maintaining intact mitochondrial membrane as preciously described (Chowdhury et al., 2000; Floryk and Houstek, 1999). The effects of modified lipoproteins on the activities of mitochondrial ETC complexes were analyzed in EC using oxygraphy with the presence of specific substrates and inhibitors. Glutamate (10 mM) + malate (5 mM), succinate (10 mM), or ascorbate (5 mM) + N,N,N',N'-tetramethyl-p-phenylendiamine dihydrochloride (TMPD, 0.5 mM) were used as substrates for Complex I, II/III, or IV, respectively. Rotenone (1 μM), antimycin A (1 μg/mL), or KCN (0.25 mM) were used as inhibitors for Complex I, III, or IV, respectively. Oligomycin (1 μM) as ATP synthase inhibitor or FCCP (1 μM) as a mitochondrial uncoupler were used. Unless mentioned, all the chemicals were obtained from Sigma. Oroboros DatLab software was used for analysis and graphic presentation of experimental data. Oxygen consumption was normalized by cell numbers and expressed as pmol/sec/10^6 cells. Respiratory control index (RCI) was determined from oxygen respiration in mitochondria of digitonin-permeabilized EC induced by adenosine diphosphate (ADP, 2 mM) (Chowdhury et al., 2000).

4.12 NAD^+/NADH assay: Nicotinamide adenine dinucleotide (NAD^+) levels and its reduced form, NADH, in cell lysates were analyzed using EnzyChrom™ NAD^+/NADH assay kits (BioAssay System Haywood, CA, USA). Changes in absorbance were measured under 565 nm using spectrophotometer.

4.13 Mitochondrial respiratory chain enzyme activity

4.13.1 NADH-ubiquinone dehydrogenase (ND) activity: ND activity (Complex I) was measured as previously described (Birch-Machin et al., 1994). Mitochondrial extracts (50 µg) were added to a buffer containing 25 mM potassium phosphate (pH 7.2), 5 mM $MgCl_2$, 2 mM KCN, 2.5 mg/ml of bovine serum albumin (BSA) (fraction V), 2 µg/ml antimycin A, 0.1 mM NADH, and 50 µM decylubiquinone. The cells were sonicated (10 seconds for 3 times on ice), which ruptures the mitochondrial membrane and allows the access of substrates to intermembrane space. ND activity measurement was started at 3 min before the addition of rotenone (2 µg/ml) and continued for another 3 min at 340 nm using Ultrospec 2000 UV-visible spectrophotometer equipped with Biochrom Swift II software (Biopharmacia Biotech, Uppsala, Sweden) (Chowdhury et al., 2000).

4.13.2 Succinate cytochrome *c* reductase (SCCR) activity: The enzymatic activity of SCCR (Complex II/III) was evaluated by monitoring the rate of reduced cytochrome *c* formation using succinate as a substrate. The formulation of reaction mixture comprised of 10 mM potassium phosphate (pH 7.4), 2 mM EDTA, 0.01% BSA (fatty acid-free), 0.2 mM ATP, 1 mM KCN, 5 µM rotenone, and 10 mM succinate. Unless indicated, all the

chemicals were used from Sigma. Sonicated cell lysates (0.2 mg protein) were incubated in the reaction mixture for 3 min and 40 μM oxidized cytochrome *c* was subsequently added as previously described (Rustin et al., 1994). Changes in absorbance were monitored at 30°C using spectrophotometer for 5 min at 550 nm (Chowdhury et al., 2000).

4.13.3 Ubiquinone cytochrome *c* reductase (UCCR) activity: The enzymatic activity of UCCR (Complex III) was evaluated using 100 μg of cell lysates. The reaction mixture contained 25 mM potassium phosphate (PH 7.4), 5 mM $MgCl_2$, 2 mM KCN, 2 ug/ml rotenone, 2.5 mg/ml BSA and 50 μM cytochrome *c* was added to cell lysates in a final volume of 1 ml. After a 2 min equilibration period, the reaction was started by the addition of 50 μM ubiquinol-2 and the increase in absorbance at 550 nm was monitored using spectrophotometer (Birch-Machin et al., 1994).

4.13.4 Cytochrome *c* oxidase activity: Cytochrome *c* oxidase (Complex IV) activity was measured at 30°C by following the rate of oxidation of reduced cytochrome *c* at 550 nm. The assay was performed in cultured cells in the presence of 40 μM reduced cytochrome *c*, 20 mM phosphate buffer, 0.1 mg of protein from cultured cells and 16 mg of lauryl maltoside/mg protein (0.16%) (Chowdhury et al., 2000; Wharton and Tzagoloff, 1967).

4.13.5 Citrate synthase (CS) activity: CS activity was used to determine the amount of functional mitochondria in cells. CS activity was determined at 30˚C in a medium

containing 150 mM Tris-HCl (pH 8.2), 0.16% of lauryl maltoside, 0.1 mM dithionitrobenzoic acid and 0.1 mg protein from EC. The reaction was started by the addition of 300 μM acetyl CoA and changes in absorbance at 412 nm were measured for 1 min. This rate was subtracted from that with an addition of 0.5 mM oxalacetic acid (Srere, 1969).

4.14 Intracellular ROS detection: EC were treated with LDL, oxLDL or glyLDL for 2 h as optimized in previous studies published from our laboratory (Zhao and Shen, 2005). After incubation, cells were washed with phosphate buffered saline. Lipoprotein-treated cells were loaded with a fluorogenic probe for ROS, MitoSox Red™ (2.5 μM, Invitrogen) for 20 or 30 min (Robinson et al., 2006). To confirm the association of ROS with mitochondria induced by oxLDL or glyLDL, cells were also labeled with MitoTracker Green (20 nM, Invitrogen) for 20 min. Cells were washed with Hanks' balanced salt solution and fixed with 3% paraformaldehyde for 15 min. Fluorescent images were captured using an Olympus IX70 inverted microscope coupled to an Olympus Fluoview Confocal imaging system (Ma et al., 2006). Quantification of MitoSox Red™ fluorescence after treatment of live cells with the dye, and subsequent fixation with 3% paraformaldehyde was performed using an iCys Laser Scanning Cytometer (CompuCyte Corp, MA) and associated system hardware. Cell nuclei were labeled using H33342 (1 μg/ml) for 30 seconds. For these studies, images were captured using a 20x objective with a scan step size of 5 μm. For all analyses, we captured red fluorescence (MitoSox), blue fluorescence (nuclei), and forward light scatter to generate shaded relief contrast images.

Mean integrated fluorescence intensity of MitoSox Red in each scan area (5 mm^2) was determined by capturing fluorescence signal from a non-overlapping matrix of ~780 circular "phantom contour" events (each 50 μm diameter). Data were captured from duplicate experiments using 3 different cell cultures. For comparison between treatments and experiments, MitoSox Red fluorescence was normalized on the basis of cell number per field, as determined by primary event counts, with contouring on DNA fluorescence (Roy Chowdhury et al., 2010).

4.15 Assessment of mitochondrial membrane potential: EC were treated with of LDL, oxLDL or glyLDL (100 μg/ml) for 12 h. After treatment with lipoproteins, cells were harvested by trypsinization, washed three times in cold phosphate buffered saline, counted and resuspended in a KCl medium (80 mM KCl, 10 mM Tris-Cl, PH 7.4, 3 mM $MgCl_2$, 1 mM EDTA, 5 mM KH_2PO_4, 10 mM succinate, 1 μM rotenone) at a protein concentration of 1 mg/ml. Digitonin was added at standardized concentration (0.02 mg digitonin/mg protein) to permeabilize cells with incubation for 5 min on ice and spin for 5 min at 600x g. The supernatant was removed and resuspended in 0.5 ml KCl medium. The permeabilised cells were incubated with 20 nM tetramethylrhodamine methyl ester (TMRM), a mitochondrial membrane potential probe (Molecular Probes, Eugene, OR) for 10 min at room temperature. Mitochondrial membrane potential ($\Delta\Psi_m$) measurements were performed using MoFloXDP flow cytometer (Beckman Coulter) equipped with an argon laser 488 nm. TMRM signal was analyzed in the FL2 channel, equipped with band pass filter 575 ± 25 nm. Approximately 23,000 cells were used for each measurement. Data were acquired and analyzed in log scale using Summit 5.2 software (Beckman

Coulter). Arithmetic mean values of TMRM fluorescence signal in arbitrary units were calculated for each condition for graphic representation (Floryk and Houstek, 1999).

4.16 Cell viability assay: Cell viability was determined using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), a colorimetric assay. HUVEC seeded in 96-well plates (1 x 10^4/well) cultured for 24 h until 70-80% confluence. Cells were incubated with oxLDL for 24-96 h with or without FTI-277. Media were replaced by fresh medium containing 0.5 mg/ml of MTT, and incubation was continued for 2 h. After 2 h incubation at 37˚C the MTT solution was removed, and the insoluble formazan crystals formed were dissolved in 200 µl of dimethyl sulfoxide (DMSO, Sigma). The absorbance was measured at 570 nm using 96-well plate FLUOstar Optima (BMG, Germany).

4.17 Streptozotocin (STZ)-diabetic mice model: Male C57BL/6J (C57) mice were obtained from the Jackson Laboratory (Bar Harbor, Maine, USA). Mice were placed in stainless steel cages in an air-conditioned room and received regular rodent chow after arrival at 4 weeks of age. Diabetes was induced with STZ (Sigma) intra peritoneal injection (150 mg/kg body wt) at 9 weeks of age. Control mice received equal volumes of sodium citrate (pH 4.5) via the identical route. Hearts and blood were collected 3 weeks after STZ or vehicle injection (n = 5 animals/group). Tissue specimens were frozen immediately after harvest and stored at -70˚C. Targeted proteins in heart homogenate were determined using Western blotting. Plasma was freshly isolated for PAI-1 and

glucose analysis. The protocol of the animal experiments was approved by the Animal Protocol Management and Review Committee in the University of Manitoba.

4.18 Statistical analysis: Data were expressed as mean ± standard error mean (SEM). The differences between two groups were evaluated by Student's t-test. Comparisons among multiple treatment groups were done with the use of one-way analysis of variance (ANOVA), followed by post-hoc Newman-Keul's test. All statistical analyses were performed using Graphpad Prism 4 (GraphPad Software, San Diego, CA, USA), with $p<0.05$ taken as significant.

5. Results

5.1 Signaling pathway for oxLDL-induced PAI-1 in EC

5.1.1 Effects of oxLDL on PAI-1 protein and mRNA: Previous studies from our group demonstrated that oxLDL significantly increased the release of PAI-1 from HUVEC or HCAEC (Ren et al., 2000). The present study investigated the effects of oxLDL (50-150 μg/ml) for 12-48 h on PAI-1 protein and mRNA levels in HUVEC. Treatment with 100 μg/ml of oxLDL for 24 h showed the maximal increase of PAI-1 protein or mRNA in EC **(Figure 5A-5C)**. Incubation with oxLDL at 100 μg/ml for 24 h induced significantly greater increase in cell-associated PAI-1 in HUVEC and HCAEC compared to F-oLDL or unmodified LDL ($p<0.05$). The biological properties of oxLDL *in-vitro* appear to be dependent on the degree to which the lipid and protein components are oxidized. The involvement of copper oxidation of LDL *in-vivo* is controversial. Markedly elevated levels of iron – most of it apparently low molecular weight ion complexes have been detected in advanced carotid lesions compared to healthy control samples. F-oLDL is a type of oxLDL modified by a physiologically relevant concentration of iron ion compared to *in-vitro* prepared oxLDL using copper ion. (Watson et al., 1995). The increased expression of PAI-1 in HCAEC after LDL, oxLDL or F-oLDL treatment was comparable to that in HUVEC **(Figure 6)**.

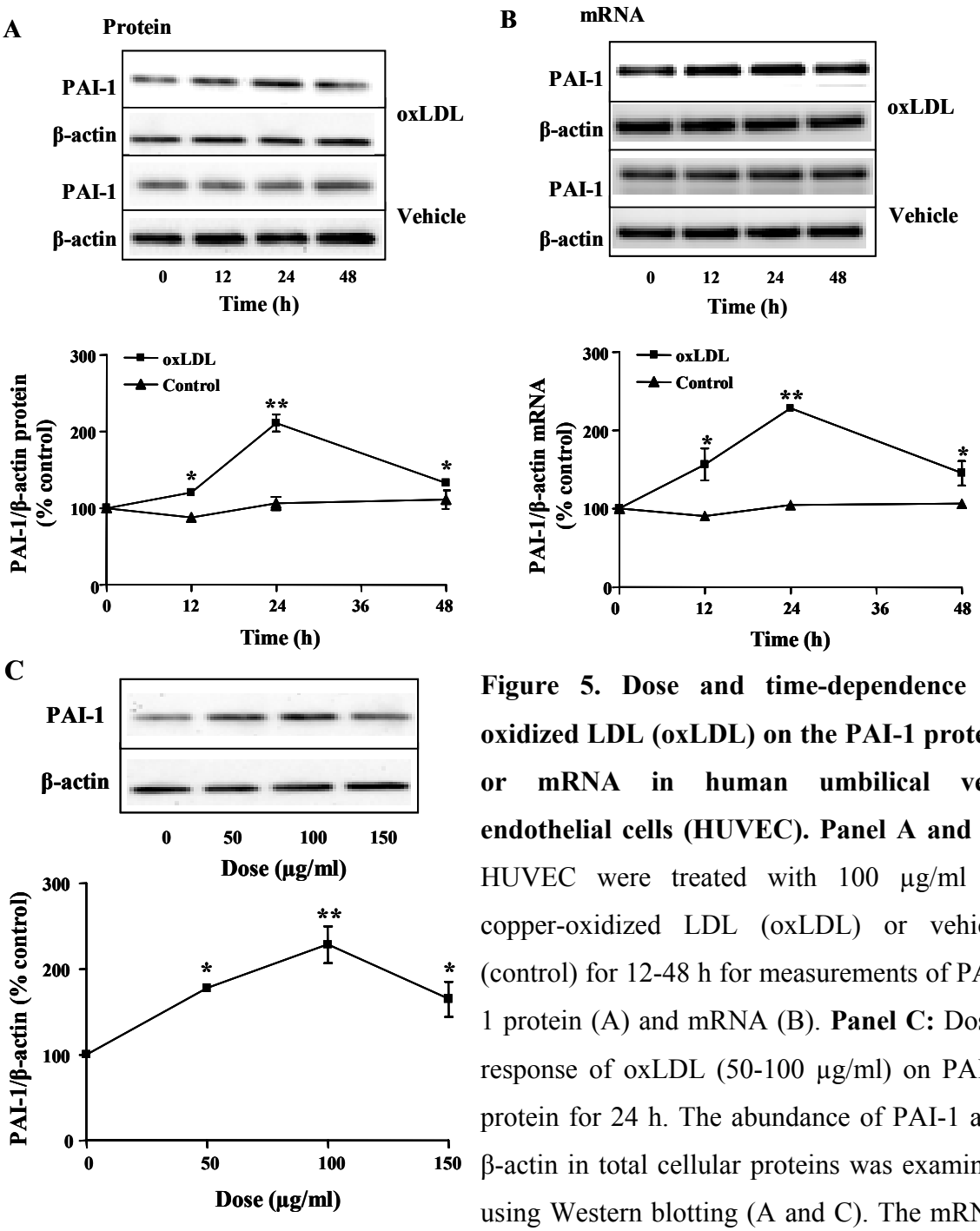


Figure 5. Dose and time-dependence of oxidized LDL (oxLDL) on the PAI-1 protein or mRNA in human umbilical vein endothelial cells (HUVEC). Panel A and B: HUVEC were treated with 100 μg/ml of copper-oxidized LDL (oxLDL) or vehicle (control) for 12-48 h for measurements of PAI-1 protein (A) and mRNA (B). **Panel C:** Dose-response of oxLDL (50-100 μg/ml) on PAI-1 protein for 24 h. The abundance of PAI-1 and β-actin in total cellular proteins was examined using Western blotting (A and C). The mRNA level of PAI-1 and β-actin was measured using RT-PCR (B). Integrative data were expressed in the percentage of controls after normalization with β-actin (mean ± SEM, n = 3 experiments). *, **: $p<0.05$ or 0.01 versus control.

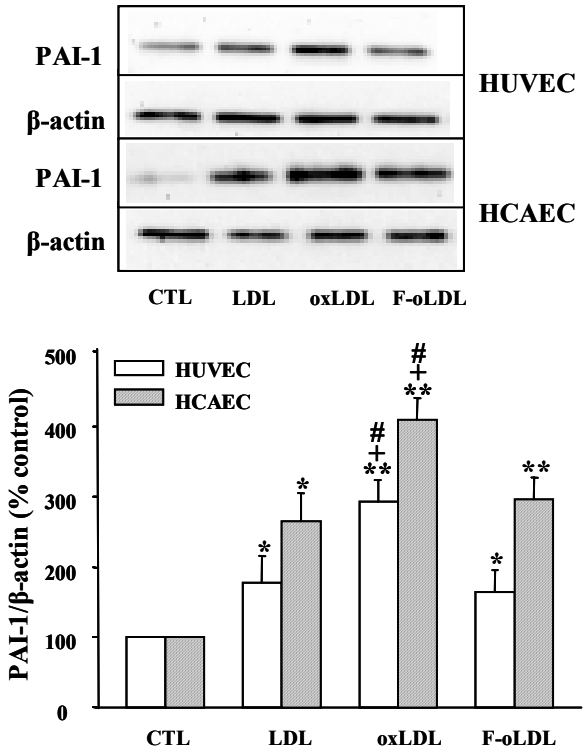


Figure 6. Effects of oxLDL on PAI-1 protein in HUVEC and human coronary artery endothelial cells (HCAEC). HUVEC or HCAEC were treated with vehicle, 100 μg/ml of LDL, oxLDL or FeSO4 modified LDL (F-oLDL) for 24 h. The abundance of PAI-1 and β-actin in total cellular proteins was examined using Western blotting. Integrative data were expressed in the percentage of controls after normalization with β-actin (mean ± SEM, n = 3 experiments). *, **: $p<0.05$ or 0.01 versus control; +: $p<0.05$ versus LDL; #: $p< 0.05$ versus F-oLDL.

5.1.2 LOX-1 mediates oxLDL-induced increase of PAI-1 in EC: Treatment with oxLDL or F-oLDL significantly increased the abundance of LOX-1 in HUVEC and HCAEC compared to vehicle (**Figure 7A**, $p<0.05$). To investigate the involvement of LOX-1 in oxLDL-induced upregulation of PAI-1 in EC, we used a blocking antibody against LOX-1. HUVEC were preincubated with LOX-1 antibody (10 μg/ml) for 30 min, and then exposed to oxLDL or LDL (100 μg/ml) for 24 h. LOX-1 antibody prevented the effect of oxLDL on the upregulation of PAI-1 in EC ($p<0.05$), and partially inhibited LDL-induced increase of PAI-1 in EC **(Figure 7B)**. LOX-1 antibody had no effect on the basal level of PAI-1. The results suggest that LOX-1 mediates, at least in part, cross-membrane signaling of oxLDL-induced increase of PAI-1 in EC.

5.1.3 Effect of LDLR antibody on oxLDL-induced PAI-1 in EC: The protein level of LDLR in EC was suppressed by LDL, oxLDL or F-oLDL treatment as expected **(Figure 8A)**. The effect of LDLR blocking antibody on oxLDL-induced PAI-1 is not known. HUVEC were pretreated with LDLR blocking antibody (5 μg/ml) for 30 min and then exposed to oxLDL or LDL (100 μg/ml) for 24 h. LDLR blocking antibody did not significantly affect LDL or oxLDL-induced PAI-1 expression in EC **(Figure 8B)**. This suggests that LDLR does not mediate LDL or oxLDL-induced PAI-1 protein expression in EC.

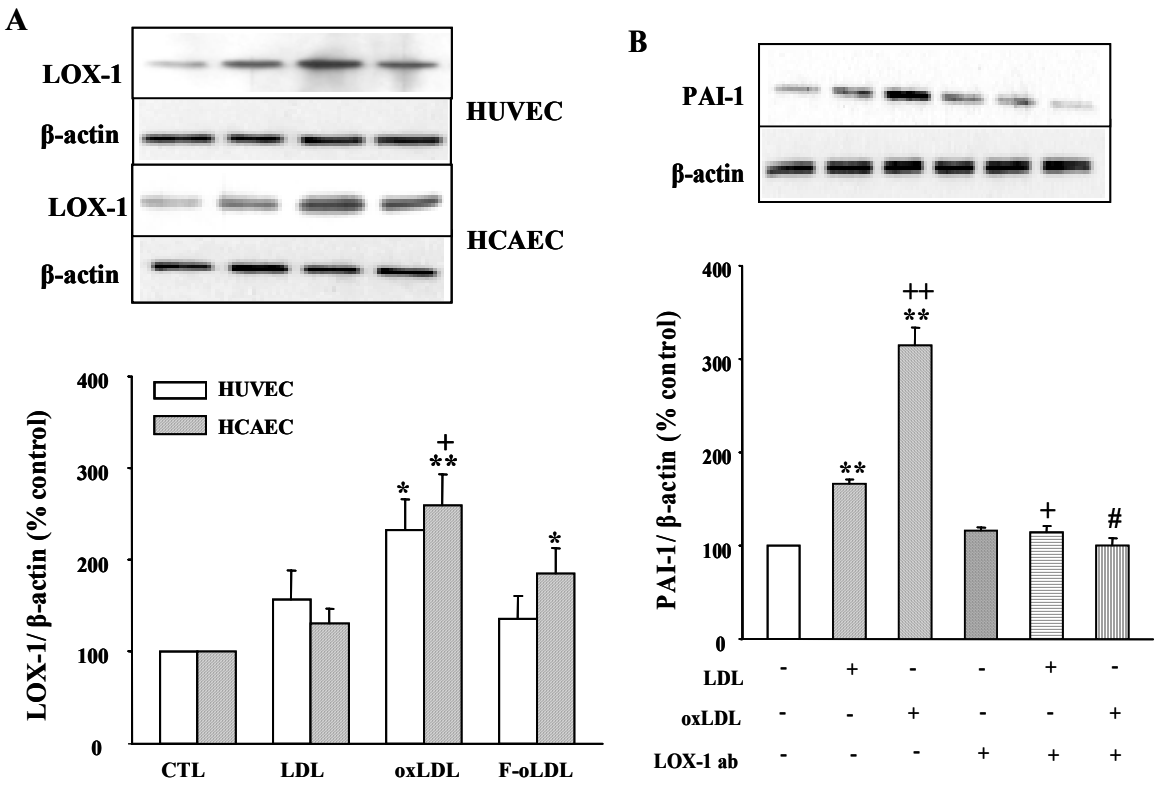


Figure 7. Involvement of lectin-like oxidized LDL receptor-1 (LOX-1) in oxLDL-induced upregulation of PAI-1 in EC. Panel A: HUVEC and HCAEC were treated with vehicle, 100 μg/ml of LDL, oxLDL or F-oLDL for 24 h. **Panel B:** HUVEC were preincubated with LOX-1 blocking antibody (LOX-1 ab) for 30 minute at 10 μg/ml and then exposed to 100 μg/ml of LDL or oxLDL for 24 h. The protein expressions of LOX-1, PAI-1 or β-actin were detected using Western blotting. Values are expressed in percentage of controls after normalization with β-actin (mean ± SEM, n = 3 experiments). *, **: $p<0.05$ or 0.01 versus control; +, ++: $p<0.05$ or 0.01 versus LDL; #: $p<0.05$ versus oxLDL.

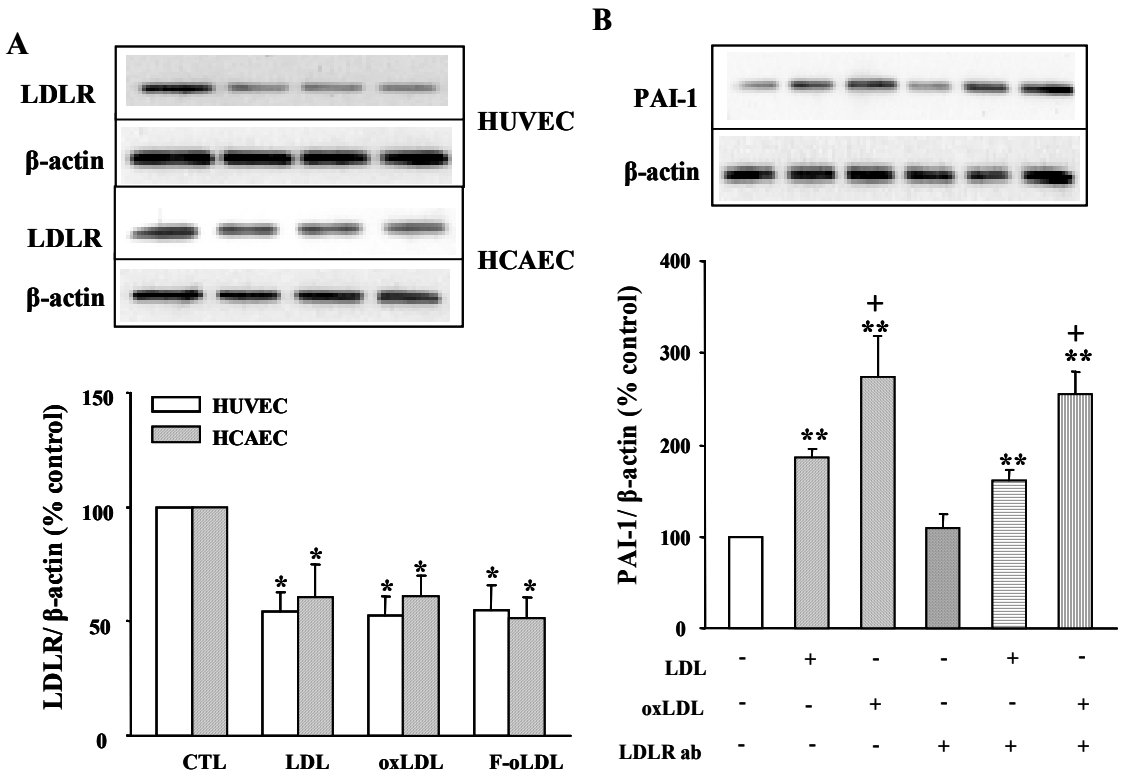


Figure 8. Effects of LDLR antibody on oxLDL-induced increase of PAI-1 abundance in EC. Panel A: HUVEC or HCAEC were treated with vehicle, 100 μg/ml of LDL, oxLDL or F-oLDL for 24 h. **Panel B:** HUVEC were pretreated with LDLR blocking antibody (LDLR ab) for 30 minute at 5 μg/ml and then exposed to 100 μg/ml of LDL or oxLDL for 24 h. The abundance of LDLR, PAI-1 or β-actin was detected using Western blotting. Values are expressed in percentage of controls after normalization with β-actin (mean ± SEM, n = 3 experiments). *, **: $p<0.05$ or 0.01 versus control; +: $p<0.05$ versus LDL.

5.1.4 Effect of oxLDL on H-Ras translocation and protein or mRNA expression in EC: Previous studies demonstrated that LDL significantly increased H-Ras abundance in the membrane fraction of EC (Zhu et al., 2001). To investigate whether oxLDL affects Ras translocation within EC, HUVEC were exposed to oxLDL (100 μg/ml) for 20 min. The expression of H-Ras in membrane fraction was significantly increased by oxLDL at 20 min interval compared to control ($p<0.05$). β-actin, an intracellular cytoskeletal protein, was used as a loading control for membrane H-Ras. We did not detect obvious change in H-Ras expression in whole-cell lysate under similar conditions **(Figure 9A)**. However, prolonged incubation with oxLDL (24 h) significantly increased H-Ras expression in EC. Further we examined the effect of FTI-277 on oxLDL-induced H-Ras protein expression after 24 h of treatment. The results demonstrated that FTI-277 inhibited LDL or oxLDL-induced H-Ras expression in HUVEC (**Figure 9B**, $p<0.05$). To determine the effect of oxLDL on H-Ras mRNA level, HUVEC were treated with 100 μg/ml of LDL or oxLDL for 24 h. The level of H-Ras mRNA was significantly increased in EC treated with oxLDL. The image for mRNA gel have been optically inverted (**Figure 9C**, $p<0.05$).

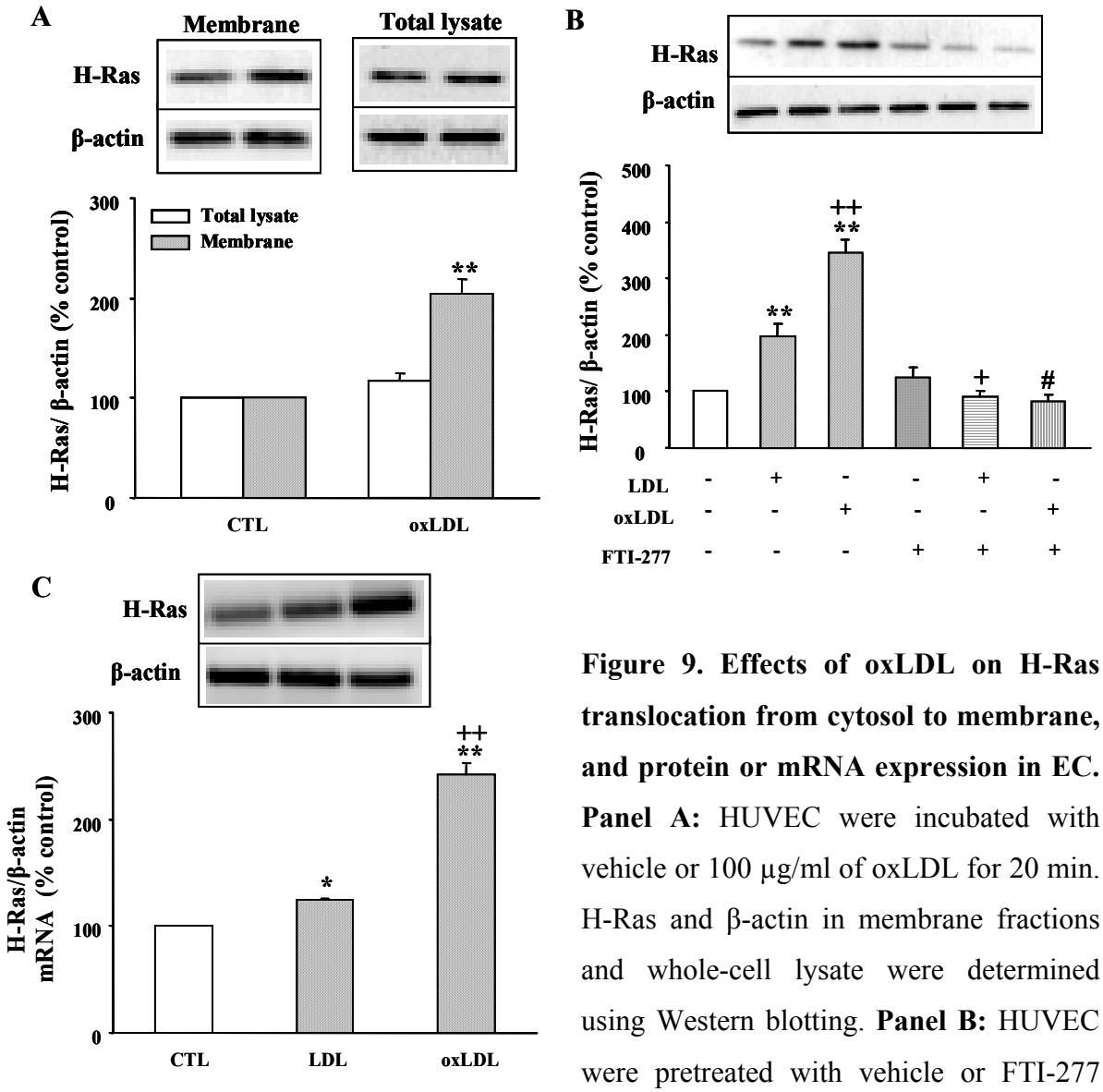


Figure 9. Effects of oxLDL on H-Ras translocation from cytosol to membrane, and protein or mRNA expression in EC. Panel A: HUVEC were incubated with vehicle or 100 μg/ml of oxLDL for 20 min. H-Ras and β-actin in membrane fractions and whole-cell lysate were determined using Western blotting. **Panel B:** HUVEC were pretreated with vehicle or FTI-277 then incubated with 100 μg/ml of LDL, oxLDL or vehicle for 24 h. H-Ras and β-actin in cellular proteins were determined using Western blotting. **Panel C:** HUVEC were treated with 100 μg/ml of LDL, oxLDL or vehicle (control or CTL) for 24 h. The mRNA levels of H-Ras and β-actin were measured using RT-PCR. The mRNA gel image was optically inverted. Values are expressed in percentage of controls after normalization with β-actin (mean ± SEM, n = 3 experiments). *, **: $p<0.05$ or 0.01 versus control; +, ++: $p<0.05$ or 0.01 versus LDL; #: $p<0.05$ versus oxLDL.

5.1.5 Involvement of H-Ras in oxLDL-induced upregulation of PAI-1 in EC: To determine the significance of H-Ras in oxLDL-induced PAI-1, HUVEC were preincubated with FTI-277 (10 μM), a farnesyltransferase inhibitor, for 30 min and then exposed to 100 μg/ml of LDL or oxLDL for 24 h. The expression of cell-associated PAI-1 and β-actin were measured using Western blotting, and the levels of PAI-1 release were examined in post-cultural medium of HUVEC. FTI-277 inhibited oxLDL or LDL-induced abundance of PAI-1 in EC (**Figure 10A**, $p<0.05$). The level of PAI-1 was significantly increased in the media of EC treated with oxLDL compared to LDL or vehicle. Incubation with FTI-277 prevented oxLDL or LDL-induced increase of PAI-1 antigen in the media of EC. The basal levels of PAI-1 were also reduced by FTI-277 treatment (**Figure 10B**, $p<0.05$).

5.1.6 Impact of H-Ras siRNA on oxLDL-induced upregulation of PAI-1: To examine the direct involvement of H-Ras in oxLDL-induced upregulation of PAI-1 in EC, we used siRNA targeting H-Ras mRNA. H-Ras siRNA blocked oxLDL or LDL-induced increases of H-Ras and PAI-1 protein in HUVEC (**Figure 11**, $p<0.05$). In EC transfected with H-Ras siRNA but without an addition of lipoproteins, the abundance of both H-Ras and PAI-1 was inhibited ($p<0.05$). SiRNA against H-Ras did not evidently affect the abundance of β-actin in EC with or without lipoprotein treatment. SiRNA for β-actin or scramble siRNA did not evidently affect the level of PAI-1 in EC. Above results suggest that H-Ras is an essential upstream mediator of oxLDL-induced PAI-1 expression in EC.

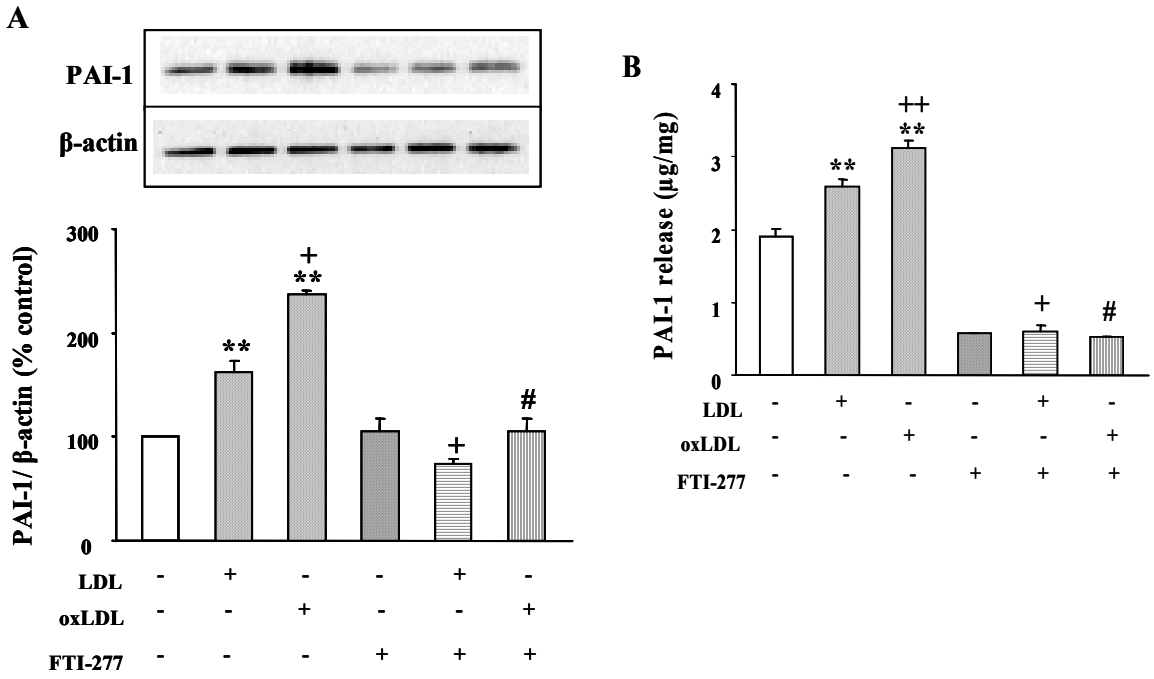


Figure 10. Inhibition of H-Ras farnesylation regulates oxLDL-induced PAI-1 in EC. Panel A: HUVEC were pretreated with vehicle or FTI-277 (10 μM) for 30 min and then stimulated with 100 μg/ml of LDL, oxLDL or vehicle for 24 h. PAI-1 and β-actin protein expressions were determined using Western blotting. **Panel B:** HUVEC were treated with FTI-277, and LDL or oxLDL as described in A. The levels of PAI-1 antigen in the post-cultural media of EC were measured using enzyme-linked immunosorbent assay kits. Values are expressed in percentage of controls after normalization with β-actin (mean ± SEM, n = 3 experiments). **: $p<0.01$ versus control; +, ++: $p<0.05$ or 0.01 versus LDL; #: $p<0.05$ versus oxLDL.

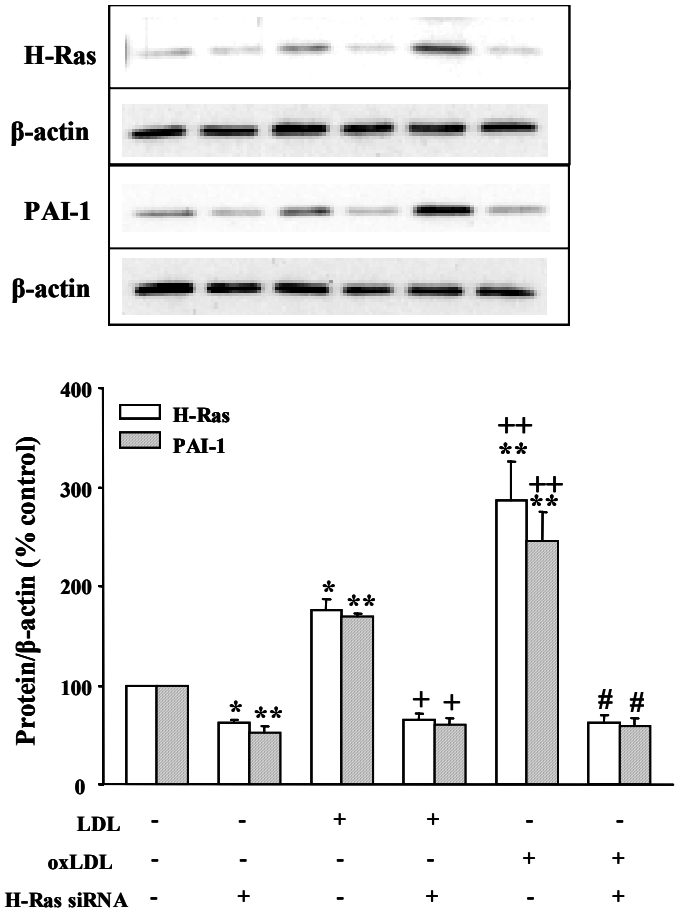


Figure 11. Effect of small interference RNA (siRNA) against H-Ras on oxLDL-induced H-Ras and PAI-1 expression in EC. HUVEC transfected with H-Ras siRNA for 48 h (first 7 h in serum-free medium and remaining time in the presence of 10% serum) followed by stimulation with vehicle (control) or with an addition of 100 μg/ml LDL or oxLDL for 24 h. H-Ras, PAI-1 and β-actin in cellular proteins were detected using Western blotting. Values are expressed in percentage of controls after normalization with β-actin (mean ± SEM, n = 3 experiments). *, **: $p<0.05$ or 0.01 versus control; +, ++: $p<0.05$ or 0.01 versus LDL; #: $p<0.05$ versus oxLDL.

5.1.7 Involvement of LOX-1 in oxLDL-induced H-Ras expression in EC: Relationship between LOX-1 and H-Ras has not been studied. In order to determine the interactions between LOX-1 and H-Ras induced by oxLDL, HUVEC were pretreated with LOX-1 blocking antibody (10 μg/ml) for 30 min and then exposed to 100 μg/ml of oxLDL or LDL for 24 h. LOX-1 antibody partially, but significantly, inhibited the effect of oxLDL or LDL on the expression of H-Ras in HUVEC (**Figure 12**, $p<0.05$). The results demonstrate that LOX-1 mediates the effects on H-Ras in EC induced by oxLDL.

5.1.8 Effects of oxLDL on phosphorylated Raf-1 (pRaf-1) in EC: Raf-1 is considered as one of the important downstream effectors of H-Ras signal transduction. The effects of LDL or oxLDL (100 μg/ml) on pRaf-1 were determined through incubations for 2-120 min in HUVEC. The maximum increase of pRaf-1 induced by oxLDL was detected at 10 min compared to baseline or LDL at the same condition ($p<0.05$). LDL-induced a delayed increase of pRaf-1 expression at 60 min compared to baseline (**Figure 13A**, $p<0.05$). F-oLDL at 100 μg/ml for 10 min significantly increased Raf-1 phosphorylation in HUVEC and HCAEC compared to control ($p<0.05$), but the effect of F-oLDL in HUVEC was weaker than that seen for oxLDL (**Figure 13B,** $p<0.05$). The findings suggest that oxidized LDLs induce greater phosphorylation of Raf-1 more rapidly than LDL.

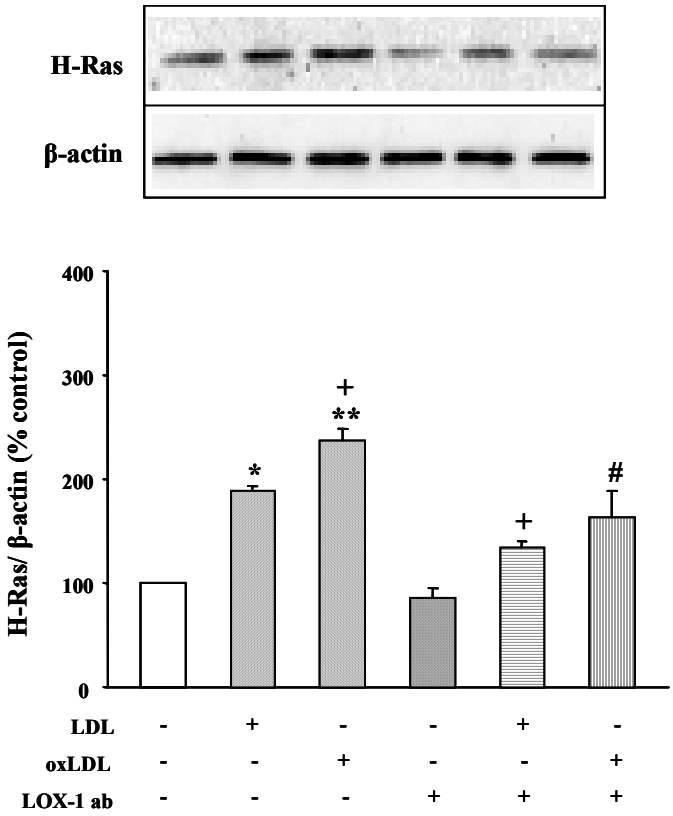


Figure 12. Effect of LOX-1 antibody on oxLDL-induced H-Ras expression in EC. HUVEC were pretreated with LOX-1 antibody (ab) (10 μg/ml) for 30 min and then exposed to 100 μg/ml of LDL or oxLDL for 24 h. H-Ras and β-actin expression in cellular proteins was determined using Western blotting. Values are presented in percentage of control after normalization with β-actin protein (mean ± SEM, n = 3 experiments). *, **: $p<0.05$ or 0.01 versus control; +: $p<0.05$ versus LDL; #: $p<0.05$ versus oxLDL.

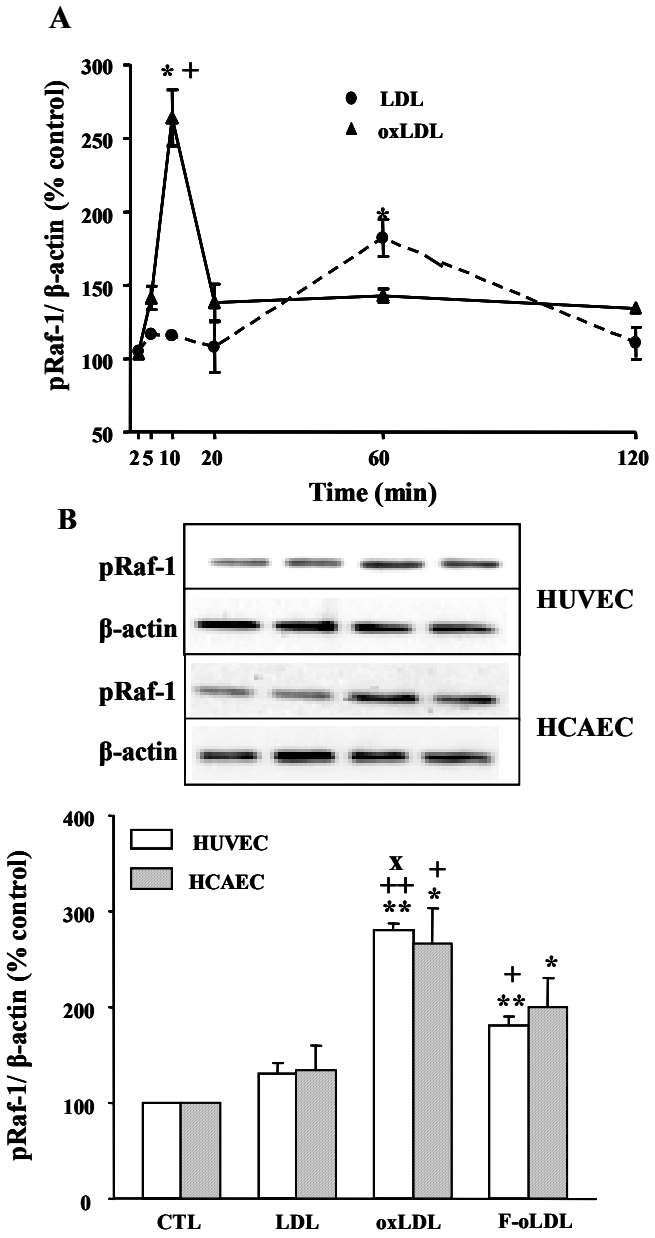


Figure 13. Effect of oxLDLs on Raf-1 phosphorylation in EC. Panel A: Time course for LDL or oxLDL-induced Raf-1 phosphorylation in HUVEC. HUVEC were treated with 100 µg/ml of LDL or oxLDL for 2-120 min. **Panel B:** HUVEC or HCAECs were incubated with LDL, oxLDL or F-oLDL (100 µg/ml) for 10 min. The abundance of pRaf-1 or β-actin in total cellular proteins was analyzed using Western blotting. Values are presented in percentage of controls after normalization with β-actin (mean ± SEM, n = 3 experiments). *, **: $p<0.05$ or 0.01 versus control; +, ++: $p<0.05$ or 0.01 versus LDL; x: $p<0.05$ versus F-oLDL.

5.1.9 Effect of Raf-1 inhibitor (GW 5074) on oxLDL-induced pRaf-1 or PAI-1 in EC: To investigate the effect of Raf-1 inhibitor on oxLDL-induced pRaf-1, HUVEC were preincubated with Raf-1 inhibitor (1 μM) for 30 min and then exposed to 100 μg/ml of oxLDL for 10 min or LDL for 60 min. Raf-1 phosphorylation induced by oxLDL or LDL was significantly suppressed by Raf-1 inhibitor (**Figure 14A and 14B**, $p<0.05$). Previous studies from our laboratory demonstrated that oxLDL (100 μg/ml for 24 h) significantly increased the steady state of mRNA level of PAI-1 or the release of PAI-1 from EC (Ma et al., 2006; Ren et al., 2000). To determine the involvement of Raf-1 in oxLDL-induced PAI-1 at mRNA level, HUVEC were preincubated with Raf-1 inhibitor (1 μM) for 30 min and then stimulated with 100 μg/ml of oxLDL or LDL for 24 h. Raf-1 inhibitor significantly reduced the level of PAI-1 mRNA induced by oxLDL or LDL (**Figure 14C**, $p<0.05$). The results suggest that Raf-1 activation is essential for oxLDL-induced PAI-1 production in EC at mRNA level.

5.1.10 Effects of oxLDL on ERK-1/2 activation in EC: Several lines of evidence have shown that ERK-1/2 is a common downstream signaling effector of Raf-1. Previous studies demonstrated activation of ERK-1/2 in vascular cells treated with oxLDL (Yang et al., 2001). The present study demonstrated time-dependence of ERK-1/2 activation in EC induced by oxLDL, HUVEC were treated with oxLDL or LDL (100 μg/ml) for 5-120 min. Treatment with oxLDL resulted in a peak of ERK-1/2 phosphorylation at 20 min. LDL significantly increased ERK-1/2 phosphorylation after 60 min of incubation **(Figure 15A)**. F-oLDL induced increase in ERK-1/2 phosphorylation that was similar to that induced by oxLDL in HUVEC and HCAEC (**Figure 15B**, $p<0.05$).

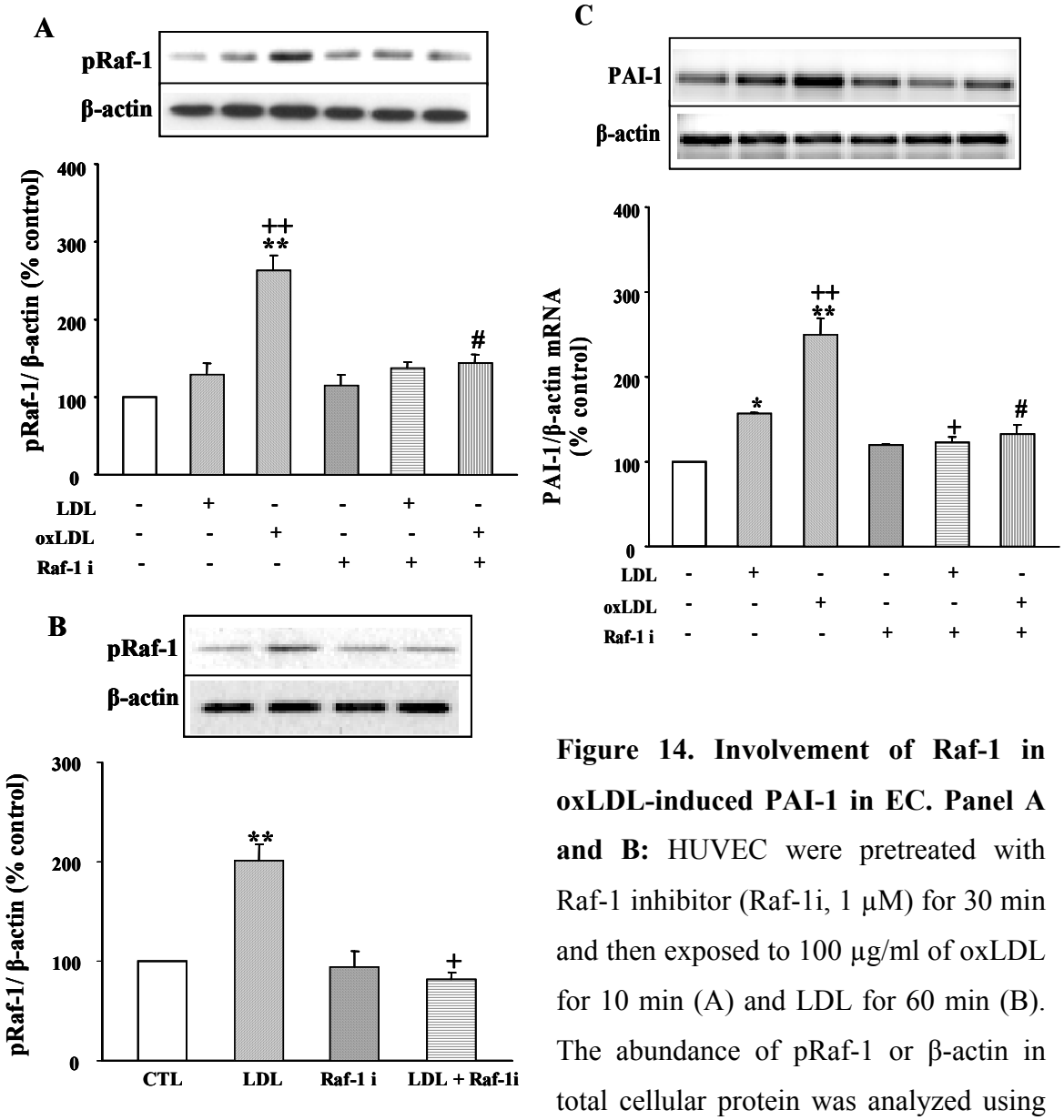


Figure 14. Involvement of Raf-1 in oxLDL-induced PAI-1 in EC. Panel A and B: HUVEC were pretreated with Raf-1 inhibitor (Raf-1i, 1 μM) for 30 min and then exposed to 100 μg/ml of oxLDL for 10 min (A) and LDL for 60 min (B). The abundance of pRaf-1 or β-actin in total cellular protein was analyzed using Western blotting. **Panel C:** EC were pretreated with vehicle or Raf-1 inhibitor (Raf-1i, 1 μM) for 30 min and then exposed to 100 μg/ml of LDL, oxLDL or vehicle for 24 h. PAI-1 and β-actin mRNA level was measured using RT-PCR. Values are presented in percentage of control after normalization with β-actin protein or mRNA (mean ± SEM, n = 3 experiments). *, **: $p<0.05$ or 0.01 versus control; +: $p<0.05$ versus LDL; #: $p<0.05$ versus oxLDL.

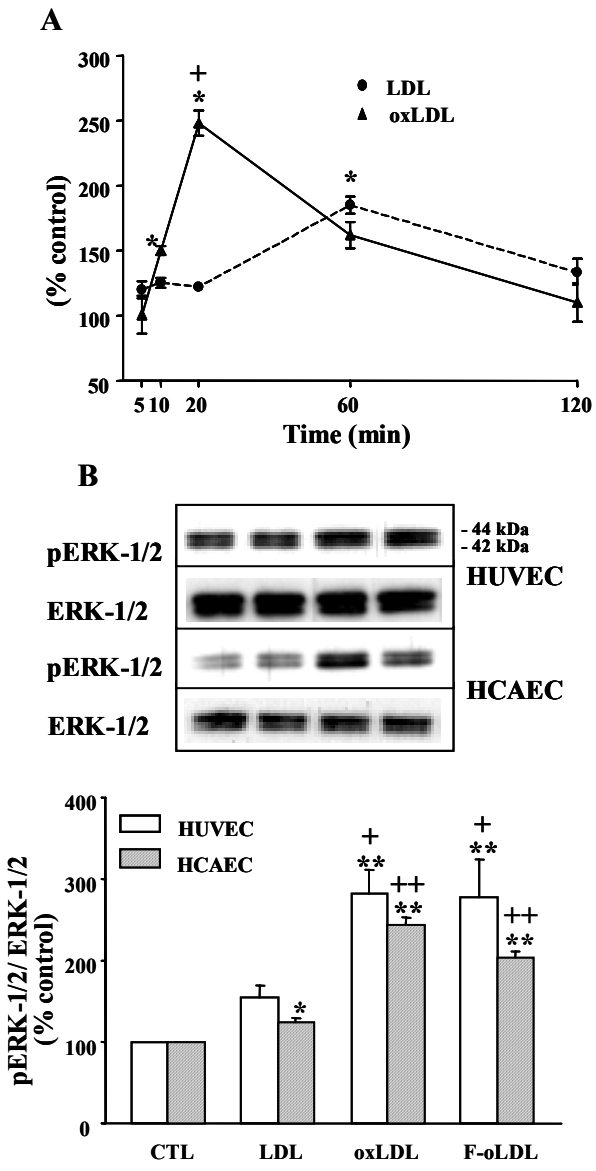


Figure 15. Effects of oxLDL on ERK-1/2 phosphorylation in EC. Panel A: Time course for LDL or oxLDL-induced ERK-1/2 phosphorylation in HUVEC. HUVEC were treated with vehicle, 100 µg/ml of LDL or oxLDL for 5-120 min. **Panel B:** HUVEC or HCAEC were treated with vehicle, LDL, oxLDL or F-oLDL (100 µg/ml) for 20 min. The abundances of pERK-1/2 and ERK-1/2 in total cellular proteins were analyzed using Western blotting. Values are presented in percentage of controls after normalization with total ERK-1/2 or β-actin (mean ± SEM, n = 3 experiments). *, **: $p<0.05$ or 0.01 versus control; +, ++: $p<0.05$ or 0.01 versus LDL.

5.1.11 Involvement of ERK-1/2 in oxLDL-induced increase of PAI-1: The increase in ERK-1/2 phosphorylation induced by 100 μg/ml of oxLDL (20 min) or LDL (60 min) was significantly inhibited by ERK-1/2 inhibitor, PD98059 (10 μM) in EC (**Figure 16A and 16B**, $p<0.05$). Treatment with PD98059 inhibited LDL or oxLDL (100 μg/ml for 24 h) induced PAI-1 expression in EC (**Figure 16C**, $p<0.05$). The results suggest that ERK-1/2 is required for oxLDL-induced PAI-1 production in EC.

5.1.12 Effect of antioxidant on oxLDL-induced H_2O_2 release: Previous studies in our group demonstrated that treatment with oxLDL significantly increased ROS release from EC (Zhao and Shen, 2005). The present study investigated effect of 80 μmol/L butylated hydroxytoluene (BHT), a potent antioxidant, on oxLDL-induced H_2O_2 in EC. The levels of H_2O_2 in the post-culture medium of EC treated with BHT-oxLDL were significantly lower than in cultures treated with oxLDL without the presence of BHT (**Figure 17**, $p<0.05$).

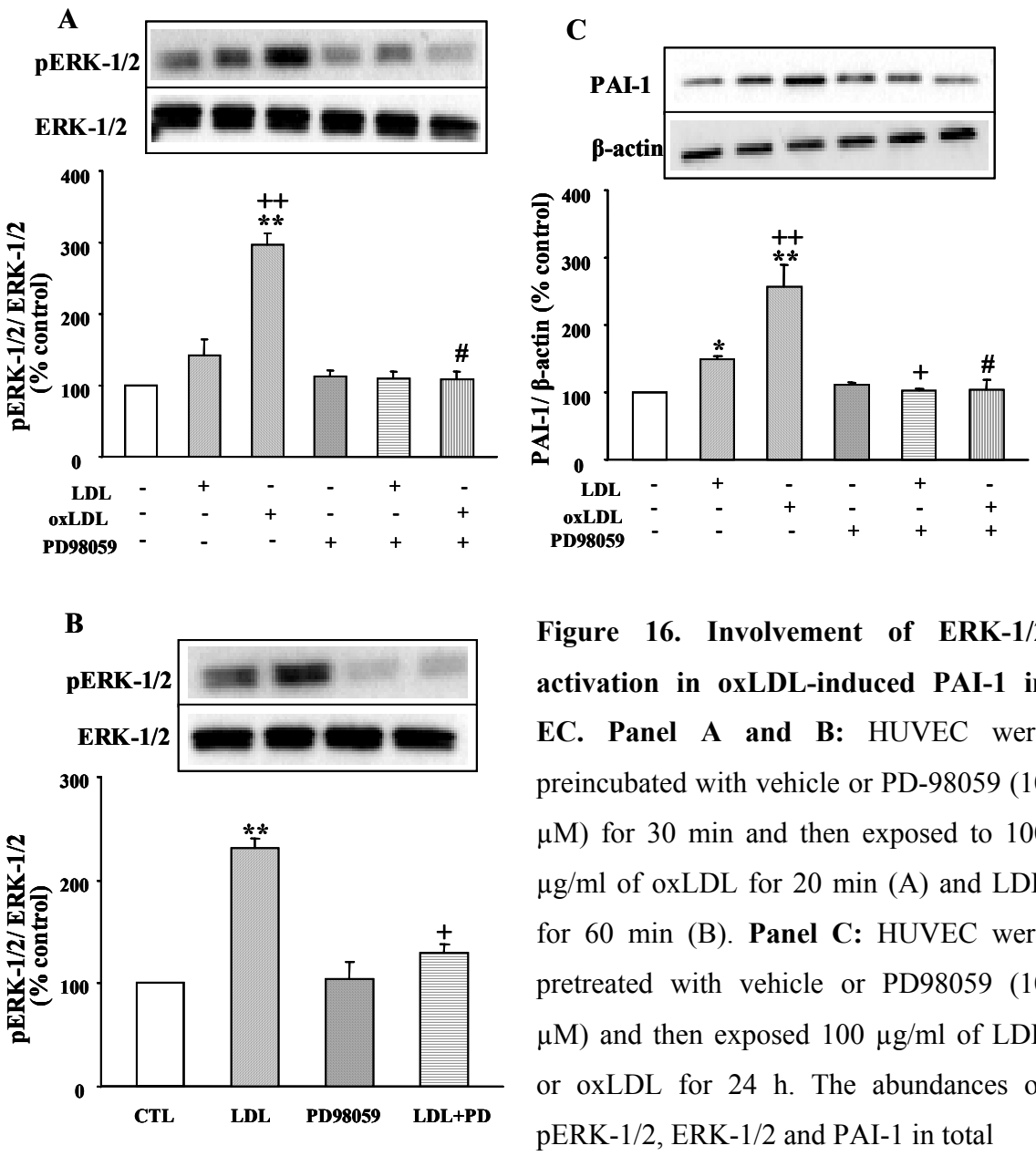


Figure 16. Involvement of ERK-1/2 activation in oxLDL-induced PAI-1 in EC. Panel A and B: HUVEC were preincubated with vehicle or PD-98059 (10 μM) for 30 min and then exposed to 100 μg/ml of oxLDL for 20 min (A) and LDL for 60 min (B). **Panel C:** HUVEC were pretreated with vehicle or PD98059 (10 μM) and then exposed 100 μg/ml of LDL or oxLDL for 24 h. The abundances of pERK-1/2, ERK-1/2 and PAI-1 in total cellular proteins were analyzed using Western blotting. Values are presented in percentage of controls after normalization with total ERK-1/2 or β-actin (mean ± SEM, n = 3 experiments). *, **: $p<0.05$ or 0.01 versus control; +, ++: $p<0.05$ or 0.01 versus LDL; #: $p<0.05$ versus oxLDL.

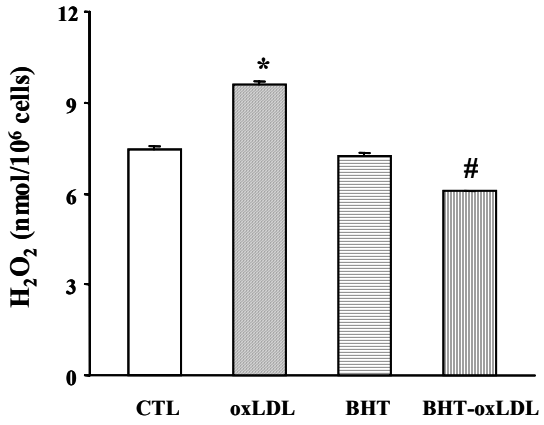


Figure 17. Effect of butylated hydroxytoluene (BHT) on oxLDL-induced hydrogen peroxide (H_2O_2) release from EC. HUVEC were treated with vehicle (CTL), 100 µg/ml oxLDL, 80 µmol/l BHT or 100 µg/ml BHT-oxLDL for 2 h. H_2O_2 release in media were determined as described in the Methods. Values are presented in nmol/10^6 cells (mean ± SEM, n = 3 experiments). *: $p<0.05$ versus control; #: $p<0.05$ versus oxLDL.

5.2 Transmembrane signaling mechanisms involved in glyLDL-induced expression of HSF1 and PAI-1 in EC

5.2.1 Involvement of RAGE in glyLDL-induced PAI-1 in EC: Previous studies by our group demonstrated that treatment with glyLDL (100 μg/ml) for 24 h increased the generation and upregulation of PAI-1 in EC (Ren and Shen, 2000; Zhao and Shen, 2007). To investigate the effect of glyLDL on RAGE expression, HUVEC and HCAEC were treated with glyLDL (100 μg/ml) for 24 h. Incubation with glyLDL significantly increased the abundance of RAGE in EC compared to LDL or vehicle (**Figure 18A**, $p<0.05$). To determine the involvement of RAGE in glyLDL-induced PAI-1, HUVEC were pretreated with RAGE blocking antibody or control goat IgG (10 μg/ml) for 30 min and incubated with glyLDL (100 μg/ml) for 24 h. RAGE antibody prevented glyLDL-induced upregulation of PAI-1 in EC (**Figure 18B**, $p<0.05$). Control goat IgG had no effect on glyLDL-induced PAI-1 expression in EC **(Figure 18C)**. The results suggest that RAGE mediates glyLDL-induced increase of PAI-1 in EC.

5.2.2 Effects of glyLDL on H-Ras translocation or protein expression in EC: To examine effects of glyLDL on H-Ras translocation, HUVEC were treated with glyLDL (100 μg/ml) for 5-60 min **(Figure 19A)**. The translocation of H-Ras to membrane was detected as early as 5 min and reached a peak at 15 min. The abundance of H-Ras in membrane fraction was significantly increased by glyLDL compared to LDL or control after 15 min (**Figure 19B**, $p<0.05$). Furthermore, we examined effect of glyLDL on H-Ras expression in whole-cell lysates, no evident difference in H-Ras was detected in EC treated with glyLDL for 15 min interval (**Figure 19B**). The effects of glyLDL

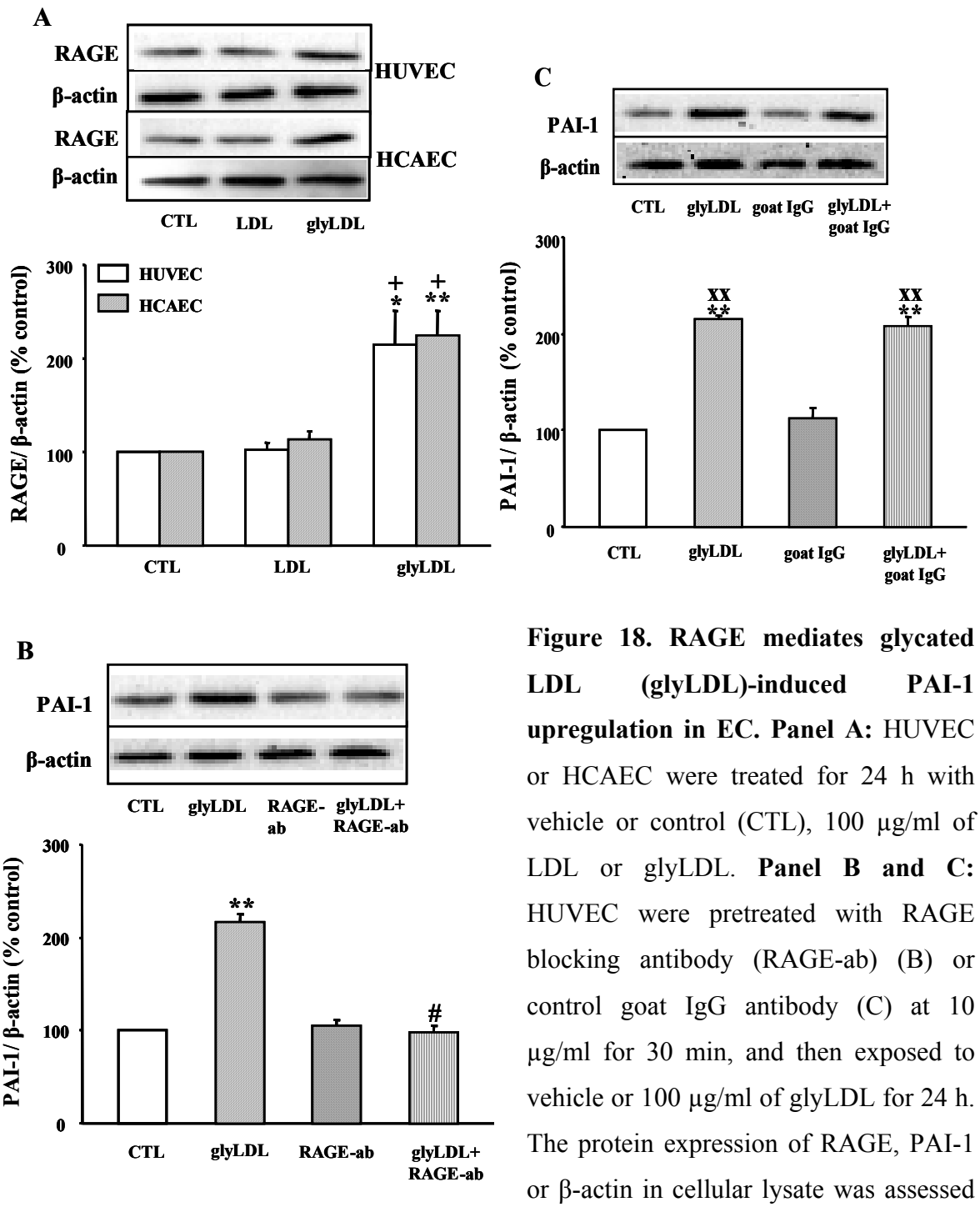


Figure 18. RAGE mediates glycated LDL (glyLDL)-induced PAI-1 upregulation in EC. Panel A: HUVEC or HCAEC were treated for 24 h with vehicle or control (CTL), 100 μg/ml of LDL or glyLDL. **Panel B and C:** HUVEC were pretreated with RAGE blocking antibody (RAGE-ab) (B) or control goat IgG antibody (C) at 10 μg/ml for 30 min, and then exposed to vehicle or 100 μg/ml of glyLDL for 24 h. The protein expression of RAGE, PAI-1 or β-actin in cellular lysate was assessed using Western blotting.

Values are expressed in percentage of controls after normalization with β-actin (mean ± SEM, n = 3 experiments). *, **: $p<0.05$ or 0.01 versus control; +: $p<0.05$ versus LDL; #: $p<0.05$ versus glyLDL; xx: $p<0.01$ versus goat IgG.

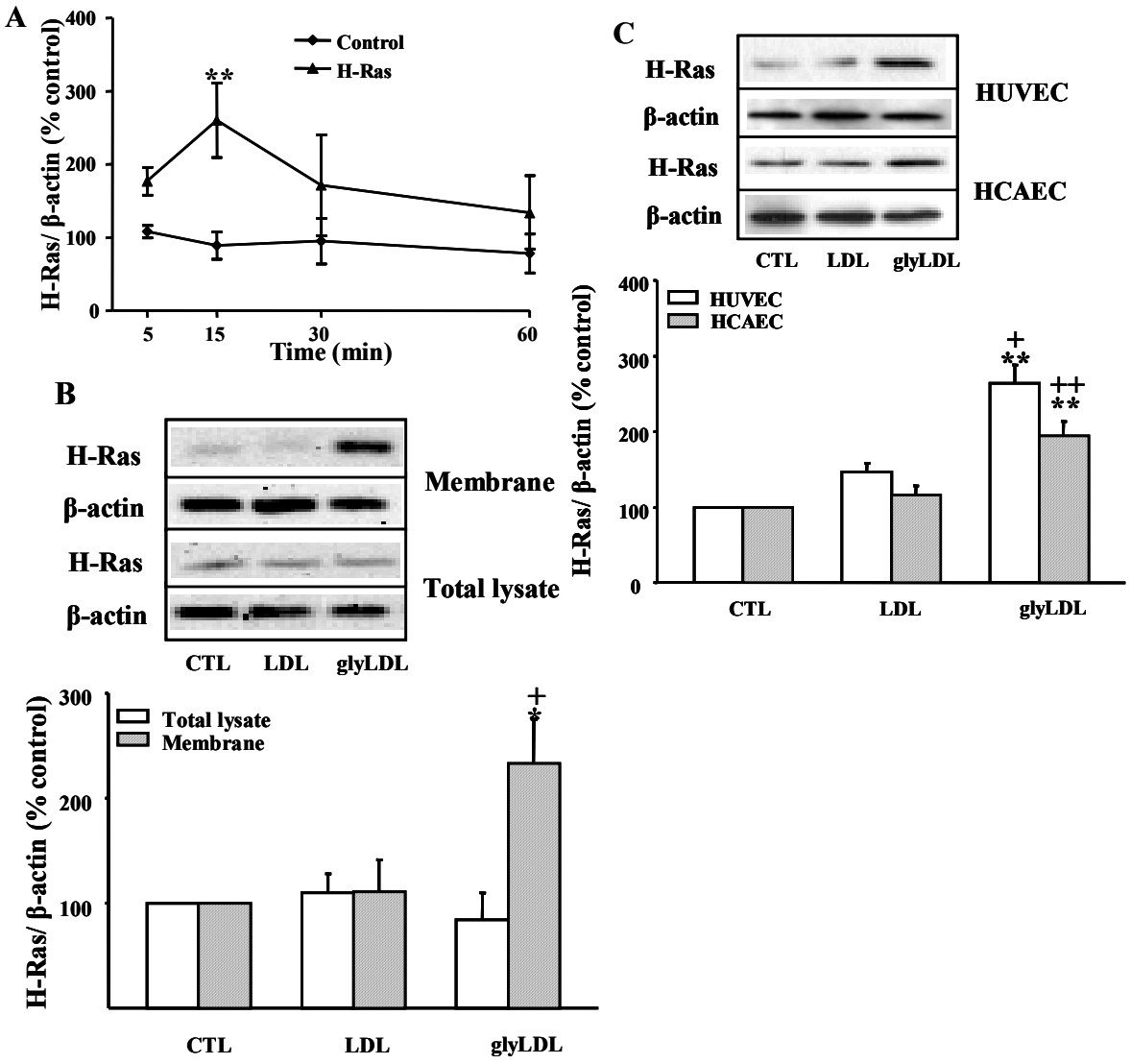


Figure 19: Effects of glyLDL on H-Ras translocation or protein expression in EC. **Panel A:** Time course of glyLDL-induced H-Ras membrane translocation in HUVEC. Cells were treated with 100 µg/ml of glyLDL or vehicle control for 5-60 min. **Panel B:** HUVEC were cultured with vehicle, 100 µg/ml of LDL or glyLDL for 15 min. Abundance of H-Ras and β-actin in membrane fractions and whole-cell lysate was determined using Western blotting. **Panel C:** HUVEC were treated for 24 h with vehicle or control (CTL), 100 µg/ml of LDL or glyLDL. The abundance of H-Ras at protein level was detected using Western blotting. Values are expressed in percentage of controls after normalization with β-actin (mean ± SEM, n = 3 experiments). *, **: $p<0.05$ or 0.01 versus control; +, ++: $p<0.05$ or 0.01 versus LDL.

(100 μg/ml) for 24 h were studied on the abundance of H-Ras in HUVEC and HCAEC. GlyLDL significantly increased the abundance of H-Ras protein in both HUVEC and HCAEC compared to LDL or control after a 24 h of treatment (**Figure 15C**, $p<0.05$). The results suggest that glyLDL rapidly increase H-Ras translocation and elevated the content of H-Ras in EC after prolonged incubation.

5.2.3 Interactions between H-Ras, HSF1 or PAI-1 induced by glyLDL: H-Ras is considered as an important upstream messenger mediating signaling between membrane receptor and downstream kinases. Previous studies from our group demonstrated that HSF1 mediates glyLDL-induced PAI-1 upregulation in EC (Zhao and Shen, 2007). To examine whether H-Ras is involved in glyLDL-induced increase of HSF1 or PAI-1, HUVEC were preincubated with FTI-277 (20 μM), a farnesyltransferase inhibitor, for 30 min and then exposed to 100 μg/ml of LDL or glyLDL for 24 h for PAI-1 detection or 6 h for HSF1 detection. FTI-277 inhibited glyLDL or LDL-induced increase in cell-associated HSF1 or PAI-1 in EC (**Figure 20A**, $p<0.05$). Moreover, interactions between H-Ras, HSF1 or PAI-1 induced by glyLDL were verified using H-Ras-specific siRNA. HUVEC were transfected with H-Ras siRNA or transfection reagent alone, and then exposed to LDL or glyLDL for 6 h (HSF1) or 24 h (PAI-1). H-Ras siRNA inhibited glyLDL or LDL-induced increases of HSF1 and PAI-1 in EC (**Figure 20B**, $p<0.05$). In HUVEC transfected with H-Ras siRNA without an addition of LDL or glyLDL, the levels of cell-associated HSF1 and PAI-1 were inhibited ($p<0.05$). H-Ras siRNA did not evidently alter the abundance of β-actin in EC with or without lipoprotein treatment. Scramble siRNA did not evidently affect the level of HSF1 or PAI-1 in EC (**Figure 24B**).

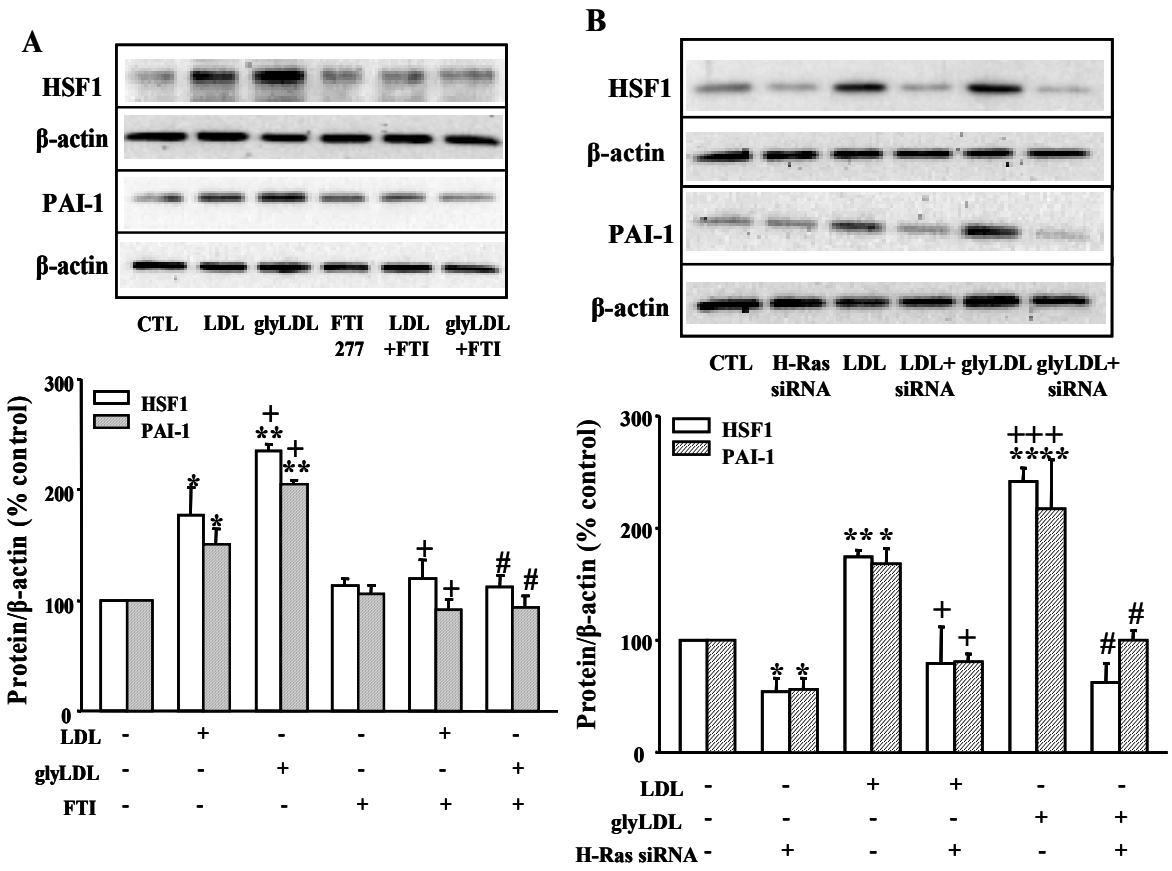


Figure 20: Involvement of H-Ras in glyLDL-induced HSF1 or PAI-1 in EC. Panel A: HUVEC were preincubated with vehicle or 20 μM FTI-277 (FTI) for 30 min and then exposed to 100 μg/ml of LDL, glyLDL or vehicle (CTL) for 6 h (HSF1) or 24 h (PAI-1). HSF1, PAI-1 and β-actin in cellular proteins were determined using Western blotting. **Panel B:** HUVEC were transfected with siRNA against H-Ras or transfection reagent alone for 48 h (first 7 h in serum-free medium and remaining time in the presence of 10% serum) and treated with vehicle (CTL) or with an addition of 100 μg/ml LDL or glyLDL for 6 h (HSF1) or 24 h (PAI-1). The protein abundance of HSF1, PAI-1 and β-actin was analyzed using Western blotting. Values are expressed in percentage of controls after normalization with β-actin (mean ± SEM, n = 3 experiments). *, **: $p<0.05$ or 0.01 versus control; +, ++: $p<0.05$ versus LDL; #: $p<0.05$ versus glyLDL.

The results indicate that H-Ras regulates glyLDL-induced HSF1 and PAI-1 expression in EC.

5.2.4 Effect of glyLDL on NOX2 in EC: Previous studies demonstrated that the activation of RAGE induced by AGE increased NOX activity in EC (Wautier et al., 2001). The dose and time-dependence of glyLDL (25-150 μg/ml) on the expression of NOX2 was characterized in EC for up to 2 h compared with vehicle control. Treatment with glyLDL (100 μg/ml) for 1 h reached the maximal increase in the abundance of NOX2 in HUVEC **(Figure 21A and 21B)**. The abundance of NOX2 induced by glyLDL was increased in HUVEC or HCAEC compared to LDL or control (**Figure 21C**, $p<0.05$). The results for the first time demonstrated that glyLDL increased the expression of a NOX subunit in EC.

5.2.5 NOX mediates glyLDL-induced H-Ras translocation and upregulation of HSF1 or PAI-1: The relationship between NOX2 and H-Ras has not been known. To investigate the involvement of NOX in H-Ras activation, we examined the effect of NOX inhibitor (DPI) on the translocation of H-Ras in HUVEC induced by glyLDL. The results demonstrated that DPI significantly reduced the membrane translocation of H-Ras in EC induced by glyLDL (**Figure 22A**, $p<0.05$). To examine the role of NOX in glyLDL-induced expression of HSF1 and PAI-1, HUVEC were pretreated with DPI and then exposed to LDL or glyLDL at optimized condition (Zhao and Shen, 2007). Treatment with DPI significantly reduced the abundance of NOX2, HSF1 and PAI-1 in EC induced by glyLDL or LDL (**Figure 22B**, $p<0.05$).

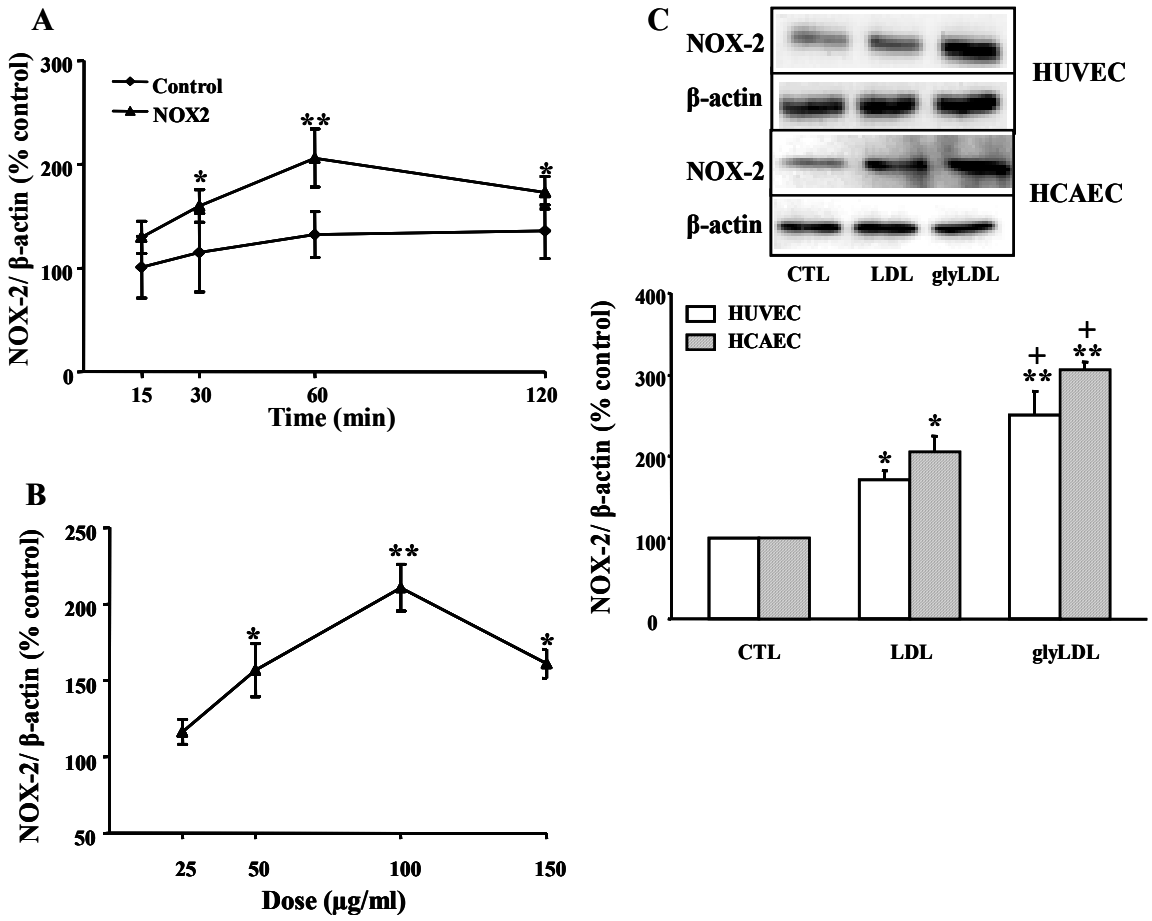


Figure 21. Dose and time-dependence of glyLDL on NOX2 expression in HUVEC. Panel A and B: HUVEC were treated with 100 µg/ml of glyLDL or control for 15-120 min (A), or 25-150 µg/ml of glyLDL or vehicle control (CTL) for 1 h (B). **Panel C:** HUVEC or HCAEC were treated with vehicle (CTL), 100 µg/ml of LDL or glyLDL for 1 h. The abundance of NOX2 or β-actin in EC was determined using Western blotting. Values are presented in percentage of control after normalization with β-actin protein (mean ± SEM, n = 3 experiments). *, **: $p<0.05$ or 0.01 versus control; +: $p<0.05$ LDL.

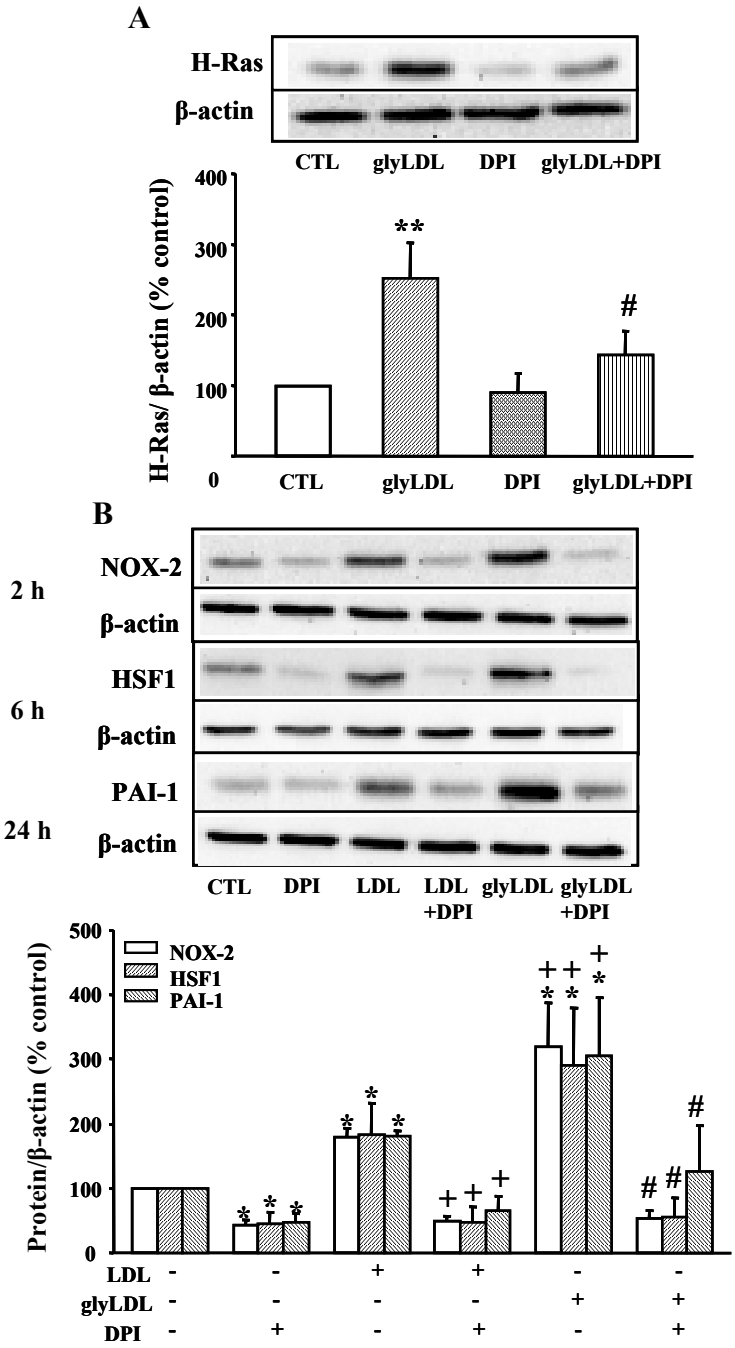


Figure 22. Involvement of NOX in glyLDL-induced H-Ras translocation and upregulation of HSF1 or PAI-1. Panel A: HUVEC were pretreated with diphenyleneiodonium chloride (DPI, 10 μM) for 4 h, and then stimulated with 100 μg/ml of glyLDL for 15 min. Abundance of H-Ras and β-actin in membrane fractions was determined using Western blotting. **Panel B:** HUVEC were pretreated with DPI, and then stimulated with 100 μg/ml of LDL, glyLDL or vehicle for 1 h (NOX2), 6 h (HSF1) or 24 h (PAI-1). NOX2, HSF1, PAI-1 and β-actin in cellular proteins were determined using Western blotting. Values are presented in percentage of control after normalization with β-actin protein (mean ± SEM, n = 3 experiments). *, **: $p<0.05$ or 0.01 versus control; +: $p<0.05$ versus LDL; #: $p<0.05$ versus glyLDL.

5.2.6 Effect of NOX inhibitor on glyLDL-induced ROS production: Previous studies in our laboratory demonstrated that glyLDL increased ROS generation from EC (Zhao and Shen, 2005). To examine whether NOX is involved in glyLDL-induced ROS production, EC were preincubated with NOX inhibitor (DPI) and then exposed to 100 μg/ml of LDL or glyLDL for 2 h. DPI inhibited increased levels of H_2O_2 in the culture media of EC induced by LDL or glyLDL (**Figure 23**, $p<0.05$).

5.2.7 Involvement of $p22^{phox}$ in glyLDL-induced expression of NOX2, HSF1 and PAI-1 in EC: $p22^{phox}$ is an essential component of NOX complexes and regulate NOX activity in cell. The involvement of NOX in glyLDL-induced upregulation of HSF1 and PAI-1 in EC was verified using $p22^{phox}$ siRNA. SiRNA against $p22^{phox}$ blocked the increase of the abundance of $p22^{phox}$, NOX2, HSF1 and PAI-1 in HUVEC induced by glyLDL or LDL. In EC transfected with $p22^{phox}$, but without an addition of lipoprotein, the abundance of NOX2, HSF1 and PAI-1 was partially inhibited (**Figure 24A**, $p<0.05$). Scramble siRNA did not evidently affect the expression of the targeted proteins (**Figure 24B**). The results confirmed the involvement of NOX in glyLDL-induced HSF1 and PAI-1 expression in EC.

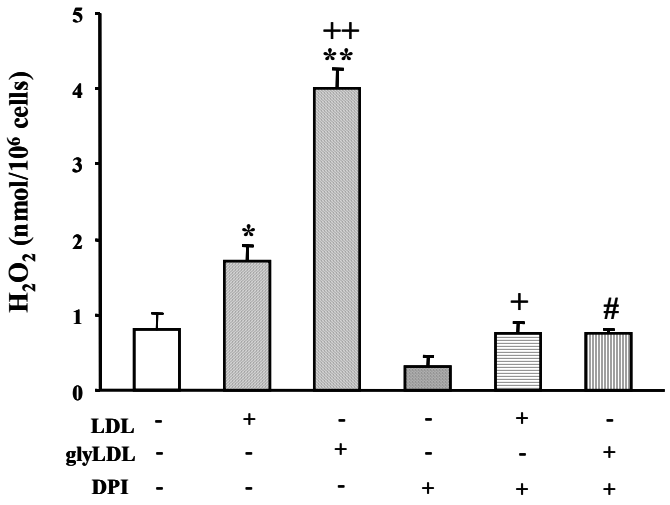


Figure 23. Involvement of NOX in glyLDL-induced H_2O_2 production in EC. HUVEC were preincubated with 10 μM DPI for 4 h and then exposed to vehicle, 100 μg/ml of LDL or glyLDL for 2 h. Hydrogen peroxide (H_2O_2) release in media was determined as described in Materials and Methods. Values are presented in percentage of control after normalization with β-actin protein (mean ± SEM, n = 3 experiments). *, **: $p<0.05$ or 0.01 versus control; +, ++: $p<0.05$ or 0.01 versus LDL; #: $p<0.05$ versus glyLDL.

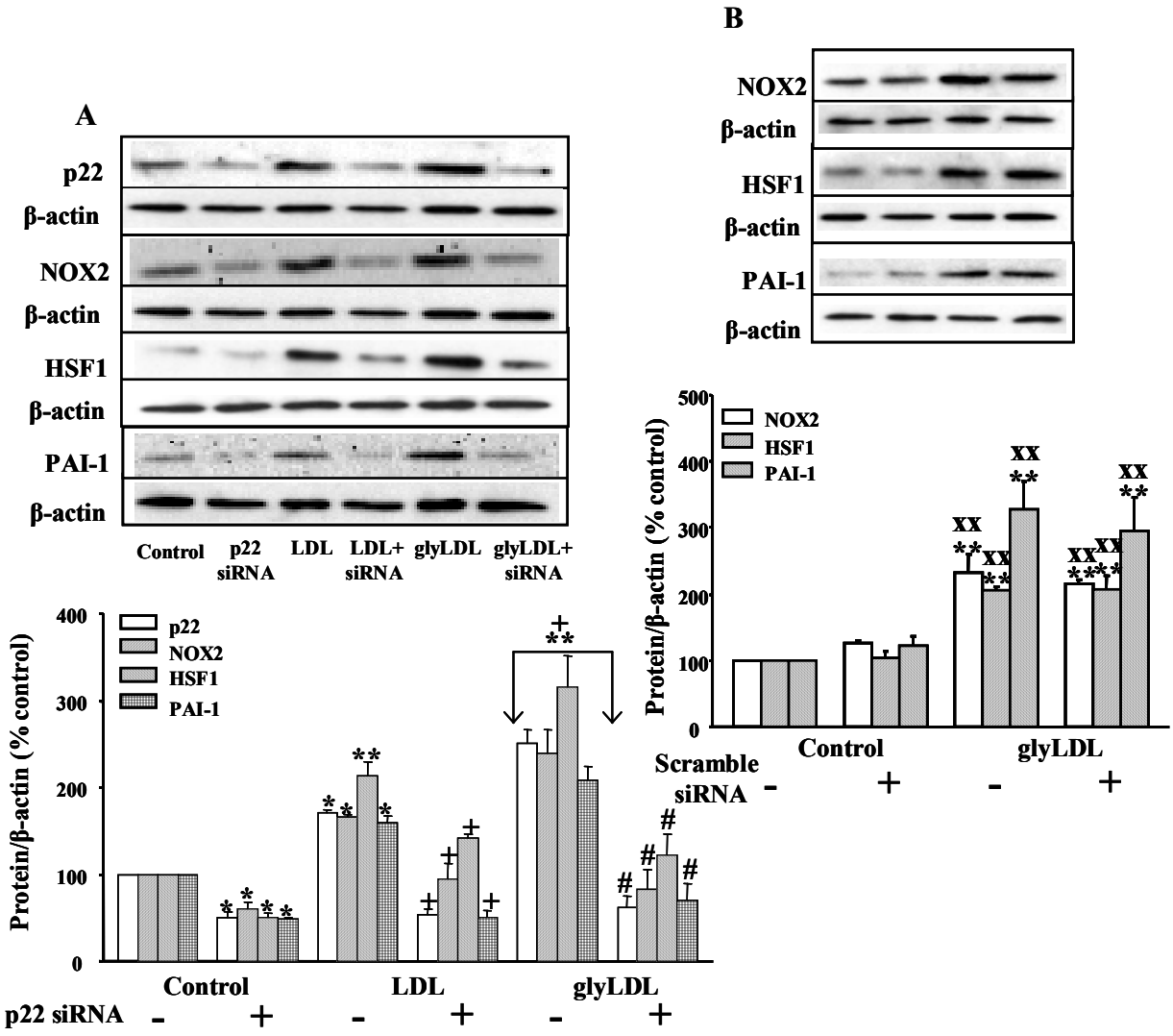


Figure 24. p22^{phox} mediates glyLDL-induced expression of NOX2, HSF1 and PAI-1 expression in EC. Panel A: The transfection of HUVEC were done with or without p22^{phox} (p22) siRNA for 7 h and then treated with vehicle (control), LDL or glyLDL (100 μg/ml) for 1 h (NOX2 and p22), 6 h (HSF1) or 24 h (PAI-1). **Panel B:** HUVEC were transfected with or without scramble siRNA for 7 h and then exposed to glyLDL for 1 h (NOX2), 6 h (HSF1) or 24 h (PAI-1). The protein expression of p22, NOX2, HSF1, PAI-1 or β-actin in EC was analyzed using Western blotting. Values are presented in percentage of control after normalization with β-actin protein (mean ± SEM, n = 3 experiments). *, **: $p<0.05$ or 0.01 versus control; +: $p<0.05$ versus LDL; #: $p<0.05$ versus glyLDL, xx: $p<0.01$ versus scramble siRNA.

5.2.8 Effect of glyLDL on pRaf-1: The present study examined time course of Raf-1 phosphorylation in EC induced by glyLDL (100 μg/ml) through incubations up to 60 min. The peak of increased abundance of pRaf-1 was detected at 5 min after the start of glyLDL treatment (**Figure 25A**). HUVEC were preincubated with Raf-1 inhibitor (1 μM) for 30 min and then exposed to 100 μg/ml of glyLDL or LDL for 5 min. LDL had no evident effect on the level of pRaf-1 at 5 min. Raf-1 inhibitor significantly inhibited pRaf-1 formation induced by glyLDL (**Figure 25B**, $p<0.05$). The findings suggest that glyLDL accelerated the phosphorylation of Raf-1 in EC compared to LDL.

5.2.9 Raf-1 regulates glyLDL-induced increase of PAI mRNA in EC: To investigate the involvement of Raf-1 phosphorylation in glyLDL-induced PAI-1, the effect of Raf-1 inhibitor was determined on glyLDL-induced PAI-1 mRNA using RT-PCR. HUVEC were preincubated with Raf-1 inhibitor (1 μM) for 30 min and then stimulated with 100 μg/ml of glyLDL or LDL for 24 h. Raf-1 inhibitor suppressed the level of PAI-1 mRNA induced by glyLDL or LDL (**Figure 26**, $p<0.05$). The results suggest that the Raf-1 is required for glyLDL-induced upregulation of PAI-1 mRNA in EC.

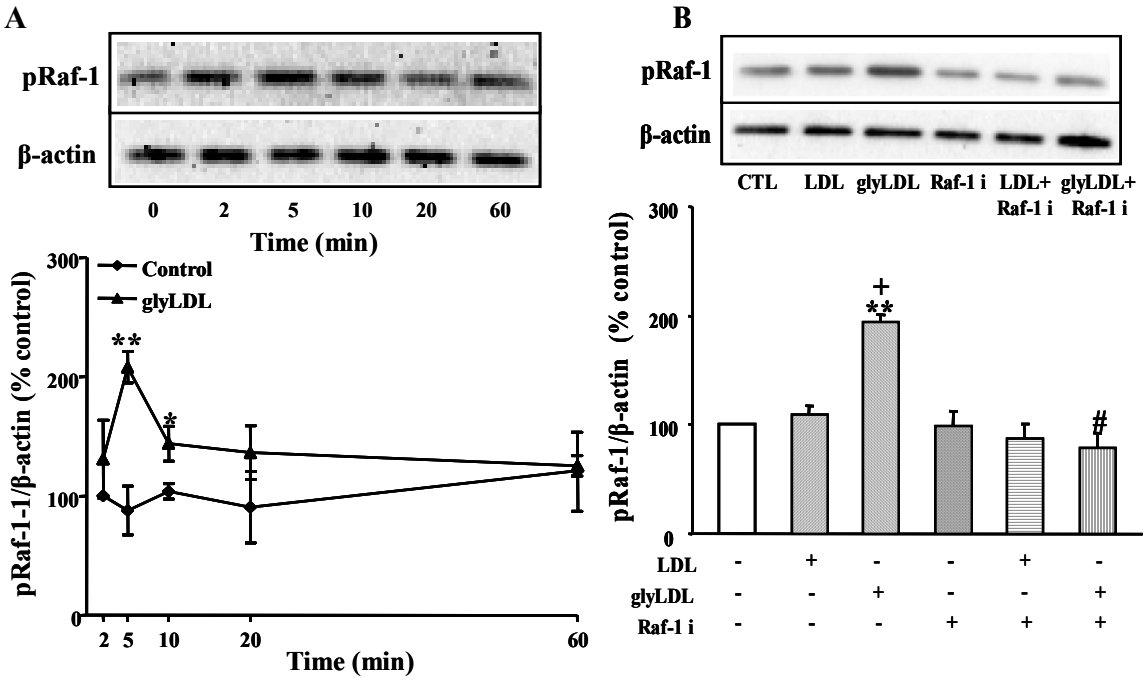


Figure 25. Effect of glyLDL on Raf-1 phosphorylation (pRaf-1) in EC. Panel A: Time course for glyLDL induced Raf-1 phosphorylation in HUVEC. Cells were treated with 100 μg/ml of glyLDL or vehicle control for 2-60 min. **Panel B:** HUVEC were pretreated with Raf-1 inhibitor (Raf-1i, 1 μM) for 30 min and then exposed to vehicle (CTL), 100 μg/ml of LDL or glyLDL for 5 min. The abundance of pRaf-1 or β-actin in total cellular proteins was analyzed using Western blotting. Values are presented in percentage of controls after normalization with β-actin protein (mean ± SEM, n = 3 experiments). *, **: $p<0.05$ or 0.01 versus control; +: $p<0.05$ versus LDL; #: $p<0.05$ versus glyLDL.

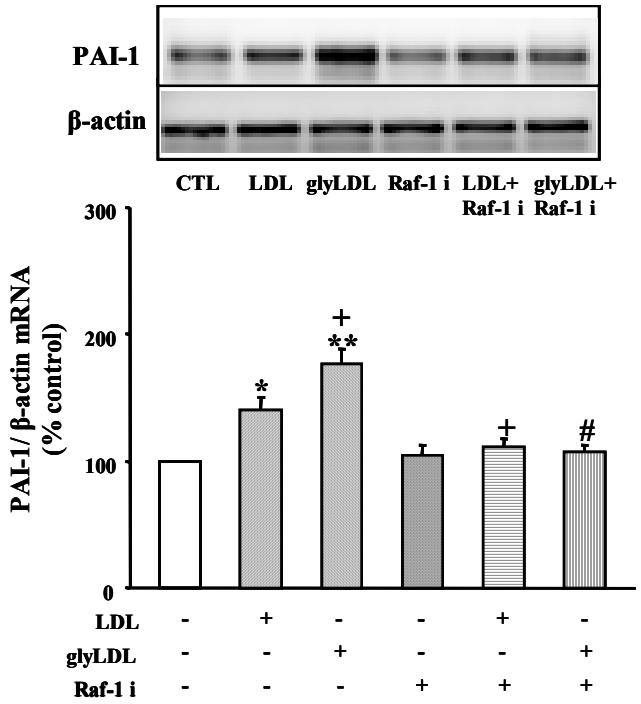


Figure 26. Involvement of Raf-1 in glyLDL-induced PAI-1 in EC. HUVEC were pretreated with vehicle or Raf-1 inhibitor (Raf-1i) (1 μM) for 30 min and then exposed to 100 μg/ml of LDL, glyLDL or vehicle for 24 h. PAI-1 and β-actin mRNA level was measured using reverse transcriptase-PCR. Values are presented in percentage of controls after normalization with β-actin mRNA (mean ± SEM, n = 3 experiments). *, **: $p<0.05$ or 0.01 versus control; +: $p<0.05$ versus LDL; #: $p<0.05$ versus glyLDL.

5.2.10 Effects of glyLDL on ERK-1/2 activation in EC: ERK-1/2, a key member of MAP kinase family, is a common downstream signaling effector of Raf-1. Previous studies demonstrated an activation of ERK-1/2 in vascular cells treated with oxLDL (Sangle et al., 2008; Yang et al., 2001). The present study demonstrated the time-dependence of ERK-1/2 activation in EC induced by glyLDL. HUVEC were treated with glyLDL or LDL (100 μg/ml) for 5-120 min. Treatment with glyLDL accelerated ERK-1/2 phosphorylation within 5 min and reached a peak at 15 min. LDL significantly increased ERK-1/2 phosphorylation after 60 min of incubation **(Figure 27)**.

5.2.11 Interactions between signaling mediators and PAI-1 in STZ-induced diabetes mice model: The role of RAGE, H-Ras, NOX, HSF1 and PAI-1 was studied in the hearts of STZ-diabetic mice. The protein expression of RAGE, H-Ras, NOX, HSF1 and PAI-1 in the hearts of diabetic mice was significantly increased compared to that in control mice (**Figure 28A**, $p<0.05$). Plasma levels of glucose were significantly increased in STZ-induced diabetic mice compared to control mice (**Figure 28B**). Significant increase in PAI-1 mRNA expression was detected in hearts of STZ-diabetic mice compared to control mice (**Figure 28C**, $p<0.05$). Plasma levels of PAI-1 were significantly increased in diabetic mice compared to control mice. The levels of glucose positively correlated with RAGE, NOX, H-Ras, HSF1 and PAI-1 in hearts of the mice (**Table 1A and B**, $r = 0.72$-0.96, $p<0.05$ or 0.01). The results suggest that the expression of RAGE, NOX, H-Ras, HSF1 and PAI-1 was increased in the cardiovascular tissue of diabetic mice, which supports our findings in cultured human EC treated with glyLDL.

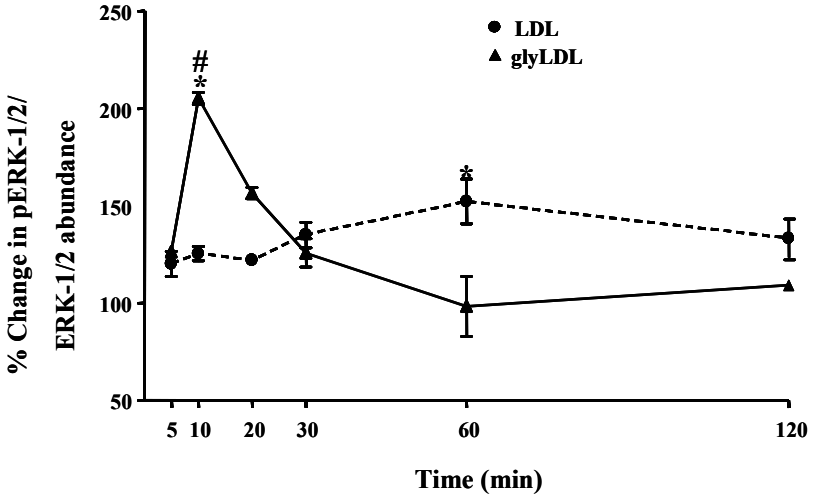


Figure 27: Effect of glyLDL on ERK-1/2 activation in EC. HUVEC were with vehicle, 100 µg/ml of LDL or glyLDL for 5-120 min. pERK-1/2 and ERK-1/2 abundance was measured using Western blotting. Values are presented in percentage of controls after normalization with ERK-1/2 protein (mean ± SEM, n = 3 experiments). *: $p<0.05$ versus control; #: $p<0.05$ versus LDL.

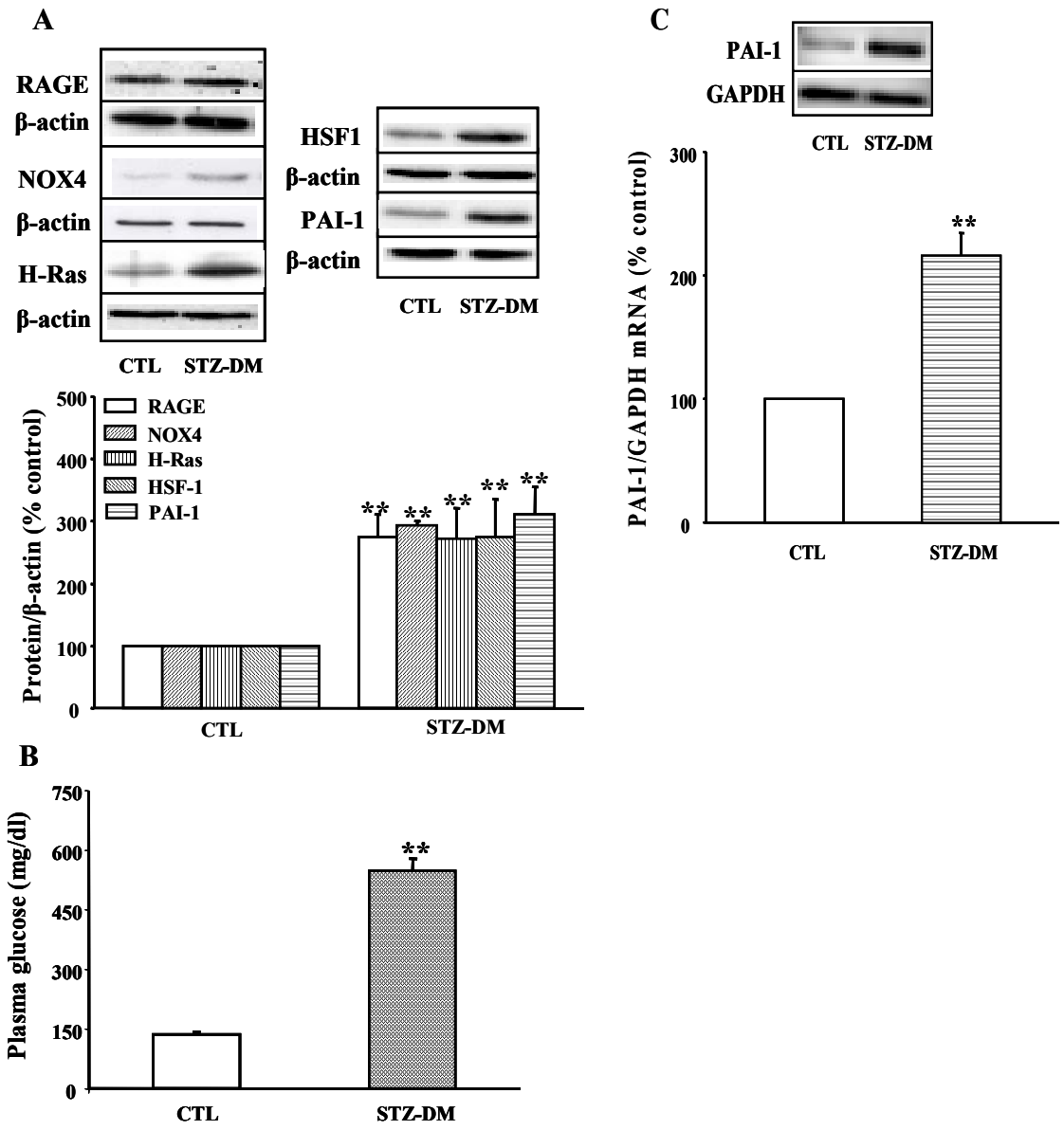


Figure 28. Effect of STZ-induced diabetes on signaling mediators, oxidative stress regulators and PAI-1 in mice. Panel A: The protein expression of RAGE, H-Ras, NOX, HSF1 and PAI-1 was determined in the hearts of control (CTL) and STZ-DM mice using Western blotting and mouse-specific antibodies. **Panel B:** Plasma glucose levels in CTL and STZ-DM mice. **Panel C:** PAI-1 mRNA levels were determined in hearts of control and STZ-DM mice using RT-PCR. Values in Fig A and C are presented in percentage of control after normalization with β-actin protein or GAPDH mRNA (mean ± SE, n = 5 animals/group). **: $p<0.01$ versus control.

Table 1A. Effect of STZ-induced diabetes on glucose, PAI-1, HSF1, RAGE, NOX and H-Ras in mice.

	Control	STZ-DM
pGlucose (mg/dl)	135.2 ± 10.4	549.8 ± 37**
pPAI-1 (µg/ml)	3.1 ± 0.5	7.7 ± 0.6**
hHSF-1 (fold)	0.9 ± 0.0	2.7 ± 0.6**
hPAI-1 (fold)	0.9 ± 0.1	3.1 ± 0.5**
hNOX4 (fold)	1.0 ± 0.0	2.9 ± 0.1**
hH-Ras (fold)	1.0 ± 0.1	2.7 ± 0.5**
hRAGE (fold)	1.0 ± 0.1	2.7 ± 0.4**
PAI-1mRNA (fold)	1.0 ± 0.0	2.2 ± 0.2**

p: plasma; h: heart; STZ-DM: Streptozotocin-induced diabetes mellitus.
Data in the tables are fold change compared to control except pGlucose and pPAI-1.
Mean ± SE (n=5 animals/group). **: $p < 0.01$.

Table 1B. Correlation between RAGE, H-Ras, NOX4, HSF1, PAI-1 and glucose in diabetic and control mice

	pGlucose	pPAI-1	hHSF1	hPAI-1	NOX4	hH-Ras
pPAI-1	0.85**					
hHSF1	0.89**	0.90**				
hPAI-1	0.91**	0.90**	0.96**			
hNOX4	0.96**	0.86**	0.94**	0.91**		
hH-Ras	0.72*	0.76*	0.24	0.88**	0.73**	
hRAGE	0.76*	0.87**	0.44	0.54	0.82**	0.64*

Data in the tables are r values (n=10 animals). *, **: $p<0.05$ or 0.01.

5.3 Effects of oxLDL on mitochondrial function in porcine aortic EC.

5.3.1 Oxygen consumption in mitochondria of aortic EC: The present study examined oxygen consumption in multiple respiratory chain complexes in PAEC treated with oxLDL using oxygraphy in the presence or absence of complex-specific substrates and inhibitors. PAEC were used in mitochondrial study due to the similarity between human and porcine cardiovascular systems, and the rapid growth of PAEC. Digitonin was used for the permeabilization of EC, which enables the evaluation of the function of respiratory chain complexes in intact mitochondria of living cells. The function of Complex I was determined as rotenone-sensitive respiration in the presence of NADH-dependent substrates. Complex II/III function was assessed as succinate-antimycin A-sensitive respiration in the presence of flavin-adenine dinucleotide (FADH)-dependent substrates. Ascorbate and TMPD was used as a substrate for Complex IV activity and verified by the addition of KCN (a Complex IV inhibitor). The thin curves in **Figure 29** represent the amount of oxygen in the oxygraphy chamber, while thick curves demonstrate the amount of oxygen consumed by living cells and verified with the addition of complex-specific inhibitors (rotenone for Complex I, antimycin A for Complex III or KCN for Complex IV) (**Figure 29 A-C**).

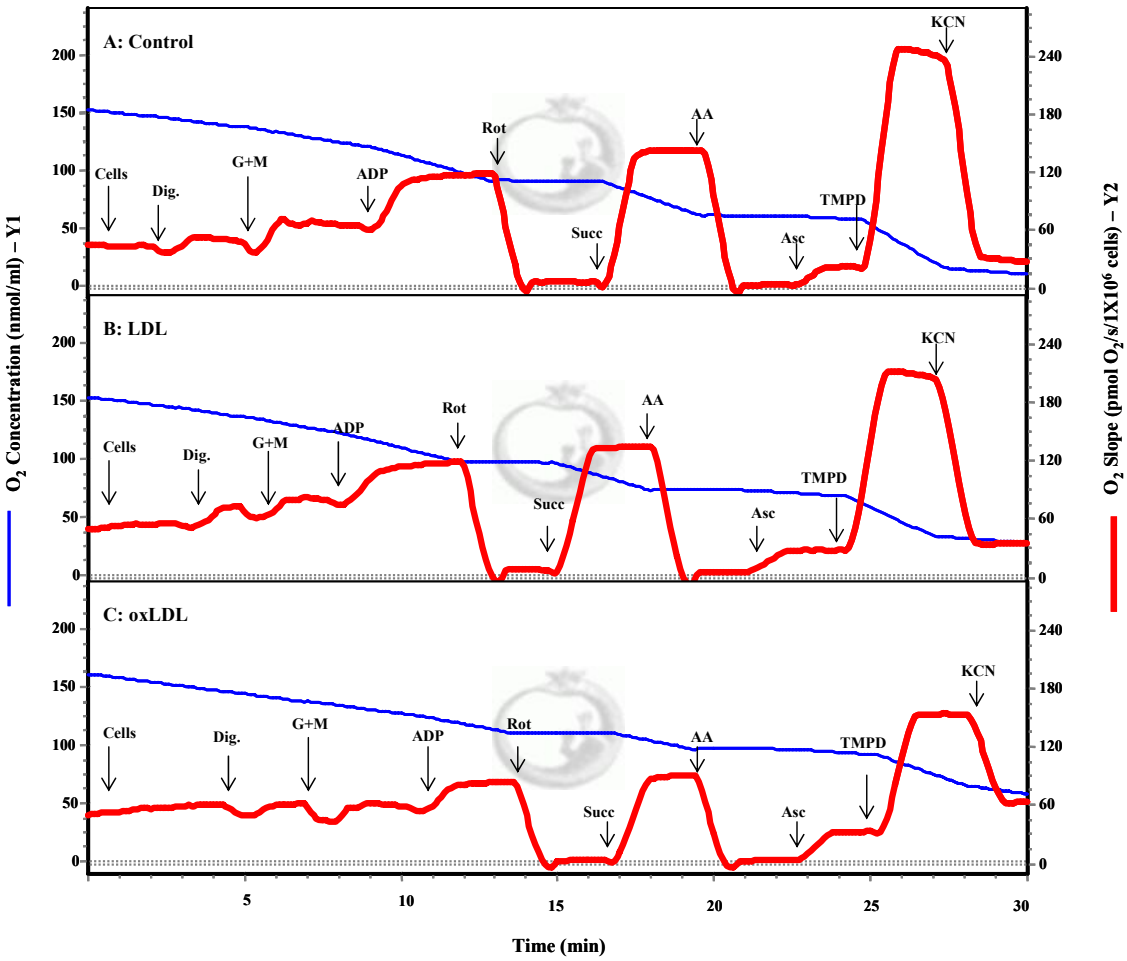


Figure 29. Oxygen consumption in mitochondrial respiratory chain of porcine aortic EC (PAEC) treated with oxLDL or LDL. EC were treated with 100 µg/ml of LDL or oxLDL for 12 h. The consumption of oxygen was assessed in PAEC treated with vehicle (**A**), LDL (**B**) or oxLDL (**C**) using oxygraphy as described in Materials and Methods. Arrows: addition of following substrates or inhibitors. Dig: digitonin (25 µg/10^6 cells); G: glutamate (10 mM); M: malate (5 mM); ADP: adenosine diphosphate (2 mM); Rot: rotenone (1 µM); Succ: succinate (10 mM); AA: antimycin A (1 µg/mL); Asc: ascorbate (5 mM); TMPD: N,N,N',N'-tetramethyl-p-phenylendiamine dihydrochloride (0.5 mM); KCN: potassium cyanide (0.25 mM).

5.3.2 Effect of oxLDL on oxygen consumption and respiratory control index (RCI) in mitochondria: PAEC were stimulated with LDL or oxLDL (100 μg/ml) for 2-24 h. Treatment with oxLDL reduced oxygen consumption in EC compared to control cells (**Figure 30A-C**). Oxygen consumption in Complex I, II/III and IV of EC was significantly reduced by oxLDL after 12-24 h of incubation compared to control (**Figure 30A-C**, $p<0.05$). Treatment with LDL moderately, but not significantly, reduced oxygen consumption in Complex I, II/III or IV (**Figure 30A-C**). OxLDL enhanced effect on the reduction of oxygen consumption in Complex I-IV compared to LDL when treated for 24 h (**Figure 30A and 30C,** $p<0.05$). RCI was assessed from amounts of oxygen consumed in presence or absence of ADP, which reflects the tightness of coupling between respiration and oxidative phosphorylation in mitochondria. Treatment with LDL or oxLDL for 12 or 24 h significantly reduced RCI in PAEC compared to control ($p<0.05$). The results suggests that oxLDL for 24 h induced a significantly greater reduction of RCI in EC compared to that with LDL for equivalent period (**Figure 30D**, $p<0.05$).

5.3.3 Effect of uncoupler on oxLDL-induced oxygen consumption in mitochondrial respiratory chain: PAEC were treated with 100 μg/ml of oxLDL or control for 12 h. After treatment, cells were trypsinized, resuspended in KCl medium and used for oxygen consumption assessment using oxygraphy as described in Methods. Treatment with oxLDL significantly reduced ADP-induced oxygen consumption in EC compared to control. The consumption of oxygen induced by ADP was reduced in presence of oligomycin in control and oxLDL-treated cells. Uncouplers block oxidative phosphorylation by dissipating the H^+ electrochemical gradient. FCCP, an uncoupler of

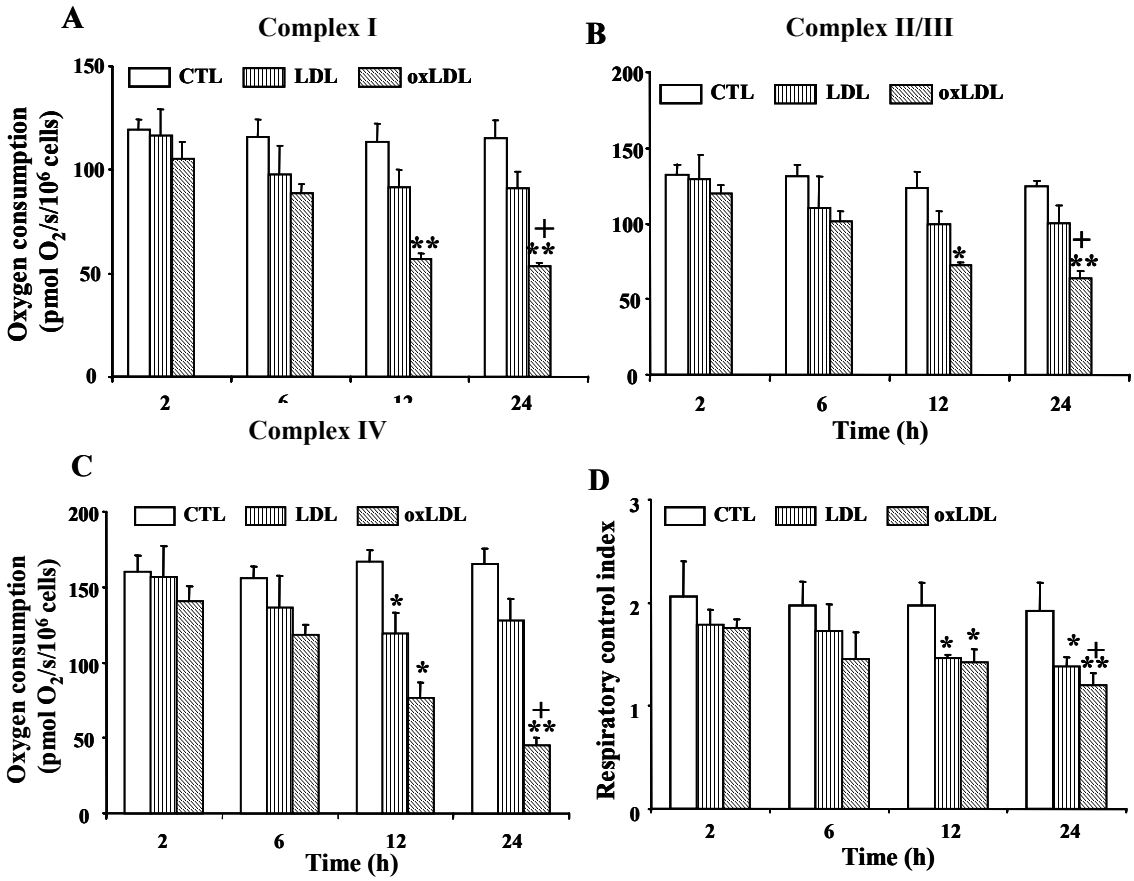


Figure 30. Effect of oxLDL on oxygen consumption in mitochondrial Complexes I-IV and respiratory control index (RCI) in PAEC. PAEC were treated with 100 µg/ml of LDL or oxLDL for 2-24 h. The consumption of oxygen in live EC was measured using oxygraphy as described in legend of Figure 24. **Panel A:** Oxygen consumption in Complex I assessed in the presence of glutamate + malate + ADP followed by the addition of rotenone. **Panel B:** Complex II/III respiration was assessed with succinate-coupled oxygen consumption followed by the addition of antimycin A. **Panel C:** Complex IV respiration measured with KCN-sensitive oxygen consumption induced by ascorbate + TMPD. **Panel D:** RCI was measured from oxygen consumption in PAEC. RCI: + ADP (glutamate + malate)/-ADP (glutamate + malate). Values were expressed in pmol/s/10^6 cells (mean ± SEM, n = 3 experiments using different batches of LDL) after justification with cell numbers. *, **: $p<0.05$ or 0.01 versus control; +: $p<0.05$ versus LDL.

mitochondria, increased oxygen consumption with greater extent in control cells (210.9 ± 27.3 pmol O^2/s/1X10^6 cells, n=3) compared to oxLDL (102.6 ± 3.1 pmol O^2/s/1X10^6 cells, n=3) (**Figure 31**).

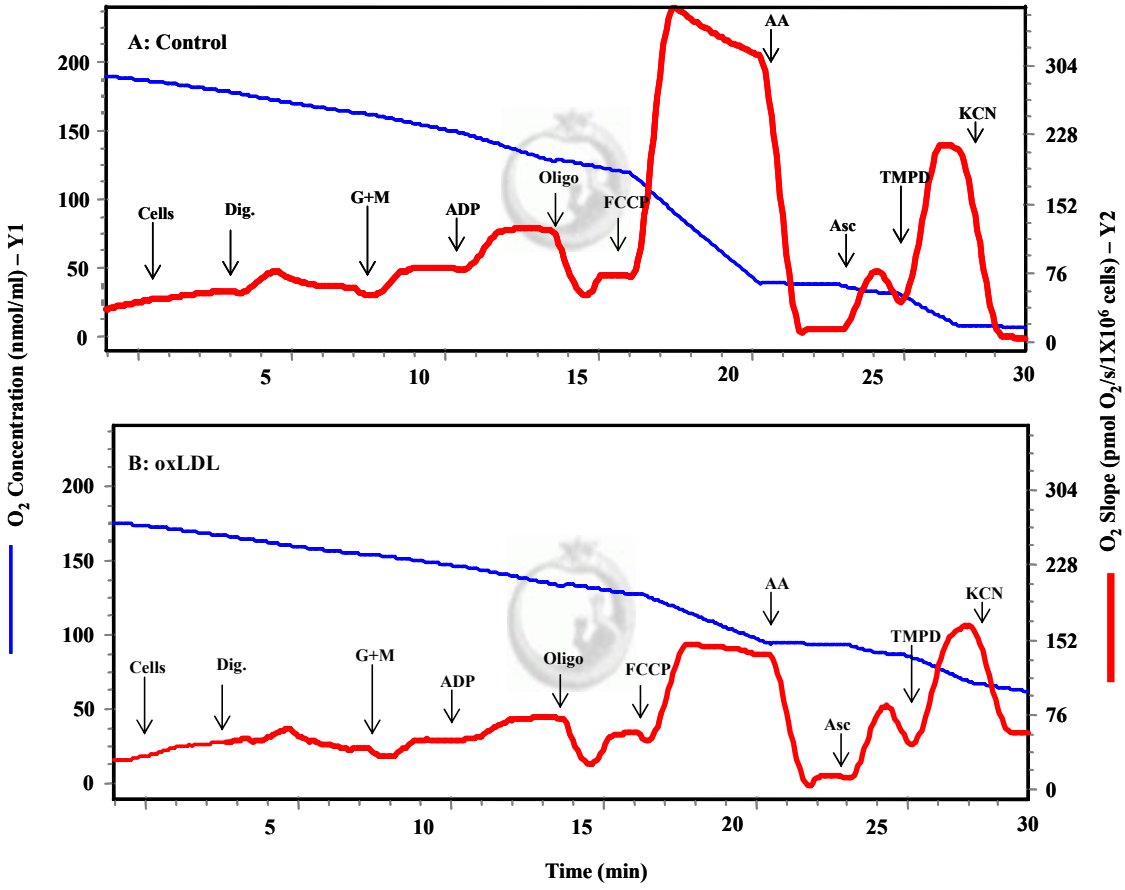


Figure 31. Effect of uncoupler on oxLDL-induced mitochondrial oxygen consumption in PAEC. EC were incubated with 100 µg/ml of oxLDL or control for 12 h. Oxygen consumption was assessed in PAEC treated with vehicle control (**A**), or oxLDL (**B**) using oxygraphy as described in Materials and Methods. Arrows: addition of following substrates, uncoupler or inhibitors. Dig: digitonin (25 µg/10^6 cells); G: glutamate (10 mM); M: malate (5 mM); ADP: adenosine diphosphate (2 mM); oligo: oligomycin (1 µM); FCCP: carbonylcyanide p-trifluoromethoxyphenylhydrazone (1 µM); AA: antimycin A (1 µg/mL); Asc: ascorbate (5 mM); TMPD: N,N,N',N'-tetramethyl-p-phenylendiamine dihydrochloride (0.5 mM); KCN: potassium cyanide (0.25 mM).

5.3.4 Effect of oxLDL on ND activity and NAD^+/NADH ratio: Incubation of PAEC with oxLDL or LDL (100 μg/ml) for 12 h or 24 h significantly decreased ND activity in PAEC compared to control ($p<0.05$ or 0.01). OxLDL showed a greater inhibition of ND activity compared to LDL (**Figure 32A**, $p<0.05$). Complex I is considered as one of intracellular sources of NAD^+ being converted from NADH (Grivennikova and Vinogradov, 2006). To verify the involvement of Complex I, we analyzed the effect of oxLDL on the abundance of NAD^+, NADH and its ratio in total cell lysates in PAEC. Treatment with oxLDL (100 μg/ml) for 6, 12 or 24 h significantly reduced NAD^+/NADH ratio compared to control ($p<0.05$). LDL at the same concentration reduced NAD^+/NADH ratio after 12 or 24 h of incubation compared to control ($p<0.05$). OxLDL induced significantly greater reduction in NAD^+/NADH compared to LDL after 12 or 24 h of incubation (**Figure 32B**, $p<0.05$).

5.3.5 Effect of oxLDL on SCCR, UCCR, cytochrome *c* oxidase and CS activity: Treatment with oxLDL (100 μg/ml) for 12 or 24 h significantly reduced the activity of SCCR in PAEC compared to control or LDL (**Figure 33A**, $p<0.05$ or 0.01). A significant decrease in UCCR activity was detected in PAEC induced by LDL or oxLDL for 6, 12 or 24 h ($p<0.05$ or 0.01). However, oxLDL significantly reduced UCCR activity in EC after 6, 12 or 24 h of incubation compared to LDL (**Figure 33B**, $p<0.05$). The activity of cytochrome *c* oxidase was significantly reduced in PAEC after treatment with LDL or oxLDL for 6, 12 or 24 h ($p<0.05$ or 0.01). OxLDL induced a greater decrease in cytochrome *c* oxidase activity in EC after 6-24 h of incubation compared to LDL (**Figure 33C**, $p<0.05$). CS, a mitochondrial matrix-soluble enzyme is relatively insensitive to the

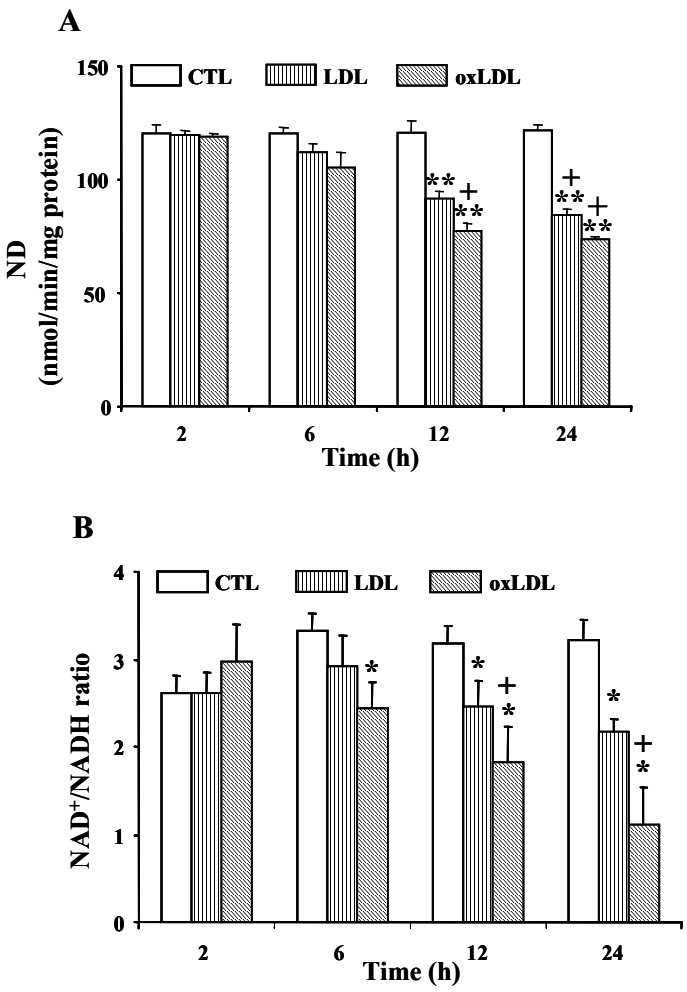


Figure 32. Effect of oxLDL on Complex I (ND) activity or NAD^+/NADH ratio in PAEC. Confluent aortic EC were treated for 2-24 h with 100 µg/ml of LDL or oxLDL. Cell lysates were then assayed for NADH-ubiquinone dehydrogenase (ND) activity (**A**) and NAD^+/NADH ratio (**B**) using spectrophotometer. Values were expressed in nmol/min/mg protein except NAD^+/NADH ratio (mean ± SEM, n = 3 experiments using different batches of LDL) after justification with cellular proteins. *, **: $p<0.05$ or 0.01 versus control; +: $p<0.05$ versus LDL.

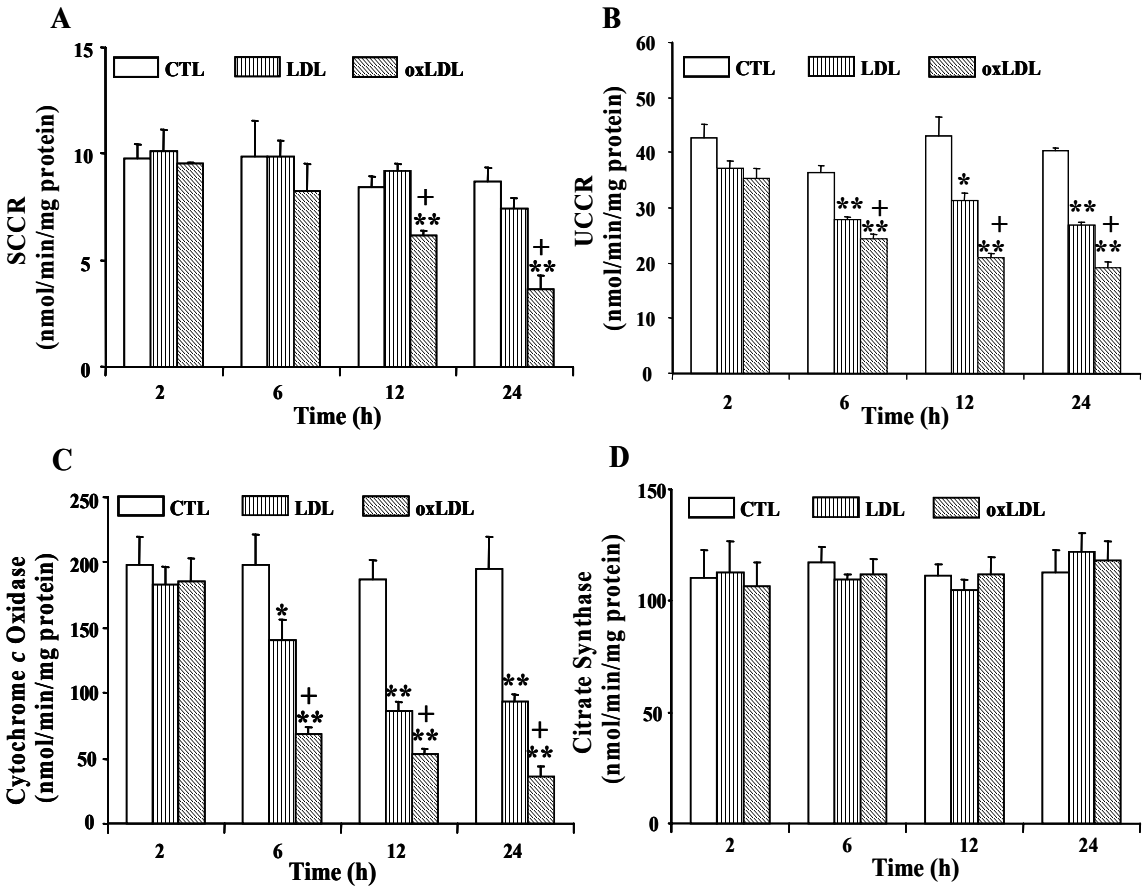


Figure 33. Effect of oxLDL on mitochondrial respiratory chain activities (Complexes II-IV) in PAEC. EC were incubated with 100 µg/ml of LDL or oxLDL for 2-24 h. Cell lysates were then assayed for succinate cytochrome *c* reductase (SCCR) activity (**A**), ubiquinone cytochrome *c* reductase (UCCR) activity (**B**), cytochrome *c* oxidase activity (**C**), and citrate synthase activity (**D**) using spectrophotometer. Values were expressed in nmol/min/mg protein (mean ± SEM, n = 3 experiments using different batches of LDL) after justification with cellular proteins. *, **: $p<0.05$ or 0.01 versus control; +: $p<0.05$ versus LDL.

effects of oxidants (Masaki et al., 1995) and confirms the abundance of mitochondrial mass in cells (Wharton and Tzagoloff, 1967). CS activity was determined in PAEC as an internal control for the activity of other mitochondrial enzymes. The results from the present study demonstrated that CS activity was not significantly altered in EC after exposure to LDL or oxLDL (100 μg/ml, 2-24 h) (**Figure 33D**).

5.3.6 Effects of mildly oxLDL on ND, SCCR, UCCR or cytochrome *c* oxidase activity: To compare the effect of mildly oxidized LDL (moLDL) with oxLDL, we incubated EC with 100 μg/ml of moLDL or oxLDL for 12 h. The results demonstrated a moderate decreases in ND, UCCR and cytochrome *c* oxidase activity, but not SCCR activity, induced by moLDL in PAEC compared to control (**Figure 34A-D**, $p<0.05$ or 0.01) in extents similar to unmodified LDL. Incubation with 100 μg/ml of oxLDL for 12 h induced significantly greater inhibition on the activities of ND, SCCR, UCCR or cytochrome *c* oxidase compared to moLDL or LDL (**Figure 34A-D**, $p<0.05$ or 0.01).

5.3.7 Effects of antioxidant on oxLDL-induced impaired activities of ND and UCCR: Previous studies in our group demonstrated that BHT, a potent antioxidant, at 80 μM prevented oxLDL-induced H_2O_2 production in EC (Zhao et al., 2009). In order to determine the involvement of antioxidant in oxLDL-mediated effects, we examined effect of BHT on oxLDL-induced impairment of the activities of ND and UCCR in PAEC. BHT treatment normalized oxLDL-induced reduced activities of ND and UCCR in EC (**Figure 35A and B**, $p<0.05$ or 0.01).

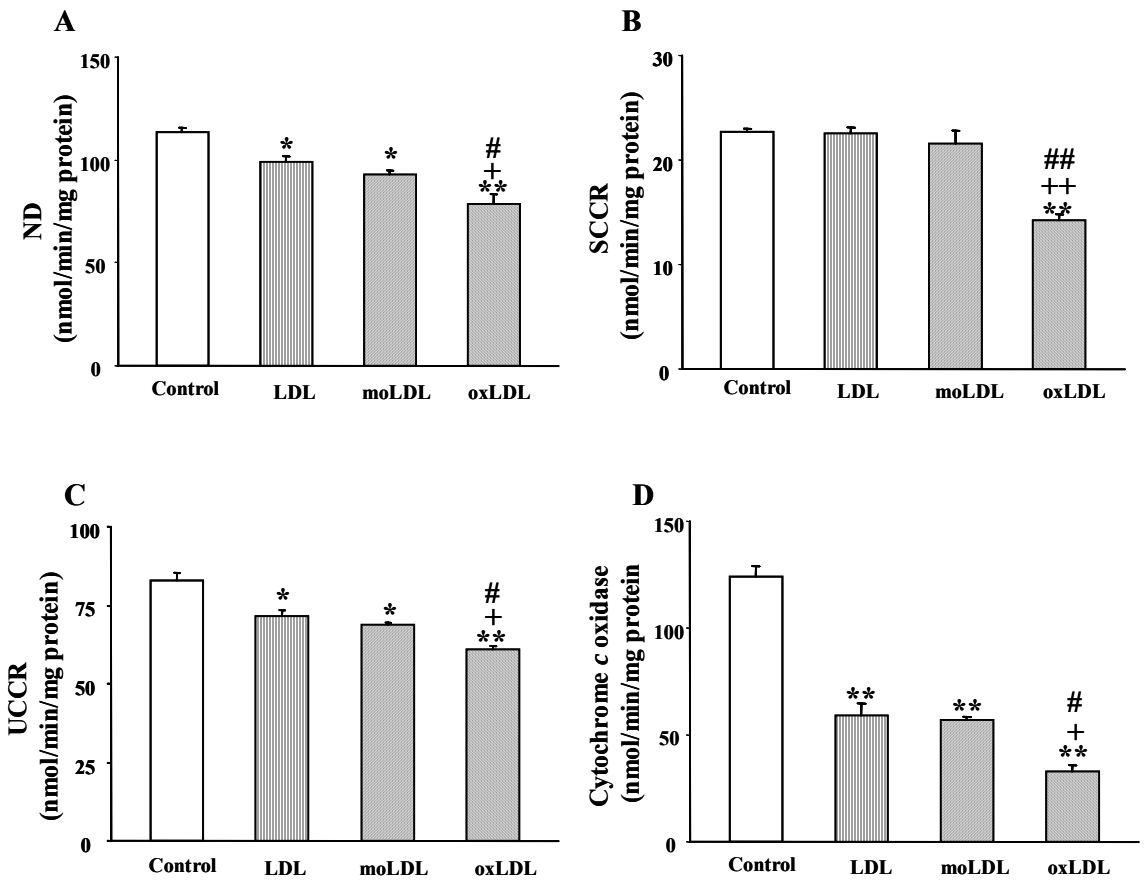


Figure 34. Effect of mildly oxidized LDL (moLDL) on mitochondrial respiratory chain enzyme activities in PAEC. EC were treated with 100 µg/ml of moLDL, oxLDL or LDL for 12 h. Cell lysates were then assayed for NADH-ubiquinone dehydrogenase (ND) activity (**A**), succinate cytochrome *c* reductase (SCCR) activity (**B**), ubiquinone cytochrome *c* reductase (UCCR) activity (**C**), and cytochrome *c* oxidase activity (**D**) using spectrophotometer. Values were expressed in nmol/min/mg protein (mean ± SEM, n = 3 experiments using different batches of LDL) after justification with cellular proteins. *, **: $p<0.05$ or 0.01 versus control; +, ++: $p<0.05$ or 0.01 versus LDL; #, ##: $p<0.05$ or 0.01 versus moLDL.

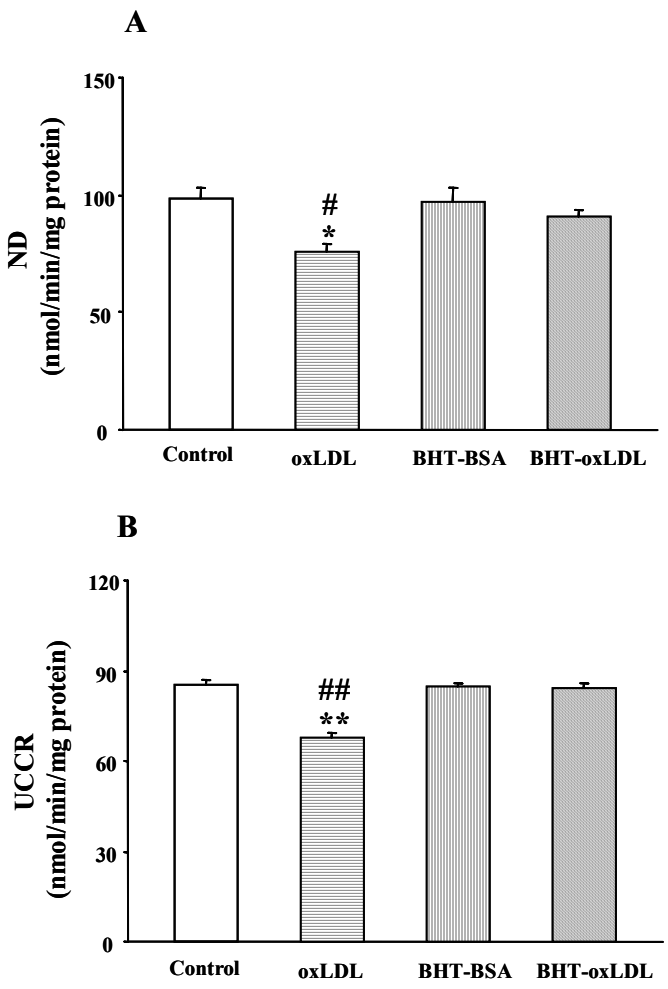
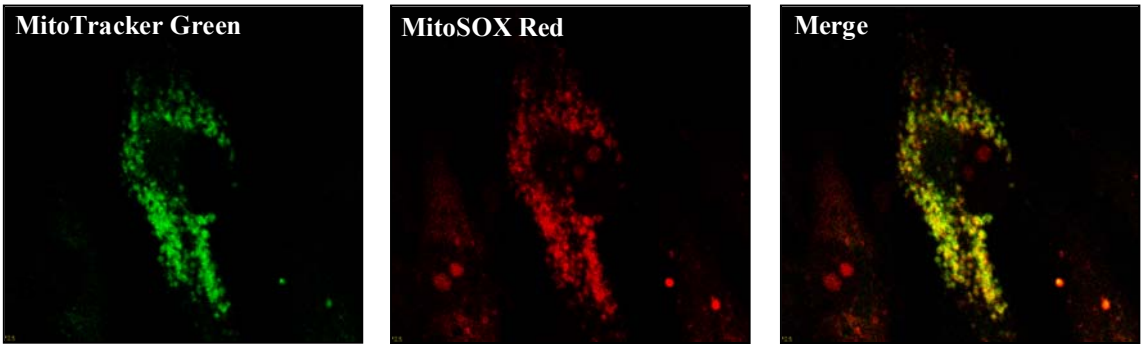


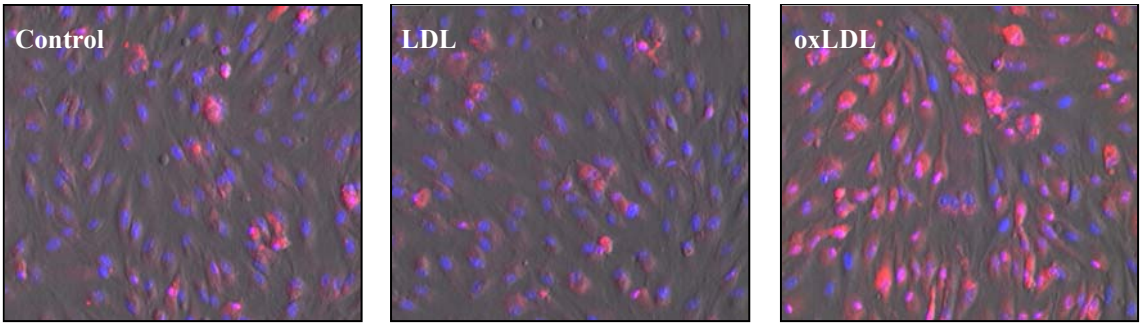
Figure 35. Effect of BHT on reduced activities of Complex I and III induced by oxLDL in PAEC. PAEC were incubated with 100 μg/ml of oxLDL, BHT-BSA or BHT-oxLDL for 12 h with. Cell lysates were used for the analysis of NADH-ubiquinone dehydrogenase (ND) activity (**A**) and ubiquinone cytochrome *c* reductase (UCCR) activity (**B**) using spectrophotometer. Values were expressed in nmol/min/mg protein (mean ± SEM, n = 3 experiments using different batches of LDL) after justification with cellular proteins. *, **: $p<0.05$ or 0.01 versus control; #, ##: $p<0.05$ or 0.01 versus BHT-oxLDL.

5.3.8 Relationship between oxLDL-induced ROS and mitochondria: Previous studies from our laboratory demonstrated that oxLDL increased ROS release from HUVEC (Zhao and Shen, 2005), but the role of mitochondria in oxLDL-induced ROS generation remains unclear. To verify involvement of mitochondria, PAEC were treated with 100 μg/ml of LDL or oxLDL for 2 h and then labeled with MitoSox Red, a marker of ROS. High resolution confocal microscopy was used to confirm that labeling with MitoSox Red was specific for mitochondria-derived ROS (**Figure 36A**). The ROS signal was entirely overlapping with staining by MitoTracker Green, a mitochondria-specific dye. This confirms the validity of MitoSox Red for measuring mitochondria-specific ROS generation. We next used quantitative imaging by laser scanning cytometry to measure the increase in mitochondria-derived ROS after oxLDL treatment. Treatment with oxLDL doubled the mitochondria-associated ROS in PAEC compared to control or LDL-treated cultures (**Figure 36B-D**, $p<0.05$). The effect of oxLDL on the release of ROS from PAEC has not been documented. PAEC were treated with 100 μg/ml of LDL or oxLDL for 2 h. OxLDL also significantly increased the release of H_2O_2 from PAEC compared to LDL or control (**Figure 36E**, $p<0.05$).

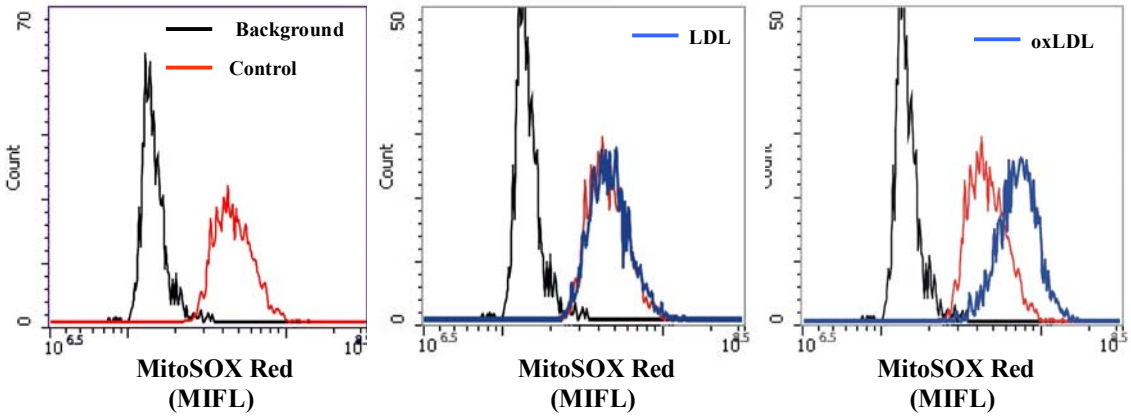
A


MitoTracker Green
MitoSOX Red
Merge

B


Control
LDL
oxLDL

C


Background
Control
LDL
oxLDL
Count
MitoSOX Red
(MIFL)

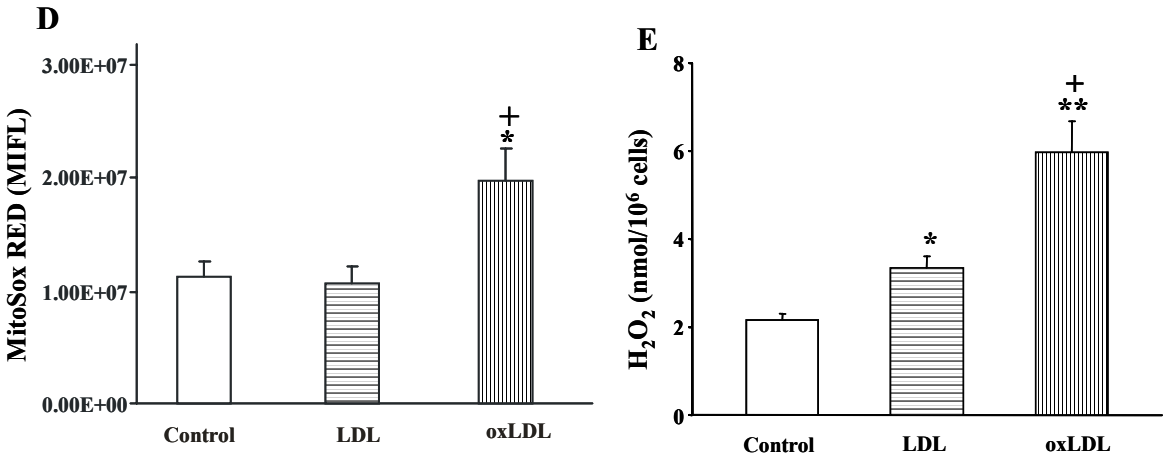


Figure 36. Effects of oxLDL on mitochondria-derived ROS generation from PAEC. **Panel A:** EC were stained with both MitoTracker Green (left panel) and MitoSox Red (middle panel). The confocal images were captured as described in Methods. A merged image shows significant overlapping in staining (yellow in right panel), and confirms specificity of MitoSox staining for mitochondria-derived ROS. **Panel B:** EC were stimulated with vehicle (control), LDL or oxLDL (100 μg/ml) for 2 h, and then incubated with MitoSox Red (red staining) prior to fixation and staining of DNA with H33342 dye (blue staining) as described in the Methods. Shaded relief contrast images shown are typical of individual scan fields captured using an iCys LSC for vehicle-treated (left panel), LDL-treated (middle panel) and oxLDL treated (right panel) cultures. **Panel C:** The mean integrated fluorescence (MIFL) was assessed from individual scan areas of histograms (MitoSox Red) for each condition in the corresponding upper panels (row B). Autofluorescence (black line) and control MIFL (red line) were shown in each panel. Middle panel indicate the signal measured after LDL treatment (blue line), while right panel displayed the signal measured after oxLDL treatment (blue line). **Panel D:** Comparison between control and lipoprotein-induced MitoSox Red MIFL in PAEC. The MIFL was assessed by using laser scanning cytometry. **Panel E:** Confluent PAEC were treated with 100 μg/ml of LDL or oxLDL for 2 h. H_2O_2 levels in media were determined as described in the Methods. Values were expressed in mean integrated fluorescence per cell except H_2O_2 levels expressed in nmol/10^6 cells (mean ± SEM, n = 3 experiments using different batches of LDL). *, **: $p<0.05$ or 0.01 versus control; +: $p<0.05$ versus LDL.

5.3.9 Effect of oxLDL on mitochondrial membrane potential: PAEC were treated with LDL or oxLDL (100 μg/ml) for 12 h. OxLDL or LDL induced evident shift in mitochondrial membrane potential ($\Delta\Psi_m$) compared to control (**Figure 37A**). The level of relative TMRM intensity induced by LDL or oxLDL was significantly lower than that in vehicle treated cultures (**Figure 37B**, $p<0.05$ or 0.01). The results indicated that oxLDL reduced $\Delta\Psi_m$ in EC, which is consistent to the alteration of ETC enzyme activity in oxLDL-treated EC described in Figs 28 and 29.

5.3.10 FTI-277 protects oxLDL-induced ROS production and cell death in HUVEC: OxLDL is potentially cytotoxic in HUVEC. In order to examine the effect of oxLDL on cell viability, HUVEC were treated with oxLDL for 24-84 h. Cell viability was determined by the MTT colorimetric assay. The results demonstrate that incubation of EC with 100 μg/ml oxLDL reduces cell viability 60-84 h after incubation ($p<0.05$ or 0.01). OxLDL had no cytotoxic effect on cell viability after 24 or 48 h of treatment (**Figure 38A**). Previous study demonstrated that FTI-277 inhibits H_2O_2-induced oxidative stress in EC (Cuda et al., 2002). To verify effects of FTI-277 on oxLDL-induced oxidative stress, EC were pretreated with FTI-277 (20 μM) and then exposed to 100 μg/ml of LDL or oxLDL for 2 h. Treatment with FTI-277 significantly blocked oxLDL-induced H_2O_2 release from EC (**Figure 38B**, $p<0.05$ or 0.01). To assess the effect of FTI-277 on oxLDL-induced cell death, HUVEC were pretreated with FTI-277 and then exposed to 100 μg/ml of oxLDL for 72 h. FTI-277 significantly inhibited cytotoxic effects induced by oxLDL in EC after a prolonged exposure (**Figure 38C**, $p<0.05$ or 0.01). The results of above suggest that oxLDL (100 μg/ml) ≤ 48 h do not affect viability

of EC. Inihibition of H-Ras farnesylation using FTI-277 may partially inhibit oxLDL-induced oxidative stress in EC.

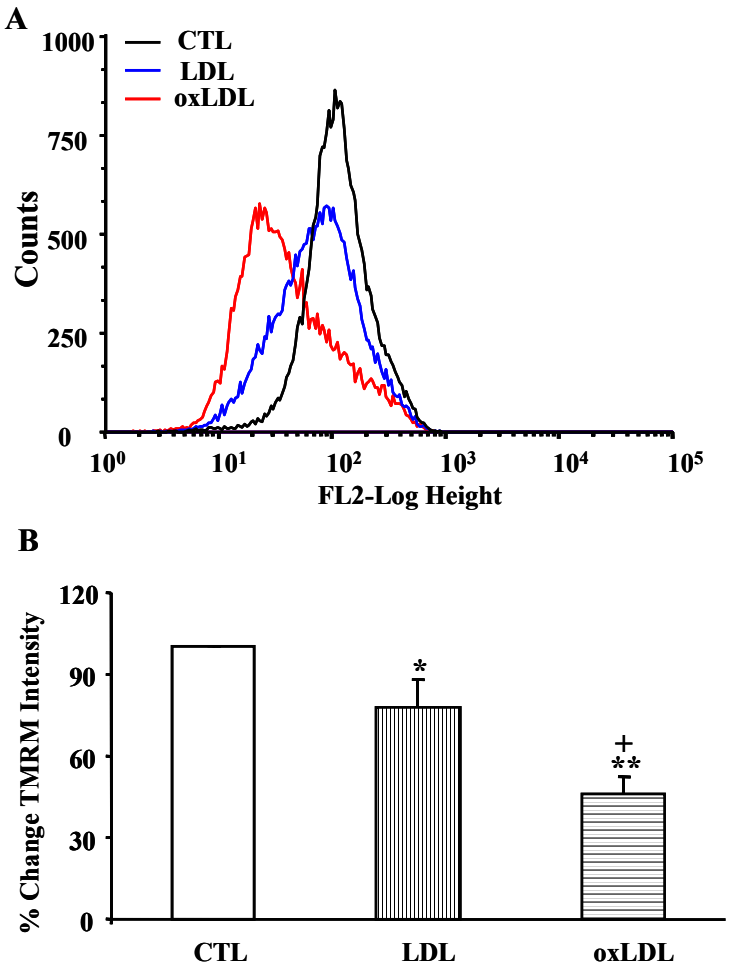


Figure 37. Impairment of mitochondrial membrane potential induced by oxLDL in PAEC. Panel A: PAEC were treated with 100 µg/ml of LDL or oxLDL for 12 h and then treated with 0.02 mg/mg protein of digitonin. The cells were incubated with TMRM as described in the Methods. Cytofluorometric analysis was done on an EPICS Altra Beckman Coulter flow cytometer. TMRM signal was detected in the FL2 channel. **Panel B**: High intensity signal for TMRM was calculated from change in median induced by control, LDL or oxLDL. Values were expressed in % change versus control (mean ± SEM, n = 3 experiments). *, **: $p<0.05$ or 0.01 versus control; +: $p<0.05$ versus LDL.

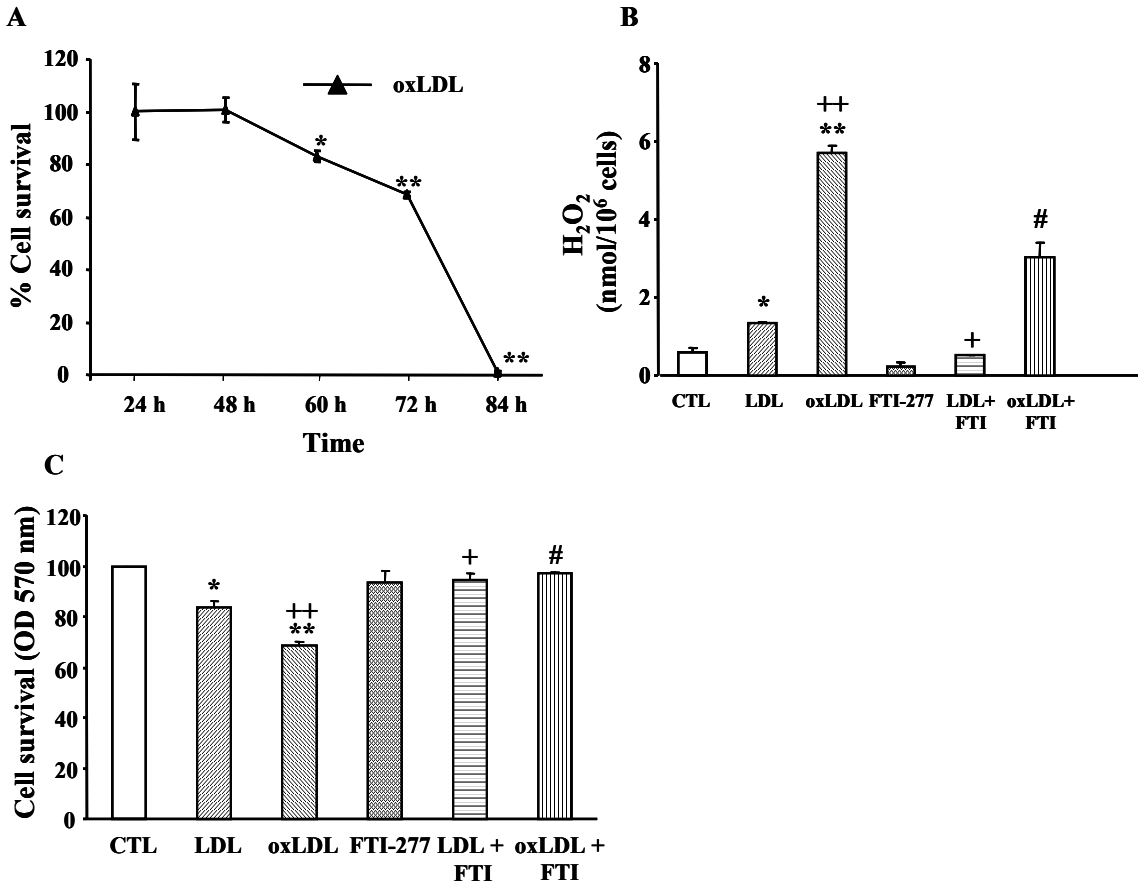


Figure 38. Effect of Ras inhibitor (FTI-277) on oxLDL-induced H_2O_2 or cell death in HUVEC. Panel A: Time course for oxLDL-induced cell death in HUVEC assessed by using MTT assay as described in Methods. **Panel B:** Confluent HUVEC were treated with medium + vehicle (control) or 100 μg/mL of LDL or ox-LDL with or without addition of FTI-277 for 2 hrs. H_2O_2 levels in media were determined as described in Methods. **Panel C:** HUVEC were treated with 100 μg/mL of oxLDL in presence or absence of Ras Inhibitor (20 μM) for 72 h. Values were expressed in mean ± SEM. *, **: $p<0.05$ versus control; +, ++: $p<0.05$ versus LDL; #: $p<0.05$ versus oxLDL.

5.4 Effects of glyLDL on mitochondrial respiratory chain activity in aortic EC.

5.4.1 Effects of glyLDL on oxygen consumption in mitochondria of PAEC: The oxygen consumption in mitochondria of PAEC after the treatment with glyLDL or LDL was assessed using oxygraphy with additions of complex-specific substrates and inhibitors as described in section 5.3.1 on page 96 (**Figure 39**).

PAEC were treated with 100 μg/ml of LDL or glyLDL for 2-24 h. Cells were harvested freshly and used for measurement of oxygen consumption. Treatment with glyLDL significantly reduced Complex I, II/III or IV-specific oxygen consumption in EC after 12-24 h of incubation compared to control (**Figure 40A-C**, $p<0.05$). LDL treatment had no significant effect on Complex I or II/III-specific oxygen consumption compared to control (**Figure 40A and B**). Incubation with LDL significantly reduced Complex IV-specific oxygen consumption after 12 h compared to control (**Figure 40C**, $p<0.05$). GlyLDL showed enhanced effects on reduction of oxygen consumption in Complex I and IV after 24 h of incubation with PAEC compared to LDL (**Figure 40A and C**, $p<0.05$).

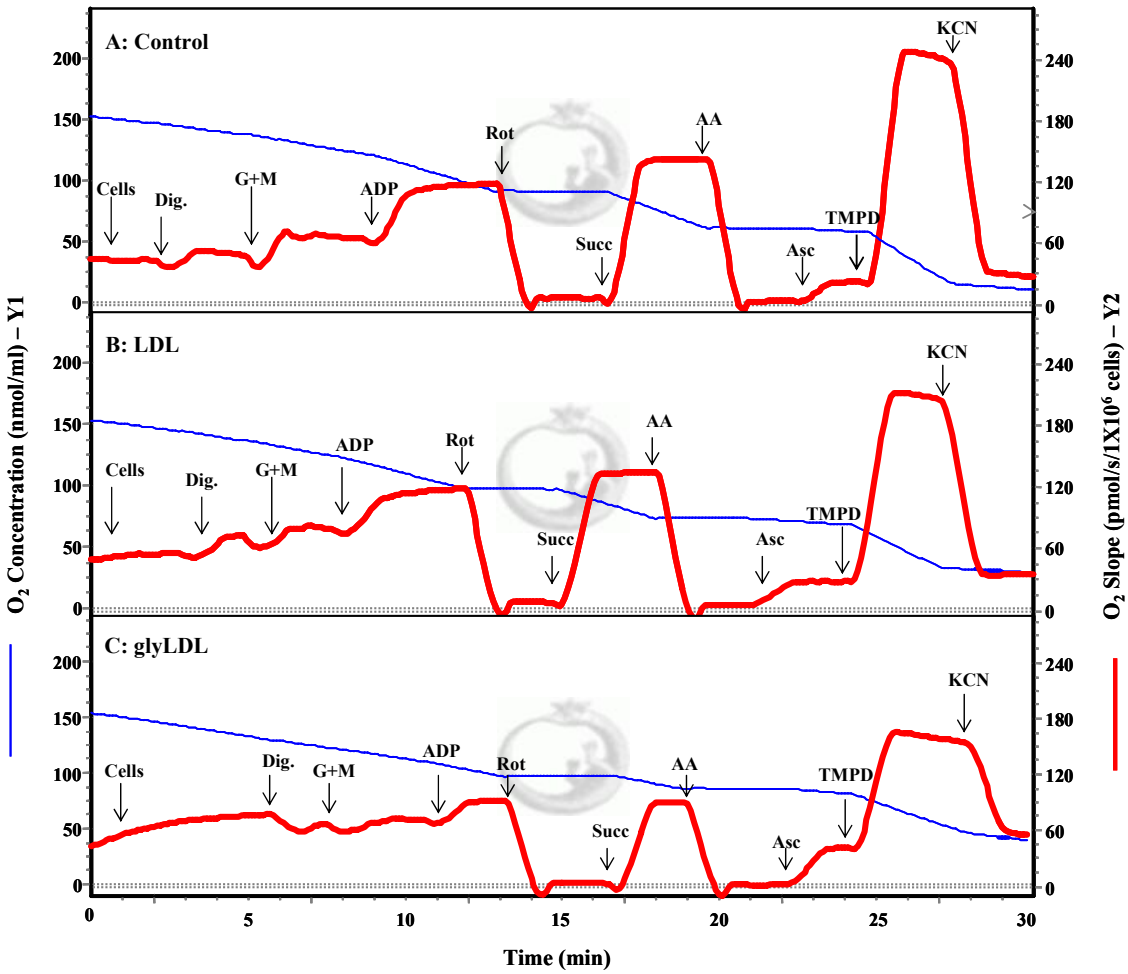


Figure 39. Oxygen consumption profile in mitochondria of PAEC induced by glyLDL. PAEC were stimulated with LDL or glyLDL (100 µg/ml) for 12 h. Cells were homogenized and used for the assessment of oxygen consumption using oxygraphy after treatment with vehicle (**A**), LDL (**B**) or glyLDL (**C**) as described in Materials and Methods. Dig: digitonin; G: glutamate; M: malate; ADP: adenosine diphosphate; Rot: rotenone; Succ: succinate; AA: antimycin A; Asc: ascorbate; TMPD: N,N,N',N'-tetramethyl-p-phenylendiamine dihydrochloride; KCN: potassium cyanide. Y1 (thin line): Decline of oxygen concentration in chamber of oxygraphy containing live EC. Y2 (thick line): Oxygen consumption in response to substrates for mitochondrial respiratory chain Complexes I-IV.

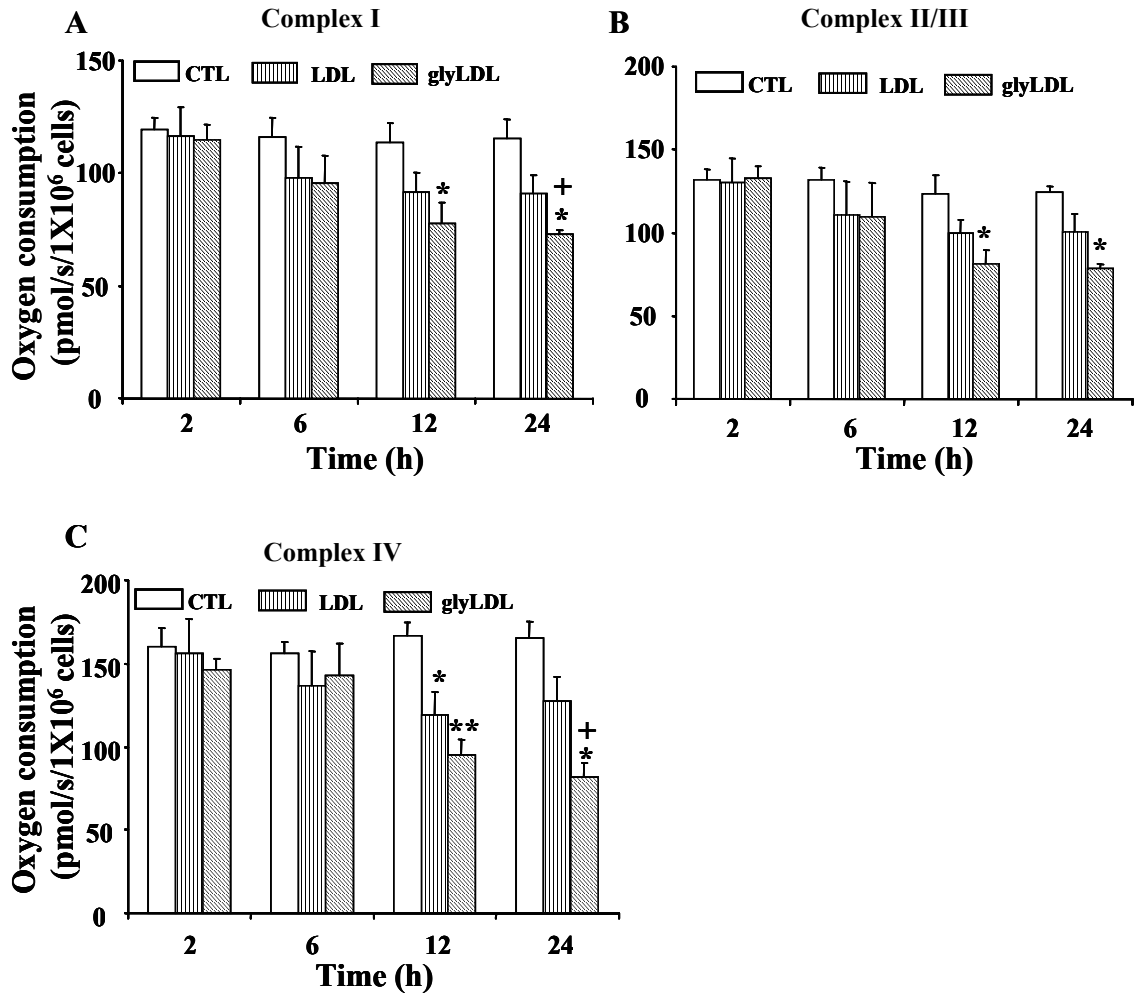


Figure 40. Effect of glyLDL on oxygen consumption in mitochondrial respiratory chain complexes (Complex I-IV) of PAEC. PAEC were treated with 100 µg/ml of LDL or glyLDL for 2, 6, 12 or 24 h. Oxygen consumption was assessed using oxygraphy as described in the legend of Figure 35. **Panel A:** Complex I-specific oxygen consumption was measured using glutamate (10 mM) + malate (5 mM) as substrate and rotenone as an inhibitor. **Panel B:** Complex II/III activity was assessed with antimycin A-sensitive oxygen consumption induced by succinate (10 mM). **Panel C:** Complex IV activity measured with KCN-sensitive oxygen consumption induced by ascorbate (5 mM) + TMPD (0.5 mM). Values were expressed in pmol/s/10^6 cells (mean ± SEM, n = 3 experiments) using different batches of LDL from various donors, same as below) after justification with cell numbers. *, **: $p<0.05$ or 0.01 versus control; +: $p<0.05$ versus LDL.

5.4.2 Effects of glyLDL on RCI: Treatment with glyLDL (100 μg/ml) for 12 or 24 h significantly reduced RCI compared to control ($p<0.05$). LDL at the same concentration for 12 h, but not 24 h, significantly reduced RCI ($p<0.05$). GlyLDL treatment for 24 h induced a significantly greater inhibition on RCI compared to LDL (**Figure 41**, $p<0.05$).

5.4.3 Effects of glyLDL on ND activity and NAD^+/NADH ratio in PAEC: Incubation of PAEC with 100 μg/ml of glyLDL or LDL for 12 h or 24 h significantly reduced the activity of Complex I compared to control ($p<0.05$ or 0.01). GlyLDL reduced ND activity in EC compared to LDL (**Figure 42A**, $p<0.05$). Complex I is a main user for NADH in EC (Grivennikova and Vinogradov, 2006). To verify the effect of glyLDL on ND activity, we analyzed the abundance of NAD^+, NADH and its ratio in total cell lysates in PAEC. GlyLDL or LDL (100 μg/ml) significantly reduced the ratio of NAD^+/NADH in total cellular lysates of EC after 12 or 24 h compared to control ($p<0.05$ or 0.01). Treatment with glyLDL for 24 h induced a significantly greater reduction in NAD^+/NADH ratio compared to LDL (**Figure 42B**, $p<0.05$).

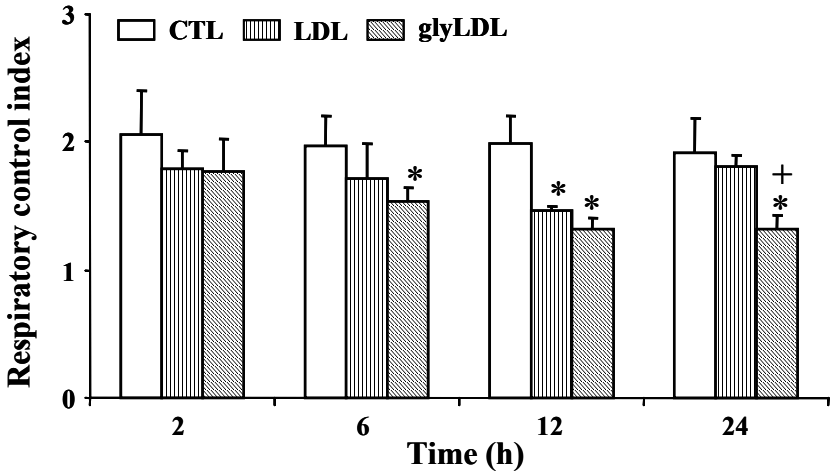


Figure 41. Effect of glyLDL on RCI in PAEC. PAEC were exposed to LDL or glyLDL (100 µg/ml) for 2-24 h. RCI was assessed from oxygen consumption in PAEC with the addition of glutamate + malate + ADP using oxygraphy. Values were expressed in ratio (mean ± SEM, n = 3 experiments). *: $p<0.05$ versus control; +: $p<0.05$ versus LDL.

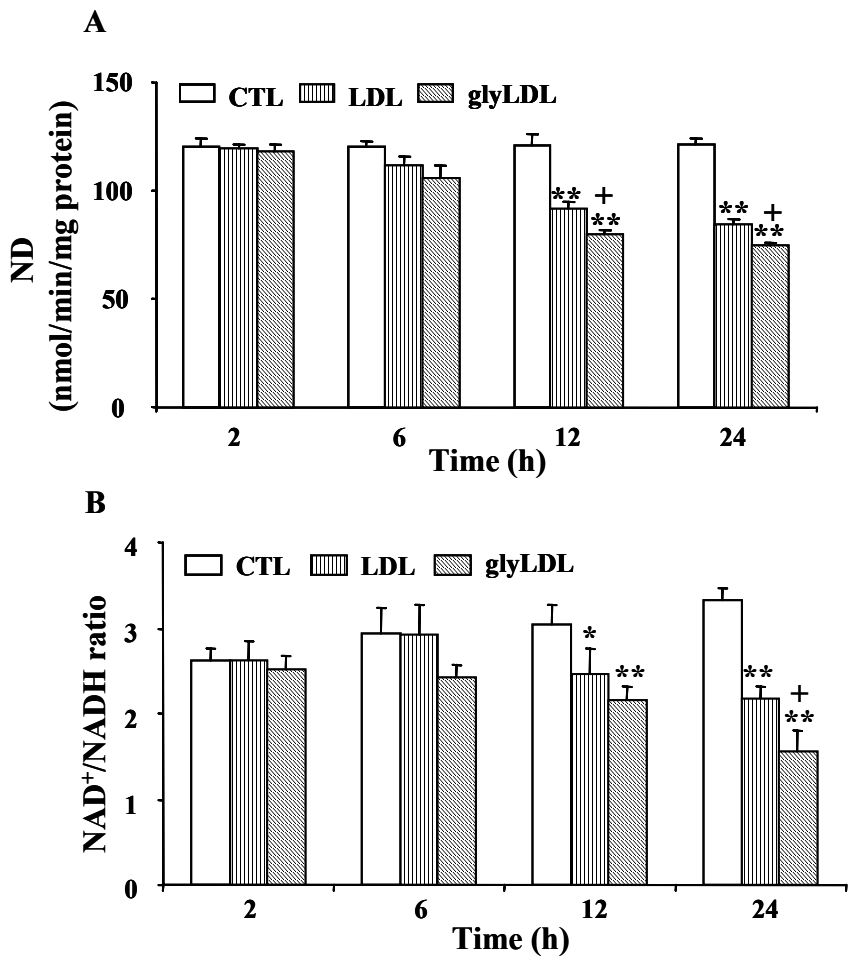


Figure 42. ND activity and NAD^+/NADH ratio in PAEC induced by glyLDL. Cells were treated for 2-24 h with 100 µg/ml of LDL or glyLDL. Cell lysates were then used for the following assays: **Panel A:** NADH-ubiquinone dehydrogenase (ND) activity, **Panel B:** NAD^+/NADH ratio using spectrophotometer. Values were expressed in nmol/min/mg protein (mean ± SEM, n = 3 experiments) after justification with cellular proteins. *, **: $p<0.05$ or 0.01 versus control; +: $p<0.05$ versus LDL.

5.4.4 Effects of glyLDL on SCCR, UCCR, cytochrome *c* oxidase or CS activity: PAEC were treated with LDL or glyLDL (100 μg/ml) for 2-24 h. A significant decrease in SCCR activity was detected in PAEC treated with glyLDL for 12 or 24 h compared to control, while LDL had no effect on SCCR activity in EC (**Figure 43A**, $p<0.05$). The activity of UCCR was significantly reduced in PAEC after treatment with LDL or glyLDL for 6, 12 or 24 h ($p<0.05$ or 0.01). GlyLDL significantly reduced UCCR activity in EC after 12 or 24 h of incubation compared to LDL (**Figure 43B**, $p<0.05$ or 0.01).

Treatment of EC with LDL or glyLDL significantly reduced the activity of cytochrome *c* oxidase after 6-24 h. GlyLDL induced greater decrease in cytochrome *c* oxidase activity after 6 or 24 h of treatment compared to LDL (**Figure 43C**, $p<0.05$). Sodium cyanoborohydride was used as a reducing agent during glycation of LDL with glucose. To verify the effect of sodium cyanoborohydride present during glycation on Complex IV, we examined the effect of LDL treated with 50 mM sodium cyanoborohydride but without an addition of glucose on the activity of cytochrome *c* oxidase. The results demonstrated that LDL-modified with sodium cyanoborohydride at tested concentration had similar effects as unmodified LDL on the activity of cytochrome *c* oxidase after 12 h treatment. However, glyLDL had significantly greater effects compared to native LDL or sodium cyanoborohydride-modified LDL (**Figure 43D**, $p<0.05$). CS, a mitochondrial matrix-soluble enzyme, has been used as a control for mitochondrial enzyme activity. There was no change in CS activity in lipoprotein-treated EC (data not shown).

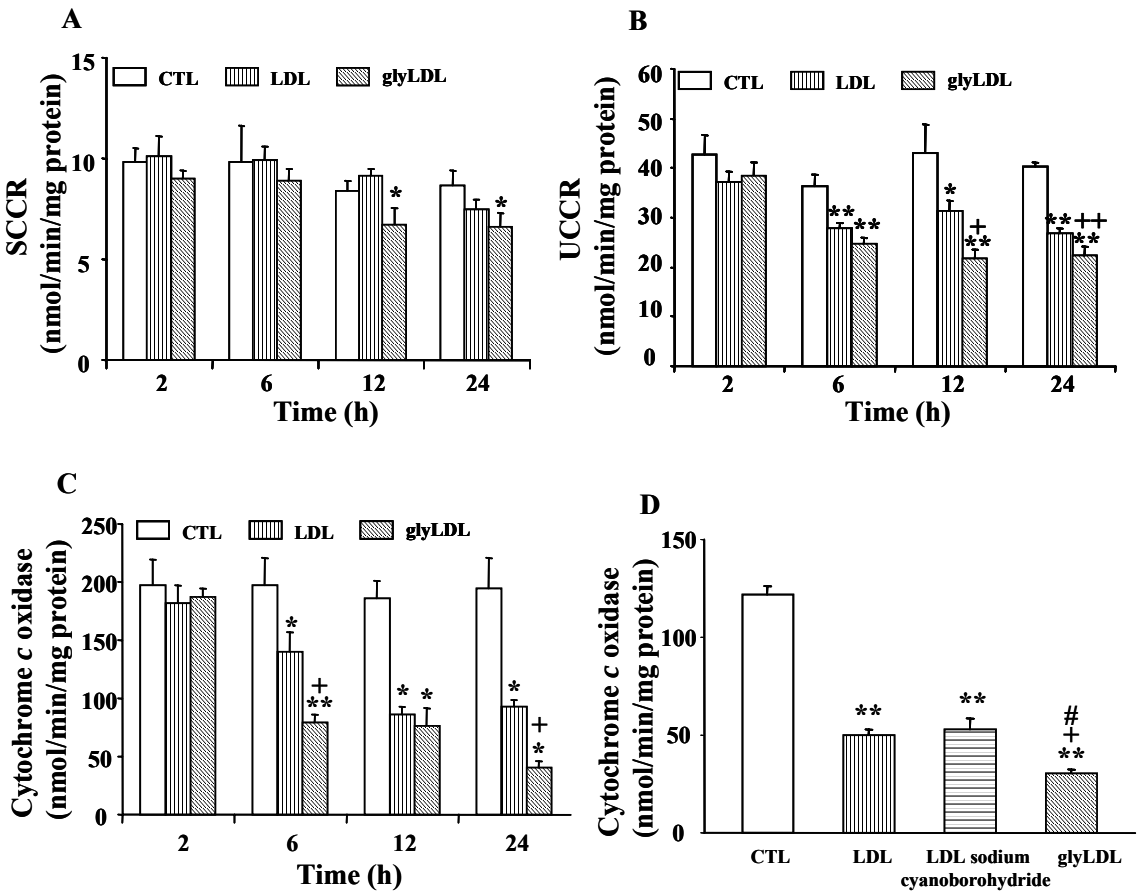
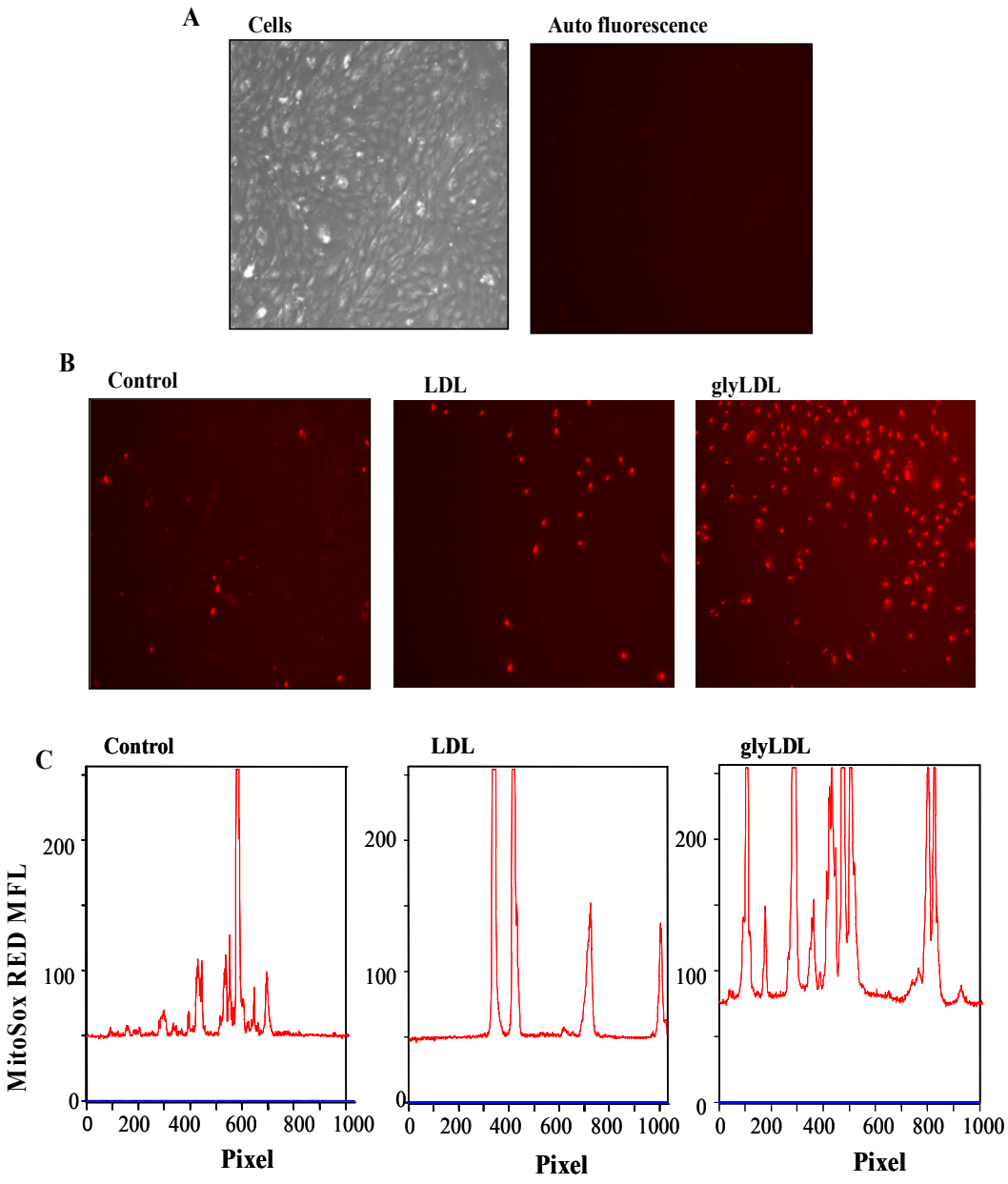


Figure 43. Effect of glyLDL on SCCR, UCCR, and cytochrome *c* oxidase activity in PAEC. Cells were treated for 2-24 h with 100 µg/ml of LDL, glyLDL or for 12 h with 100 µg/ml of LDL modified with sodium cyanoborohydride. Cell lysates were then used for following assays. **Panel A:** succinate cytochrome *c* reductase (SCCR) activity, **Panel B:** Ubiquinone cytochrome *c* reductase (UCCR) activity, and **Panel C/D:** cytochrome *c* oxidase activity using spectrophotometer. Values were expressed in nmol/min/mg protein (mean ± SEM, n = 3 experiments) after justification with cellular proteins. *, **: $p<0.05$ or 0.01 versus control; +, ++: $p<0.05$ or 0.01 versus LDL; #: $p<0.05$ versus LDL sodium cyanoborohydride.

5.4.5 Effect of glyLDL on the abundance of ROS in EC: To verify the effect of glyLDL on mitochondria-derived ROS production in EC, PAEC were treated with LDL or glyLDL (100 μg/ml) for 2 h and then labeled with a MitoSox Red. The release of ROS in mitochondria was visualized by using QCapture Pro imaging system. The results demonstrated that treatment with LDL or glyLDL increased the abundance of MitoSox Red in EC compared to control. Moreover, glyLDL demonstrated greater increase in the abundance of MitoSox Red compared to LDL (**Figure 44A-D**, $p<0.05$).

To determine the relationship between ROS and mitochondria, PAEC were labeled with MitoSox Red and MitoTracker Green (a marker of mitochondria). High resolution confocal microscopy was used to determine the relationship between MitoSox Red and mitochondria. Indeed, the ROS signal was well overlapped with MitoTracker Green, a mitochondria-specific dye. The results suggest that glyLDL-induced ROS is closely associated with mitochondria in EC (**Figure 44E**). Previously our group demonstrated that treatment with glyLDL increased the release of H_2O_2 from HUVEC (Zhao and Shen, 2005). However, the effect of glyLDL on ROS release from PAEC has not been described. PAEC were treated with 100 μg/ml of LDL or glyLDL for 2 h. The present study demonstrated that stimulation with glyLDL induced greater release of H_2O_2 from PAEC compared to control or LDL (**Figure 44F**, $p<0.05$ or $p<0.01$).


A
Cells
Auto fluorescence
B
Control
LDL
glyLDL
C
Control
LDL
glyLDL
MitoSox RED MFL
0
100
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Pixel

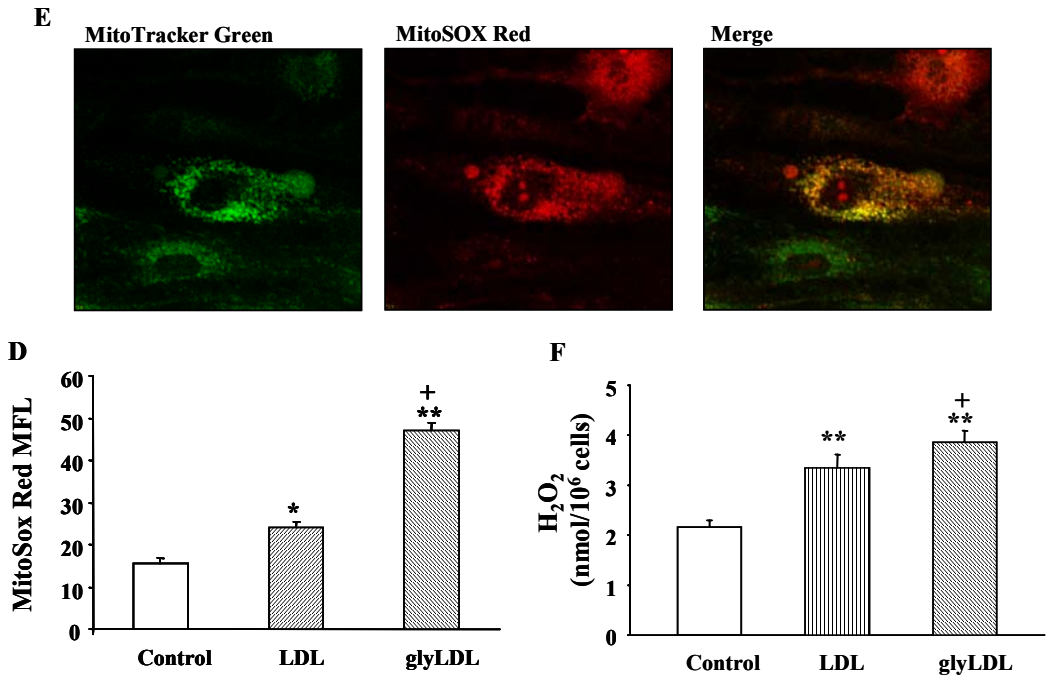

Figure 44. Effects of glyLDL on the ROS production in PAEC. PAEC were stimulated with vehicle (control), 100 μg/ml of LDL or glyLDL for 2 h. The production of ROS from mitochondria was determined in live cells using MitoSox Red as described in the Methods. **Panel A:** Cultured PAEC as a control (left panel) and background autofluorescence of cells without MitoSox Red staining (right panel). **Panel B:** Fluorescent images of EC incubated with vehicle (left), LDL (middle) or glyLDL (right), and MitoSox Red. The images were captured using Olympus IX71 inverted microscope coupled to QCapture Pro imaging system. **Panel C:** Examples of histograms from individual scan areas that show mean fluorescence (MFL) for MitoSox Red for EC treated with vehicle (left panel), LDL (middle panel) or glyLDL (right panel). **Panel D:** Comparison of integrative data of MitoSox Red MFL in EC treated with vehicle, LDL or glyLDL. **Panel E:** Confocal images after incubated with MitoTracker Green (left panel) or MitoSox Red (middle panel). A merged image shows significant overlapping of the two probes (yellow in right panel). **Panel F:** H_2O_2 levels in media were determined as described in the Methods. Values in panel D expressed in mean fluorescence per cell and panel F were expressed in nmol/10^6 cells. (mean ± SEM, n = 3 experiments). **: $p<0.01$ versus control; +: $p<0.05$ versus LDL.

5.4.6 Effect of glyLDL on mitochondrial membrane potential: PAEC were incubated with 100 μg/ml of LDL or glyLDL for 12 h. Stimulation with glyLDL or LDL induced evident shift in $\Delta\Psi_m$ compared to control (**Figure 45A**). The level of relative TMRM intensity induced by LDL or glyLDL was significantly lower than that in vehicle treated cultures (**Figure 45B**, $p<0.05$ or 0.01). The results suggest that glyLDL impaired mitochondrial respiration and electron transport in EC.

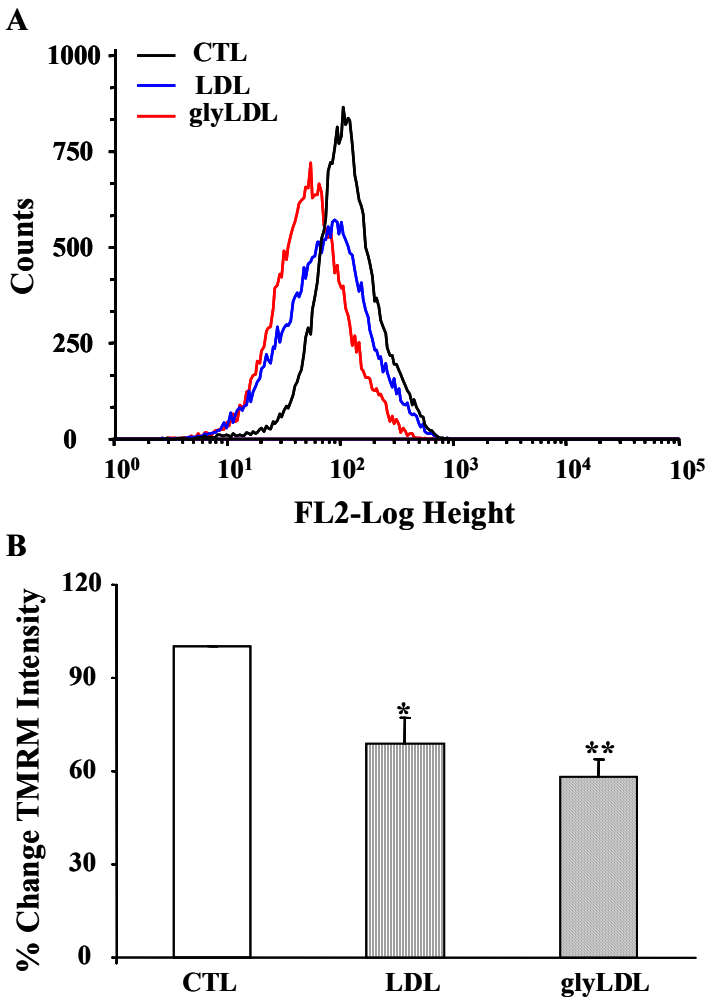

Figure 45. Effect of glyLDL on mitochondrial membrane potential in PAEC. Panel A: PAEC were incubated with LDL or glyLDL (100 μg/ml) for 12 h and then treated with 0.02 mg/mg protein of digitonin for permeabilization. The cells were incubated with TMRM as described in the Methods. Cytofluorometric analysis was done on an EPICS Altra Beckman Coulter flow cytometer. TMRM signal was detected in the FL2 channel. **Panel B:** High intensity signal for TMRM was calculated from change in median induced by control, LDL or glyLDL. Values were expressed in % change versus control (mean ± SEM, n = 3 experiments). *, **: $p<0.05$ or 0.01 versus control.

6. Discussion

Clinically, it has been shown that cholesterol lowering treatments such as statins reduced cardiovascular complications in diabetic patients. However, LDL levels in diabetic patients not always very high (Nesto, 2005). Elevated levels of oxLDL or glyLDL are evident in diabetic patients. GlyLDL undergoes modification *in-vivo* and forms glyco-oxidized LDL. These modified lipoproteins including oxLDL and glyLDL are involved in the upregulation of PAI-1 in vascular EC. The present study identified transmembrane signaling mechanisms involved in atherogenic lipoprotein-induced upregulation of PAI-1 in EC. OxLDL or glyLDL increases oxidative stress in EC via impairment of mitochondrial function and the activation of NOX. I also show that increased oxidative stress plays central role in oxLDL or glyLDL-induced expression of PAI-1 and stress response in vascular EC.

6.1 Transmembrane signaling pathway involved in oxLDL-induced expression of PAI-1 in vascular EC.

The present study demonstrated that oxLDL upregulates the expression of PAI-1 in EC via a novel transmembrane signaling pathway. We found that the stimulating effects of oxLDL on PAI-1 production in EC are mediated via a membrane receptor, LOX-1, and downstream signaling cascade that include membrane-associated, H-Ras, and the Raf-1/ERK-1/2 pathway (Sangle et al., 2008).

OxLDL exerts its biological effects via activation of its receptors on the surface of EC, macrophages, and SMC. Several types of membrane receptors are capable of

internalizing oxLDL, including SR-AI/II, SR-BI, LOX-1, CD36 and macrosialin/CD68 (Dhaliwal and Steinbrecher, 1999). LOX-1, found predominantly on EC, is the major receptor for oxLDL uptake in EC (Sawamura et al., 1997). The expression of both LOX-1 and PAI-1 are increased in atherosclerotic tissues (Arnman et al., 1994; Chen et al., 2000; Kataoka et al., 1999; Yorimitsu et al., 1993). Previous study indicated that LOX-1 antibody reduced thrombus formation in arteries of rats (Kakatani et al., 2000). The results of the present study demonstrate that oxLDL increases the abundance of LOX-1 in vascular EC. These findings are consistent with those of Sawamura et al (1997) in the same type of cells. In the present study, we demonstrated that oxLDL upregulated the expression of PAI-1 via LOX-1. LOX-1 blocking antibody efficiently suppressed oxLDL-induced PAI-1 expression. The results suggest that LOX-1 is involved in oxLDL-induced PAI-1 expression in cultured vascular EC. The findings suggest a new role of LOX-1 in atherogenic lipoproteins-induced thrombotic process.

Generally, the uptake of native LDL is regulated by LDLR. Previous studies demonstrated that oxLDL down-regulates the expression of LDLR in EC (Hu et al., 2003). However, the receptor responsible for LDL-induced PAI-1 expression in EC has not been identified. The present study demonstrated that the expression of LDLR was decreased by LDL, oxLDL or F-oLDL in EC, which may reflect negative feedback from increased intracellular cholesterol as previously described (Brown and Goldstein, 1986). LDLR antibody failed to prevent LDL or oxLDL-induced PAI-1 expression in EC, which is consistent with an earlier report by Tremoli et al (Tremoli et al., 1993). Prolonged incubation of LDL with EC, SMC or monocytes is associated with LDL oxidation.

Increased formation of lipid peroxidation products was detected in LDL after prolonged incubation with EC, and antioxidants inhibited LDL-induced PAI-1 generation and lipid peroxidation (Ren and Shen, 2000). LDL-induced PAI-1 expression in EC may result from EC-mediated oxidation of LDL (Ren and Shen, 2000; Shen, 2003). The present study demonstrated that LOX-1 blocking antibody partially inhibited LDL-induced PAI-1 expression in EC. The findings suggest that increased PAI-1 expression in EC induced by previously unmodified LDL is partially regulated via LOX-1. Though my studies did not directly assess whether cell-mediated oxidation of LDL occurred in EC culture, it seems reasonable to assume this contributed at least in part, to LOX-1 mediated effects of LDL. LOX-1 internalizes oxLDL in EC. However, involvement of internalization of oxLDL via LOX in oxLDL-induced PAI-1 is not extensively studied.

Membrane-associated small G proteins, such as Ras and Rho act as biological switches for the regulation of downstream signaling pathways in cell system (Puddu et al., 2005; Ruiz-Velasco et al., 2004). Oxidative stress increases the activation of H-Ras (Cuda et al., 2002). H-Ras mediates various signal transduction pathways that emanate membrane receptors. Farnesylation is essential for the translocation of Ras from the cytosol to the plasma membrane (Zhang and Casey, 1996). Previous studies demonstrated that LDL increased membrane translocation and activation of H-Ras in human EC (Zhu et al., 2001). The present study shows that incubation of EC with oxLDL rapidly increases translocation of H-Ras to the cell membrane. Prolonged incubation with oxLDL significantly increased mRNA and protein for H-Ras in EC. We further demonstrate that LOX-1 blocking antibody reduces oxLDL-induced H-Ras expression in EC. Farnesylation inhibitor, FTI-277, prevented oxLDL-induced PAI-1 expression and

release from EC, which was associated with reduced abundance of H-Ras. SiRNA against H-Ras efficiently blocked oxLDL-induced upregulation of H-Ras and PAI-1 in EC. This combination of findings suggests that the translocation of H-Ras to the membrane and increased H-Ras protein expression are required for oxLDL-induced upregulation of PAI-1 in cultured EC. H-Ras also mediates oxLDL-induced PAI-1 expression in other type of cells, such as mesangial cells as described previously (Song et al., 2008). LOX-1-oxLDL binding on the EC surface may regulate the activation of H-Ras in EC, though this was not directly assessed in the present study.

H-Ras is closely linked with oxidative stress. In fact, several lines of evidence suggest a regulatory role for intracellular ROS as a trigger for Ras activation (Cuda et al., 2002; Irani et al., 1997; Kowluru et al., 2004). LOX-1-oxLDL binding contributes to ROS production in EC (Cominacini et al., 2000). The uptake of oxLDL via LOX-1 may increase oxidative stress in EC, which may activate H-Ras. Previous studies by ours, and other groups demonstrated that oxLDL-induced rapid production of ROS in EC (Chen et al., 2007a; Zhao and Shen, 2005). ROS increased interactions between H-Ras and its downstream effectors (Cheng et al., 2001; Cuda et al., 2002; Kowluru et al., 2004). OxLDL-induced oxidative stress promotes apoptosis and DNA damage in endothelium (Chen et al., 2004; Wei et al., 1998). The present study indicated that BHT, a potent antioxidant, inhibited oxLDL-induced H_2O_2 production in EC. A recent study from our laboratory demonstrated that BHT inhibited PAI-1 production in EC induced by glyLDL (Zhao and Shen, 2007) or oxLDL (Zhao et al., 2009). A recent report demonstrated that Rho mediates high-glucose-induced PAI-1 expression in EC (Iwasaki et al., 2008), which

suggests the upregulation of PAI-1 in EC by other metabolic factors, may be modulated via a different type of small G-protein.

Multiple lines of evidence demonstrate close interactions between H-Ras and several downstream signal transduction pathways, such as Raf-1 (Kowluru et al., 2004), or other MAP kinases, including MEK, ERK-1/2, JNK and p38 kinase (Cuda et al., 2002; Xia et al., 1995). Activation of H-Ras recruits Raf-1 from cytosol to cell membrane, leading to the phosphorylation of Raf-1, which in turn phosphorylates and activates MEK and subsequently ERK-1/2 (Kowluru et al., 2004; Warne et al., 1993). Chen et al (2006b) reported that increased upregulation of PAI-1 in VSMC was associated with elevated phosphorylation of Raf-1. Yang et al (2001) demonstrated increased activation of the Ras/Raf/MEK/MAPK pathway by oxLDL in SMC. The results of the present study demonstrate that oxLDL increases phosphorylation of Raf-1 in EC. Raf-1 inhibitor blocked oxLDL-induced upregulation of PAI-1 and phosphorylation of Raf-1 in EC. These findings provide new evidence for a modulatory role of Raf-1 in oxLDL-induced increase of pro-thrombotic mediator in vasculature.

The present study demonstrates that the phosphorylation of ERK-1/2 is rapidly increased in EC exposed to oxLDL, which is consistent with previous studies in SMC and mesangial cells (Hong et al., 2006; Kusuhara et al., 1997). The present stydt demonstrate that PD98059, ERK-1/2 inhibitor, significantly inhibits oxLDL-induced PAI-1 expression suggesting that the activation of ERK-1/2 is required for this response.

The present study has identified the component of oxLDL responsible for upregulation of PAI-1 in EC exposed to oxLDL. Previous studies demonstrated that oxidized lipids and components of oxLDL induce LOX-1 expression (Kelly et al., 2008). Oxidized phospholipids-induced monocyte binding to EC is mediated via the activation of cAMP dependent R-Ras/PI3-kinase pathway (Cole et al., 2003). Oxidized phospholipids also activated pulmonary EC through ERK-1/2 cascade (Birukov et al., 2004). It is unclear whether oxidized lipids internalized via LOX-1 contribute to intracellular signaling for the upregulation of PAI-1 in EC. This possibility requires further investigation in subsequent studies.

The present study suggests that oxLDL activated H-Ras, Raf-1 and ERK-1/2 after 10-30 min. Previous studies from our laboratory showed that oxLDL significantly increased ROS production within 30 min and reached a peak around 2 h (Zhao and Shen, 2005). Oxidative stress activates HSF1 (Ahn and Thiele, 2003). GlyLDL or oxidized VLDL increased HSF1 expression as early as 2 h and peaked at 6 h. HSF1 mediates oxidized VLDL or glyLDL-induced PAI-1 expression in EC through its binding to a homologue of HSF1 in PAI-1 promoter (Zhao et al., 2008; Zhao and Shen, 2007). Recent studies in our laboratory demonstrated that oxLDL increased HSF1, in a similar pattern as glyLDL or oxidized VLDL (Zhao et al., 2009). Our present results indicated that oxLDL increased the levels of PAI-1 protein and mRNA which was initiated at 12 h and peaked at 24 h. NOX mediated ROS is involved in oxLDL-induced HSF1 and PAI-1 expression in EC (Zhao et al., 2009). The present study demonstrates that the increased ROS from oxLDL-treated EC may partially result from the activation of NOX. The

increased production of ROS via NOX may regulate oxLDL-induced activation of the Ras/Raf/ERK pathway, which further activates HSF1 and increases PAI-1 transcription and expression in EC at a later time interval (**Figure 46**).

The findings of the present study indicate that LOX-1, H-Ras and Raf-1/ERK-1/2 modulate oxLDL-induced PAI-1 upregulation in an interactive manner in cultured vascular EC. Further studies in atherosclerotic animal models may verify the findings *in-vivo*. Pharmacological interventions on the suggested signaling mediators potentially help to prevent atherogenic lipoprotein-induced intravascular thrombosis.

6.2 Mechanism for oxLDL-induced production of ROS from mitochondrial respiratory chain in aortic EC

Mitochondria are both sources and targets of ROS. There is growing evidence that mitochondrial dysfunction is a relevant intermediate mechanism by which cardiovascular risk factors lead to the formation of vascular lesions. OxLDL and hyperglycemia may induce the production of ROS in mitochondria of macrophages and EC. The present study for the first time demonstrated that oxLDL significantly reduced mitochondrial complex-specific oxygen consumption in PAEC. The results are consistent with the impairment of the activity of key enzymes in mitochondrial respiratory chain in PAEC induced by oxLDL (Roy Chowdhury et al., 2010).

Multiple lines of evidence suggest that oxidative stress is involved in the pathogenesis of CAD (Ballinger, 2005; Heinecke, 1998), which implies that oxLDL-

induced oxidative stress may promote the development of cardiovascular complications (Witztum and Steinberg, 1991). Previous studies from our laboratory demonstrate that oxLDL increases the generation of ROS from EC (Zhao and Shen, 2005). Mitochondria are an important source of ROS and contribute to oxidative stress in cells under pathological conditions (Turrens, 2003). The present study reveals that oxLDL increased the intracellular abundance of ROS and the release of ROS from aortic EC. ROS in PAEC treated with oxLDL were closely co-localized with the mitochondrial marker, MitoTracker. The mitochondrial dysfunction theory postulates that excess mitochondria-derived ROS contribute to functional vascular disorder (Ballinger, 2005; Madamanchi and Runge, 2007). This suggests that mitochondrial dysfunction may play a critical role in the development of atherosclerotic vascular disease.

NAD^+ and its reduced form, NADH, are mediators of various biological processes, including energy metabolism, mitochondrial electron transport, oxidative stress, aging, and cell death (Ying, 2006; 2008). NAD^+/NADH ratio is an index of cellular reducing potential and may be altered under various pathological conditions. Complex I is one of the main consumers of NADH in cells (Ying, 2008). Previous studies demonstrate that diabetes is associated with decreased NAD^+/NADH ratio in retinas of mice (Obrosova et al., 2001). Dysfunction of Complex I may affect NADH oxidation, reverse electron transfer and ROS generation (Grivennikova and Vinogradov, 2006). ROS generation in mitochondria is influenced by multiple factors, including the availability of electron donors such as NADH and $FADH_2$ (Ballinger, 2005; Puddu et al., 2009). A recent study demonstrated that the inhibition of Complex I reduced NAD^+

levels, which was associated with enhanced superoxide production (Kussmaul and Hirst, 2006). NO is involved in scavenging of superoxide via a direct radical-radical reaction to form peroxynitrite, a potent oxidant, capable of decreasing the mitochondrial electron transport and the activity of Complex I by forming S-nitrosothiols (Cassina and Radi, 1996; Dahm et al., 2006). OxLDL increases peroxynitrite levels in EC (Plant et al., 2008). The results of the present study demonstrate that compared to LDL, oxLDL significantly reduces the NAD^+/NADH ratio in EC. NAD^+ and NADH may be generated from other intracellular sources in addition to mitochondria. We hypothesize that oxLDL-induced reduction of the NAD^+/NADH ratio may be partially due to the dysfunction of mitochondrial Complex I in EC. The hypothesis is supported by our findings that oxLDL reduced ND activity (**Figure 32A**) and rotenone-inhibited mitochondrial oxygen consumption induced by malate + glutamate in EC (**Figure 29**).

Results from previous studies on the effects of oxLDL on mitochondrial Complex I activity in EC are controversial. A large volume of evidence suggests that oxLDL is pro-apoptotic (Boullier et al., 2006; Martinet and Kockx, 2001) and causes mitochondrial dysfunction (Asmis and Begley, 2003; Walter et al., 1998). Previous studies demonstrated that minimally oxidized LDL (TBARS around 8 nmol/mg protein) was anti-apoptotic for EC (Napoli et al., 2000), while oxLDL (TBARS >30 nmol/mg protein) has pro-apoptotic effects in EC (Boullier et al., 2006). Ceaser et al (2003) demonstrated that treatment with non-cytotoxic oxLDL (up to 150 μg/ml) increased activity of Complex I in HUVEC. Although the extent of oxidation of oxLDL was not provided in that report, we assume that oxLDL preparations used in that study were not extensively

oxidized, since an anti-apoptotic effect of the oxLDL was detected in EC (Ceaser et al., 2003). OxLDL preparations used in the present study were oxidized to a relatively more extensive extent (TBARS >30 nmol/mg protein), which may be assumed as apoptotic based on the results of previous studies (Boullier et al., 2006). The results from studies using this oxLDL indicated that it is pro-apoptotic with a time-dependent manner. Treatment with 100 μg/ml oxLDL beyond 60 h reduced cell viability in HUVEC. No significant change in cell viability was detected in EC treated with oxLDL (100 μg/ml) for 48 h or less, which was used in the present study. Treatment with oxLDL for 12 or 24 h caused mitochondrial dysfunction including the impairment of Complex I activity. In addition, treatment with moLDL, which is less oxidized than oxLDL, induced weaker inhibition on mitochondrial enzyme activity in PAEC, supporting our hypothesis that the effect of oxLDL on mitochondrial Complex I activity is oxidation-extent dependent. Antioxidant (BHT) treatment normalized oxLDL-induced inhibition of mitochondrial enzyme activities as well as ROS production in EC. However, the results do not contradict to the stimulating effect of non-apoptotic oxLDL on Complex I activity described in the previous report (Ceaser et al., 2003), and suggested that oxLDL may have dual effects on mitochondrial respiratory chain activity in EC partially depending on the extent of oxidation. Farnesyltransferase inhibitor (FTI-277) significantly inhibited oxLDL-induced ROS production and apoptosis in EC. The findings suggest that H-Ras may be therapeutic target for oxLDL-induced mitochondrial dysfunction in EC.

Mitochondria are considered an important target for ROS, including mitochondrial lipids, enzymes and DNA. Oxidized components of oxLDL are possible

candidates for inducing mitochondrial dysfunction and apoptosis in EC (Ballinger, 2005). Previous studies by us and other groups demonstrate that oxLDL increases the generation of ROS in EC (Zhao and Shen, 2005; Zmijewski et al., 2005). A recent study found that oxLDL-induced ROS generation from mitochondria is associated with Complex II (Mabile et al., 1997). Treatment with oxLDL significantly reduces the enzymatic activity of SCCR, a key enzyme for Complex II in EC. Oxidative stress mediates a cascade of events in EC including mitochondrial damage (Ballinger et al., 2000). Previous studies suggest that electrons derived from $FADH_2$ via Complex II can undergo "reverse electron transport" into Complex I (Liu et al., 2002). The findings from the present study suggest that Complex II may also be an important target of oxLDL in mitochondria. Previous studies demonstrate that mitochondrial Complex III is one of the major sites for ROS production in ETC (Madamanchi and Runge, 2007). The present study demonstrates that oxLDL significantly impairs enzymatic activity of UCCR in EC compared to LDL or vehicle control. Moreover, results from a cytometry-based assay demonstrate that mitochondria are an important source of ROS in EC exposed to oxLDL. The impairment of Complex II and III induced by oxLDL may bring an add-on effect to mitochondrial dysfunction in EC.

Cytochrome *c* oxidase is coded by both nuclear and mitochondrial DNA (Ceaser et al., 2003; Zhang et al., 2002). Deficiency or reduced activity of any component of the mitochondrial respiratory chain can affect ATP production; however, the dysfunction of Complex IV interrupts the final step of the mitochondrial respiration chain and promotes ROS production (Atamna et al., 2001). Impaired activity of Complex IV is associated

with aging and is believed to play a critical role in oxidative injury of senescent EC (Zhang et al., 2002). Previous study found non-apoptotic oxLDL does not significantly alter the activity of Complex IV in HUVEC (Ceaser et al., 2003). The present study is the first to show that oxLDL inhibits the activity of Complex IV in EC as determined using two independent approaches, ascorbate/TMPD-induced and KCN-sensitive oxygen consumption and cytochrome *c* oxidase activity.

In intact mitochondria of living cells, the addition of ADP causes a sudden burst of oxygen uptake, which is likely due to increased oxidative phosphorylation and the formation of ATP. The present study demonstrates that treatment with oxLDL for ≥12 h significantly reduces ADP-induced oxygen uptake and RCI in EC. RCI reflects the relationship between ATP synthesis and oxygen consumption in mitochondria. ATP levels are evidently decreased in atherosclerotic lesions and associated with hypercholesterolemia (Heinle, 1987). Previous studies demonstrate that oxLDL decreases ATP contents, mitochondrial respiration activity and mRNA expression of oxidative phosphorylation (OXPHOS) subunits in VSMC (Ahn et al., 2010). The present study demonstrate that FCCP, a mitochondrial uncoupler, reduces less than 50% of the oxLDL-induced decrease in oxygen consumption, which suggests that a large portion of oxLDL-induced decrease in mitochondrial respiration is not due to the reduction of ATP synthesis but results from dysfunction of mitochondrial electron transport (**Figure 31**). Mitochondrial dysfunction is involved in both reduced energy output and increased mitochondrial oxidative stress (Ballinger, 2005). The results of the present study suggest that oxLDL may simultaneously impair energy generation and oxygen consumption in

EC. RCI may be considered as a marker for mitochondrial dysfunction in EC induced by oxLDL.

BHT, a potent antioxidant, inhibited oxLDL-induced H_2O_2 release in vascular EC. The current studies demonstrate that BHT normalizes oxLDL-induced changes in the activity of Complex I and III. Generally, Complex I and III are considered as predominant sites in the ETC for ROS production (Madamanchi and Runge, 2007). The formation of mitochondria-derived ROS is associated with reduced mitochondrial membrane potential (Korshunov et al., 1997). Increased free cholesterol loading of macrophages is associated with mitochondrial dysfunction, as suggested by a decrease in mitochondrial membrane potential and an activation of the mitochondrial apoptosis pathway (Yao and Tabas, 2001). In the present study, we demonstrate that oxLDL attenuates mitochondrial membrane potential in aortic EC this is consistent with previous reports (Takabe et al., 2010), that indicated the effect stemmed from attenuated mitochondrial ETC enzyme activity and increased mitochondria-associated ROS in EC.

The present study indicates that oxLDL reduces the activities of multiple enzymes in mitochondrial respiratory chain complexes and corresponding complex-specific oxygen consumption in EC, which may contribute to increased ROS generation from EC. This suggests the possibility that mitochondrial dysfunction and ROS production induced by oxLDL may promote endothelial dysfunction in vasculature of patients with atherosclerotic vascular disease.

6.3 Signal transduction and transcriptional regulation of PAI-1 expression in EC induced by glyLDL

Elevated levels of glyLDL are detected in hyperlipidemic and hyperglycemic patients (Akanji et al., 2002; Tames et al., 1992). Previous studies in our laboratory demonstrate that HSF1 mediates glyLDL-induced PAI-1 production in vascular EC (Zhao and Shen, 2007). Transmembrane signaling mechanisms involved in glyLDL-induced PAI-1 are not known. The results of the present study demonstrate the following novel findings: a) RAGE is involved in glyLDL-induced PAI-1 upregulation in cultured vascular EC; b) NOX mediates glyLDL-induced HSF1 and PAI-1 upregulation in EC; c) H-Ras activation and Raf-1 phosphorylation are required for glyLDL-induced PAI-1 upregulation in EC; and, d) the abundance of RAGE, H-Ras, NOX, HSF1 and PAI-1 are increased in the hearts of STZ-diabetic mice correlating with elevated plasma glucose.

GlyLDL is generated from irreversible glycation. The process starts with non-enzymatic glycation of lysine or arginine residues in apoB and free amino residues in phospholipids on the surface of the lipid core of LDL under high levels of glucose. Previous studies demonstrate that glycation reduces the uptake of LDL by the LDLR (Lyons, 1993), but without a highly efficient uptake of glyLDL by SR (Witztum et al., 1982). GlyLDL is one of ligands for RAGE. Other non-AGE ligands binding to RAGE include beta-amyloid peptide, amphoterin and S100/calgranulins (Isoda et al., 2008; Neeper et al., 1992). Increased expressions of RAGE and PAI-1 are detected in atherosclerotic lesions or in the vascular walls of diabetic patients (Brett et al., 1993; Pandolfi et al., 2001; Sobel et al., 1998; Zhong et al., 2006). Berrou et al (2009)

demonstrated that a blocking antibody for RAGE inhibits AGE-induced PAI-1 gene expression and also decreased the PAI-1 accumulation in mesangial cells. GlyLDL treatment (100 μg/ml) used in the present study is within physiological range as previously reported (Tames et al., 1992). We demonstrate that a physiological concentration of glyLDL increased the abundance of RAGE in vascular EC, and RAGE antibody blocks glyLDL-induced PAI-1 expression. The findings indicate that RAGE mediates the transmembrane signaling of glyLDL-induced PAI-1 in EC.

Small G-proteins, such as Ras and Rho, are often associated with the activation of membrane receptors (Ruiz-Velasco et al., 2004). Hyperglycemia increases oxidative stress and H-Ras activation (Lander et al., 1997)). H-Ras exists in either an inactive GDP-bound cytosolic form or an active GTP-bound membrane-associated form. H-Ras undergoes post-translational modifications enabling its translocation from cytosol to plasma membrane; this process is called as farnesylation (Zhang and Casey, 1996). Previous studies demonstrated that LDL increases the membrane translocation of H-Ras in human EC (Zhu et al., 2001). Protein and mRNA levels of H-Ras are also increased in EC exposed to high glucose (Kowluru et al., 2004). Diabetes is associated with increased membrane expression of H-Ras in retinal EC (Kowluru and Kanwar, 2009). The results of the present study suggest that a short incubation (15 min) of EC with glyLDL increases membrane-associated H-Ras. Prolonged incubation of EC with glyLDL increases the abundance of H-Ras in cell lysate. The findings suggest that glyLDL rapidly activates H-Ras in EC, as evident by its translocation to the membrane, and increases the expression of H-Ras in EC after prolonged incubation. A previous study demonstrated

that the gene expression of H-Ras is increased in retinal EC of diabetic rats (Kowluru and Kanwar, 2009). The present study also demonstrates an increased expression of H-Ras in the hearts of STZ-diabetic mice, which may be a chronic response to hyperglycemia and hyperlipidemia in diabetic condition. FTI-277 blocked glyLDL-induced HSF1 and PAI-1 expression in EC. H-Ras siRNA effectively inhibited LDL or glyLDL-induced upregulation of HSF1 and PAI-1 in EC. A recent study suggests that small G-proteins, Ras and Rac, mediated cyclic strain stress-induced HSF1 activation and Hsp70 expression in VSMC (Xu et al., 2000). H-Ras regulates the expression of matrix metalloproteinase-9 in retinal EC, and its activation is associated with increased vascular permeability in diabetes (Kowluru, 2010). Lander et al (1997) reported that AGE/RAGE interaction-induced ROS activates the p21Ras/MAPK pathway, which results in nuclear translocation of NF-κB. The combination of findings from the present and previous studies suggests that glyLDL activates H-Ras, which is required for glyLDL-induced expression of HSF1 and PAI-1 in vascular EC.

Previous studies by our group demonstrated that glyLDL-induces a quick generation of ROS from EC (Zhao and Shen, 2005). Multiple lines of evidence suggest that intracellular ROS may activate H-Ras (Cuda et al., 2002). NOX is one of the major sources of ROS in vascular cells. The expression of NOX has been detected in EC (Griendling et al., 2000). The upregulation of NOX activity is often associated with the progression of vascular disorders related to diabetes or obesity (Lassegue and Clempus, 2003). NOX is involved in AGE-induced ROS production in human EC (Wautier et al., 2001). The present study indicates that DPI blocks glyLDL-induced H_2O_2 production in

EC, which is consistent with results from a previous study using glyco-oxidized HDL in EC (Matsunaga et al., 2003). The findings suggest the involvement of NOX in glycated-lipoprotein induced ROS production in EC. $P22^{phox}$, the only membrane-associated subunit beside the catalytic core in NOX complex, is essential for the activation of NOX. $P22^{phox}$ is detected in advanced atherosclerosis plaques, suggesting a correlation between $p22^{phox}$, superoxide production and severity of atherosclerotic lesions (Sima et al., 2009). Our recent study demonstrated that siRNA against $p22^{phox}$ suppresses the expression of NOX2 in EC induced by oxLDL (Zhao et al., 2009). The results of the present study demonstrated that $p22^{phox}$ siRNA blocks glyLDL-induced NOX2, HSF1, and PAI-1 expression in EC. DPI blocked glyLDL-induced H-Ras translocation as well as ROS production in EC. These findings suggest that NOX-mediated ROS is directly implicated in glyLDL-induced upregulation of HSF1 and PAI-1 in EC. The present study also demonstrated increased expressions of HSF1 and PAI-1, in addition to NOX, in STZ-induced diabetic mice, which provides *in-vivo* evidence for associations between NOX, HSF1 and PAI-1 under diabetic conditions. Our findings support a recent report on the increased protein and mRNA of NOX in diabetic mice (Gao et al., 2008). Mechanism for glyLDL-induced upregulation of NOX in EC remains unclear. Oxidative stress triggers Ras activation and activated Ras may further stimulate ROS production via NOX activation (Cuda et al., 2002). Thus, the translocation of Ras to the membrane is one of the important steps in activating downstream signaling pathway resulting in accelerated oxidative stress via NOX in EC. A positive feedback between Ras and NOX may play a critical role in glyLDL-induced upregulation of HSF1 and PAI-1 in EC (**Figure 46**).

H-Ras interacts with Raf-1 resulting in its translocation to the plasma membrane, and Raf-1 acts as a key effector protein of H-Ras function. Raf-1 is a common downstream target protein of Ras activation. Activated Raf-1 subsequently phosphorylates MEK/ERK-1/2 (Kowluru et al., 2004; Kyriakis et al., 1992). A recent study demonstrated the involvement of ROS, ERK-1/2 and NF-κB in AGE-induced PAI-1 mRNA (Berrou et al., 2009). Chen et al (2006b) reported that the increased expression of PAI-1 is associated with an elevated phosphorylation of Raf-1 in VSMC. The H-Ras/Raf-1 pathway is involved in hyperglycemia-induced oxidative stress and apoptosis in retinal EC (Kowluru et al., 2004). The present study demonstrates that the phosphorylation of Raf-1 is increased in EC incubated with glyLDL. Treatment with Raf-1 inhibitor blocked glyLDL-induced upregulation of PAI-1 mRNA and Raf-1 phosphorylation in EC, which implicates Raf-1 in glyLDL-induced PAI-1 production in EC. Raf-1 expression is increased in retinal EC under diabetic condition (Rayappa and Kowluru, 2008). A recent study demonstrated that the level of pRaf-1 is increased in retinal EC of diabetic rats (Kowluru et al., 2004). Our findings suggest a new functional role of Raf-1 in terms of glyLDL-induced PAI-1 production in vascular EC. The direct target for activated Raf-1/ERK-1/2 induced by glyLDL in EC remains unclear.

The upregulation of PAI-1 mRNA in glyLDL-treated EC may be a consequence of increased transcription of PAI-1 gene, decreased degradation of PAI-1 mRNA or both. Our previous data demonstrate that glyLDL, oxLDL or oxidized VLDL increased the transcription of PAI-1 gene (Zhao et al., 2008; 2009; Zhao and Shen, 2007). The increases of PAI-1 protein and mRNA in glyLDL-treated EC are consistent with our

findings in hearts of STZ-diabetic mice. The results do not exclude the possibility of the coexistence of decreased degradation of PAI-1 mRNA, which potentially contributes to elevated PAI-1 protein or mRNA as indicated by other groups (Rikitake and Liao, 2005).

Cells respond to environmental stresses, such as heat shock, ROS, and shear force, via a variety of post-translational modifications of proteins or stress responses. Stress responses in cells are chiefly mediated by Hsp. Increased expression of Hsp has been implicated in the pathogenesis of atherosclerosis (Metzler et al., 2003). The transcription of Hsp genes is regulated by HSF. Interaction between HSF and heat shock responsive elements in promoters of targeted proteins triggers the transcription of Hsp genes (Pirkkala et al., 2001). The increased expression of HSF1 is detected in human atherosclerotic lesions (Rocnik et al., 2000), which suggest that stress response is enhanced under hypercholesterolemia. Our previous study demonstrated that glyLDL, oxLDL or oxidized VLDL enhances the expression of HSF1 in vascular EC. HSF1 is required for the upregulation of PAI-1 in vascular EC through the binding of HSF1 to PAI-1 promoter induced by the modified lipoproteins (Zhao et al., 2008; 2009; Zhao and Shen, 2007). The results demonstrate that transmembrane signaling mediators, RAGE, H-Ras, Raf-1, and NOX are involved in glyLDL-induced upregulation of HSF1 and PAI-1 in EC, which provides additional insight for diabetes-associated metabolic stress-induced upregulation of stress response mediator and fibrinolytic regulator in vasculature.

Treatment with glyLDL increased H-Ras translocation as early as 5 min. The increased abundance of H-Ras reached a peak around 15 min after the start of incubation

with glyLDL. Phosphorylation of Raf-1 was increased in EC within 5-10 min of exposure to glyLDL. Increased phosphorylation of ERK-1/2 was detected at 15 min interval induced by glyLDL. Activated H-Ras may stimulate ROS production by activating NOX (Cuda et al., 2002; Kowluru et al., 2004). A recent study demonstrated that AGE-LDL increases NOX activity in EC via RAGE (Toma et al., 2009). The abundance of NOX2 in EC was increased by glyLDL and reached a peak around 1 h. The activation of NOX increases the generation of ROS (Cuda et al., 2002; Wautier et al., 2001). Our previous studies demonstrated that glyLDL significantly increase superoxide and H_2O_2 production in EC within 30 min, reaching a peak around 2 h (Zhao and Shen, 2005). Elevated ROS may trigger the activation of H-Ras (Cuda et al., 2002). Oxidative stress activates HSF1 (Ahn and Thiele, 2003). Treatment with glyLDL increased HSF1 expression in EC as early as 2 h and reached a peak at 6 h. HSF1 further regulates the transcription of PAI-1 in EC induced by glyLDL as previously described (Zhao and Shen, 2007). Taken together, glyLDL-induced PAI-1 expression in EC may be a consequence of the serial activation of RAGE, H-Ras, Raf-1/ERK-1/2, NOX and HSF1 (**Figure 46**). ROS might be a critical factor in glyLDL-mediated effects in EC as previously reported (Toma et al., 2009). Other source of ROS in glyLDL-induced oxidative stress in EC, including mitochondria, remains to be investigated.

The results of the present study indicate that glyLDL upregulates HSF1 and PAI-1 in EC via a transmembrane receptor, RAGE, distinct from that of oxLDL. NOX-mediated ROS generation appears to be required for glyLDL-induced PAI-1 and HSF1 in EC. H-Ras and Raf-1/ERK-1/2 are required for both glyLDL and oxLDL-induced PAI-1 and

HSF1 upregulation in EC. The identification of signaling mediators involved in glyLDL-induced PAI-1 production provides potential pharmacological targets for the prevention of diabetes-associated metabolic stress-induced thrombotic events.

6.4 Impairment of mitochondrial respiratory chain activity in aortic EC induced by glyLDL

Hyperglycemia and dyslipidemia are two major risk factors for diabetic vascular complications. Hyperglycemia promotes the glycation and oxidation of lipoproteins. The present study provides the first evidence that glucose-modified LDL impairs the function of the mitochondrial ETC in cultured PAEC. The major novel findings in this section of studies include: a) glyLDL reduce oxygen consumption in multiple mitochondrial complexes in EC; b) glyLDL decrease the activities of Complexes I-IV and NAD^+/NADH ratio in EC; c) glyLDL increased the abundance of mitochondria-associated ROS and the release of ROS from PAEC; and, d) glyLDL attenuated mitochondrial membrane potential in EC (Sangle et al, 2010).

Increased lines of evidence suggest the involvement of ROS in the pathogenesis of diabetes-associated vascular complications (Giugliano et al., 1996). Elevated production of ROS via the mitochondrial ETC is the prominent feature of most diabetic cardiovascular complications (Brownlee, 2001). Previous study from our laboratory demonstrated that glyLDL increases ROS generation from HUVEC (Zhao and Shen, 2005). Underlying mechanisms for glyLDL-induced ROS production remains unclear. The mitochondrial respiratory chain is one of the potential sources of intracellular

oxidative stress (Turrens, 2003). A recent study found that mitochondrial respiration was decreased in skeletal muscle of patients with type 2 DM compared with control subjects (Rabol et al., 2009). In diabetes, electron transfer and oxidative phosphorylation are uncoupled which results in increased superoxide formation and inefficient ATP synthesis (Green et al., 2004). The present studies demonstrate that glyLDL impairs mitochondrial oxygen consumption in response to substrates for Complex I, II/III or IV in aortic EC. Furthermore, glycation enhances the inhibitory effect of LDL on electron transfer by inhibiting the enzymatic activities of mitochondrial ETC complexes in EC. Impaired activity of mitochondrial complex enzymes redundant leads to the increased formation of ROS in mitochondria. This supports our hypothesis that mitochondria may be one of important sources for excess ROS generation under diabetes-associated metabolic stress.

The present study demonstrated that incubation with glyLDL significantly reduces ADP-induced oxygen uptake and RCI in EC. RCI reflects interactions between ATP synthesis and oxygen consumption in mitochondria. Impaired mitochondrial function and reduced ATP synthesis is observed in subjects with a family history of diabetes (Petersen et al., 2004). A recent study demonstrated that AGE significantly inhibits ATP production in islet β-cells (Zhou et al., 2009). Decline in the capacity for ATP production by impairment of mitochondrial function may contribute to the development of heart failure (Huss and Kelly, 2005; Rolo and Palmeira, 2006). Mitochondrial dysfunction is involved in reduced energy output and increased oxidative stress (Ballinger, 2005). The results of the present study provide additional evidence that

diabetes-associated lipoproteins simultaneously impair energy generation and oxygen consumption in mitochondria of EC.

NAD^+/NADH ratio is considered as an index of cellular reducing potential and may be altered under various pathological conditions. NAD^+ and its reduced form, NADH, are implicated in various biological processes, including mitochondrial electron transport, oxidative stress, aging and cell death. Complex I is one of the main users of NADH in cells (Ying, 2006). Decreased activity of Complex I was detected in a patient with maternally inherited type 2 DM (Chen et al., 2006a). Hyperglycemia-induced ROS overproduction induces DNA strand breakage, poly (ADP-ribose) polymerase cleavage, NAD^+ depletion, and reduced rate of electron transport and ATP formation (Pacher and Szabo, 2005). Recent studies demonstrated a decreased NAD^+/NADH ratio in retinas of diabetic mice (Obrosova et al., 2001). Coughlan et al (2009) demonstrated attenuated Complex I activity in mesangial cells exposed to AGE in both normal and high glucose environments. Inhibition of Complex I reduces NAD^+ levels, which is associated with enhanced superoxide production (Kussmaul and Hirst, 2006). A decrease in Complex I activity may retard oxidation of NADH (Kaur and Bhardwaj, 1998). Our studies for the first time demonstrate that glyLDL significantly reduces ND activity and NAD^+/NADH ratio in EC. However, NAD^+ may be generated from other intracellular sources in addition to mitochondria. We hypothesize that the decreased NAD^+/NADH ratio induced by glyLDL may partially result from an impairment of Complex I activity in EC. This hypothesis is supported by our findings that glyLDL reduces rotenone-inhibited

mitochondrial oxygen consumption responding to substrates of Complex I in EC (**Figure 39**).

Mitochondrial ETC is one of the major sources of ROS generation in EC induced by AGE (Basta et al., 2005). Mitochondria are also considered as an important target for ROS, which may damage mitochondrial lipids, enzymes and DNA with ensuing mitochondrial dysfunction. OxLDL and glyLDL are possible contributing factors for mitochondrial dysfunction and apoptosis in CAD or diabetes (Puddu et al., 2005). Previous studies by our group demonstrated that glyLDL increases the generation of ROS in EC (Zhao and Shen, 2005). ROS are produced at Complexes I and III, but possibly also at Complex II (McLennan and Degli Esposti, 2000). Complex II is involved in ROS generation in EC exposed to high glucose (Nishikawa et al., 2000). The results of the present study demonstrate that glyLDL significantly reduces SCCR activity in EC compared to LDL or control, which provides additional evidence for the impact of diabetes-associated metabolic factors on the activity of mitochondrial Complex II in vascular EC.

Previous studies demonstrated that mitochondrial Complex III is involved in AGE-induced ROS production in EC (Basta et al., 2005). Complex III has also been reported as a target for oxidative damage induced by ROS in bovine heart mitochondria (Paradies et al., 2001). Recent studies demonstrate a significant decline in Complex III activity in diabetic rat kidney as well as retina (Kowluru et al., 2006; Munusamy et al., 2009). High glucose-induced superoxide production within mitochondria of rat renal

proximal tubular cells initiates a reduction of ATP levels and partial Complex III inactivation (Munusamy and MacMillan-Crow, 2009). These findings demonstrate that glyLDL, compared to LDL or control significantly impairs enzymatic activity of UCCR in EC. The results of the present study are indirectly supported by a recent study by Wang et al (2009), which shows that methylglyoxal decreases Complex III activity in VSMC. Moreover, we demonstrate that MitoSox stains in PAEC treated with glyLDL were closely co-localized with a mitochondrial marker. This suggests that mitochondria are a principal source of ROS in EC exposed to glyLDL.

The majority of oxygen consumed by the mitochondrion is converted to water at Complex IV (Ballinger, 2005). Impaired activity of cytochrome *c* oxidase interrupts the final step of mitochondrial respiratory chain which promotes ROS production (Atamna et al., 2001). Inhibition of electron flow at Complex IV may also increase mitochondrial ROS generation (Cassina and Radi, 1996). A recent study demonstrated that cultured human EC exposed to shear stress results in the inactivation of Complexes I-IV, at least in part, due to the formation of ROS in the mitochondria (Han et al., 2007). The dysfunction of Complex IV is associated with aging and plays a major role in oxidative injury of senescent EC (Zhang et al., 2002). The present study provides original evidence that in EC exposed to glyLDL, the activity of Complex IV enzyme is impaired as determined using two independent approaches, oxygen consumption and enzymatic activity of Complex IV. Although, KCN is a known inhibitor of Complex IV, the results of the present study indicate that LDL modified with sodium cyanoborohydride alone did

not alter Complex IV enzyme activity compared to LDL without an exposure to the reducing agent.

The formation of mitochondria-derived ROS is associated with changes in loss of mitochondrial membrane potential (Korshunov et al., 1997). A recent study demonstrated that increased ROS production in EC induced by AGE is associated with decreased mitochondrial membrane potential, indicating that the treatment with AGE induces the depolarization of mitochondrial membrane (Zhou et al., 2009). The results of the present study are consistent with this study, and demonstrate that glyLDL induced evident shift in mitochondrial membrane potential, which may result from the decrease of mitochondrial ETC enzyme activity and associated with increases of both mitochondria-associated ROS and the release of ROS from EC. The combination of results suggests that glyLDL induces ROS production and depolarization of mitochondrial membrane via the dysfunction of mitochondrial respiratory chain in EC. Impairment of mitochondrial respiration and increased ROS production induced by glyLDL may play important roles in the pathogenesis of diabetic macrovascular complications.

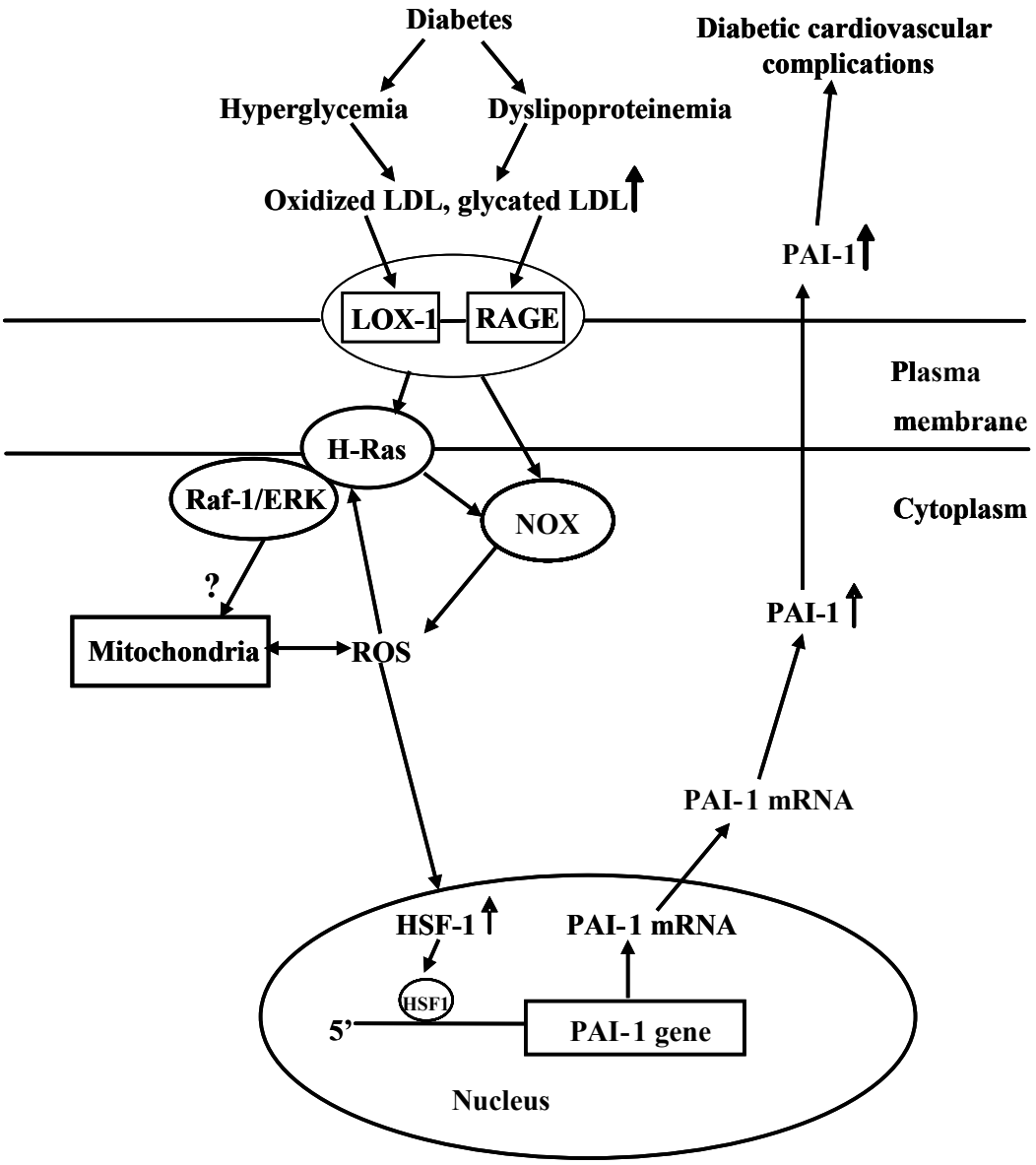

Figure 46. Scheme for signaling mechanisms involved in oxidized or glycated LDL-induced oxidative stress and PAI-1 production in vascular EC. LDL, low-density lipoprotein; LOX-1, lectin-like oxLDL receptor-1; RAGE, receptor for advanced glycation end products; ERK, extracellular signal-regulated kinase ½; NOX, NADPH oxidase; ROS, reactive oxygen species; HSF-1, heat shock factor-1; PAI-1, plasminogen activator inhibitor-1.

7. Conclusions and potential applications

Diabetes-associated LDL contributes to oxidative stress and atherosclerotic cardiovascular disease. LDL has tendency to undergo oxidation and glycation. Elevated levels of oxLDL, glyLDL or glyco-oxLDL were detected in diabetic patients. Our studies demonstrate a relationship between atherogenic lipoproteins, oxidative stress and the signaling mechanisms involved in EC. Elevated expression of PAI-1 in the vasculature is involved in intravascular thrombosis and progression of atherosclerosis. OxLDL and glyLDL-induced PAI-1 production in EC is mediated via two distinct transmembrane receptors, LOX-1 and RAGE. LOX-1 may also be involved in glyLDL-induced PAI-1 production in EC. The Ras/Raf-1 pathway is implicated in both oxLDL and glyLDL-induced PAI-1 production in EC.

Mitochondria and NOX are two major sources of ROS in cell system. Increased hyperglycemia and dyslipoproteinemia in diabetes may cause glyco-oxidation of LDL, mitochondrial dysfunction and NOX activation, further contributing to oxidative stress in the vasculature. Treatment with oxLDL or glyLDL impairs oxygen consumption, increases ROS production and reduces the activities of mitochondrial ETC enzymes leading to mitochondrial dysfunction in EC. ROS may regulate intracellular signaling related to diabetes-associated metabolic stress. The identified signaling mediators involved in oxLDL or glyLDL-induced oxidative stress, mitochondrial dysfunction, stress response, and PAI-1 production may be considered as potential drug targets for the prevention or treatment of diabetic cardiovascular complications.

The interactions between LOX-1 and RAGE induced by oxLDL or glyLDL could provide more information on signaling mechanisms. Most of the available evidence on relationship between oxLDL or glyLDL-induced oxidative stress and PAI-1 is based on *in-vitro* experiments. Moreover, subsequent studies in atherosclerotic or diabetic animal models may be useful to verify the findings *in-vivo*. Farnesyltransferase inhibitors could be a potential pharmacological target for therapeutic intervention against diabetic cardiovascular disease, but more *in-vivo* studies are needed to verify these findings. Mitochondria, NOX, or their regulators plays a critical role in sequence of cellular events that leads to a variety of diabetic complications. Further studies are required to examine effects of mitochondrial respiratory chain enzyme activators, NOX inhibitors or antioxidants on potential signaling pathways involved in oxLDL or glyLDL-induced oxidative stress in vasculature.

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9. Appendix

1. Sangle GV, Zhao R, Shen GX (2010). Involvement of RAGE, NADPH oxidase and Ras/Raf-1 pathway in glycated LDL-induced expression of heat shock factor-1 and plasminogen activator inhibitor-1 in vascular endothelial cells. Endocrinology *in press*.

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