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COMPREHENSIVE INVITED REVIEW

Oxidative Stress and Lectin-Like Ox-LDL-Receptor LOX-1 in Atherogenesis and Tumorigenesis

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Abstract

Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) has been identified as a major receptor for oxidized low-density lipoprotein (ox-LDL) in endothelial cells, monocytes, platelets, cardiomyocytes, and vascular smooth muscle cells. Its expression is minimal under physiological conditions but can be induced under pathological conditions. The upregulation of LOX-1 by ox-LDL appears to be important for physiologic processes, such as endothelial cell proliferation, apoptosis, and endothelium remodeling. Pathophysiologic effects of ox-LDL in atherogenesis have also been firmly established, including endothelial cell dysfunction, smooth muscle cell growth and migration, monocyte transformation into macrophages, and finally platelet aggregation—seen in atherogenesis. Recent studies show a positive correlation between increased serum ox-LDL levels and an increased risk of colon, breast, and ovarian cancer. As in atherosclerosis, ox-LDL and its receptor LOX-1 activate the inflammatory pathway through nuclear factor-kappa B, leading to cell transformation. LOX-1 is important for maintaining the transformed state in developmentally diverse cancer cell lines and for tumor growth, suggesting a molecular connection between atherogenesis and tumorigenesis. *Antioxid. Redox Signal*. 15, 2301–2333.

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I. Introduction

Oxidative stress plays a significant role in the genesis and progression of atherosclerosis and its complications. The well-known risk factors for atherosclerosis, such as hypertension, dyslipidemia, diabetes mellitus, and smoking, are associated with enhanced oxidative stress.

Oxidative stress is defined as excessive generation of reactive oxygen species (ROS) beyond the body's innate ability to counter their noxious effects. There is also growing evidence that oxidative stress is seen in development and growth of several tumors. The phenomenon of aging is also characterized by accumulation of products of oxidant species in endothelial cells. Some investigators have ascribed oxidant stress to the pathogenesis of neurological diseases, such as Alzheimer's disease.

Excessive generation of ROS results in the oxidation of a 52-kDa lectin-like receptor for low-density lipoprotein (LDL)-cholesterol (LOX-1). LOX-1 facilitates the internalization of ox-LDL and enhances the generation of ROS—suggesting appositive feedback loop between oxidative stress and LOX-1. This receptor gains significance because this is the only receptor of ox-LDL on endothelial lining of blood vessels. This article is a review of the role of oxidative stress and LOX-1 in atherogenesis and its complications. We also review the role of oxidative stress and LOX-1 in tumorigenesis.

II. Reactive Oxygen Species, LOX-1, and Atherogenesis

A. Identification, regulation, and physiological functions of LOX-1

Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) is a type II membrane protein with extracellular domain and a short cytoplasmic tail. This molecule was initially identified as a major receptor for oxidized low-density lipoprotein (ox-LDL) in endothelial cells. Subsequent work showed that this receptor is also expressed in monocytes, platelets, cardiomyocytes, and vascular smooth muscle cells (VSMCs) as well as in renal, pulmonary, and neuronal tissues. Structurally, it belongs to the C-type lectin family, which binds carbohydrates in a Ca^{2+}-dependent manner, and is comprised of four domains: a short N-terminal cytoplasmic domain, a single transmembrane domain, a short "neck" or stalk region, and a C-type lectin-like fold (Fig. 1). This C-type lectin-like fold is highly conserved within LOX-1 mammalian orthologs, notably at six cysteine residues that underpin the lectin-like fold by forming three intramolecular disulfide bonds. In particular, the large loop between the third and fourth cysteine of the lectin-like domain plays a crucial role for ox-LDL binding, as well as C-terminal end residues. Simultaneous mutations of these basic residues abolish the ox-LDL-binding activity of LOX-1. An electrostatic interaction between basic residues in the lectin-like domain of LOX-1 and negatively charged ox-LDL is critical for the binding activity of LOX-1. N-glycosylation of LOX-1 regulates the protein folding within the endoplasmic reticulum, secretory transport to the plasma membrane, and ligand recognition. Chen *et al.* (44) found that the positively charged amino acids within the lectin-like domain cooperatively recognize ox-LDL, which exhibits strong negative charge since the lipid peroxidation products were generated and linked to its apolipoprotein B moiety.

Expression of LOX-1 is minimal under physiological conditions but can be induced under pathological conditions,

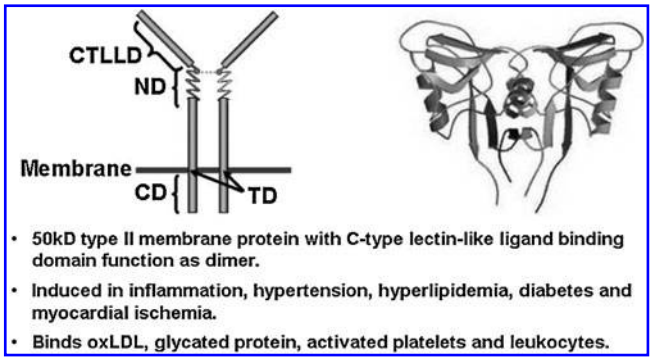

FIG. 1. Lectin-like oxidized low-density lipoprotein receptor-1 structure. LOX-1 is a 50-kDa type II membrane protein with C-type lectin-like ligand binding domain (CTLLD) that functions as a dimer. It belongs structurally to the C-type lectin family, which binds carbohydrates in a calcium-dependent manner, and is comprised of four domains: a short N-terminal cytoplasmic domain (CD), a single transmembrane domain (TD), a connecting neck domain (ND), and a lectin-like domain at the C-terminus. Some of the properties of LOX-1 are mentioned here.

such as diabetes mellitus, hypertension, myocardial ischemia, and atherosclerosis. LOX-1 is one of nontraditional scavenger receptors (SRs) that help in internalization and degradation of ox-LDL. Activation of LOX-1 is responsible for ox-LDL and angiotensin II (Ang II)-mediated cell injury and biological effects such as regulation of blood pressure. LOX-1 activation also may be the key intermediary for the cross-talk between renin-angiotensin system and dyslipidemia that is often present in hypertensive patients (37, 101, 147, 203, 248).

LOX-1 can be activated by ox-LDL, free radicals (ROS), endothelin-1, Ang II, advanced glycation end products, the L5 component of LDL, an electronegative component of LDL abundant in dyslipidemic but not in normolipidemic human plasma, and shear stress. It is interesting that ox-LDL can upregulate its own receptor at transcriptional level in human endothelial cells in a time- and concentration-dependent fashion. The upregulation of LOX-1 in response to ox-LDL can be blocked by a specific antibody or antisense to LOX-1 mRNA.

The cytokine tumor necrosis factor-alpha (TNFα), a pro-inflammatory cytokine present in large amounts in atherosclerotic regions and in the ischemic hearts, increases cell-surface expression of LOX-1 in a concentration-dependent manner with a peak time to expression of 8–12 h. TNFα also activates the transcription of LOX-1, as measured by nuclear run-off assay. Shear stress in the physiological range (1–15 dyn/cm^2) has also been shown to upregulate LOX-1 in a time-dependent fashion.

Although the expression of LOX-1 in different cells in resting state is rather small, these low levels may be important in maintenance of physiologic growth of endothelium and blood vessels (140, 187, 239). Resting LOX-1 expression may also a have a bearing on blood pressure regulation, although the resting blood pressure is similar in wild-type and LOX-1-null mice (208). There is also developing information that LOX-1-null mice may have longer lifespan (55, 103).

B. ROS, ox-LDL, and LOX-1

Upon chemical or biological perturbation, ROS are produced in various tissues and circulating cells such as leukocytes and platelets (66, 184). The NADPH oxidase system is the most potent ROS generating system in vascular endothelial cells. The prototype phagocytic NADPH oxidase is composed of membrane-bound gp91^{phox} and p22^{phox}, as well as cytosolic subunits such as p47^{phox}, p67^{phox}, and small GTPase Rac. In endothelial cells, in addition to all components of phagocytic NADPH oxidases, homologs of gp91^{phox} (Nox2), including Nox1, Nox4, and Nox5, are expressed. The molecular mechanism of NADPH oxidase activation in endothelial cells is best characterized for the Nox2-based oxidase and Nox1. In general, Nox2 oxidase activation of endothelial cells involves a translocation of cytosolic oxidase components (p47^{phox}, p67^{phox}, and GTPase Rac) to the plasma membrane and association with cytochrome b_{558}, which initiates the electron transfer process.

ROS oxidatively modify native-LDL, resulting in the formation of ox-LDL fractions. It is now amply evident that ox-LDL is much more important than native LDL in atherogenesis (184, 193). There continue to be some important questions relative to generation of ox-LDL; for example, which compartment of the vessel wall or outside the vessel wall is native-LDL oxidized, and how does ox-LDL traverse the endothelium and reside in the sub-endothelial layers to be taken up by monocytes/macrophages. Cominacini *et al.* (53) showed that ROS can induce the expression of LOX-1. In other studies, they (54) showed that LOX-1 activation *per se* can stimulate ROS generation, suggesting a positive feedback loop between ROS and LOX-1. Ox-LDL induces transcriptional upregulation of LOX-1 in isolated endothelial cells and in other cell types (137, 187). Further downstream activation of LOX-1 also results in the generation of large amounts of ROS (54, 158). Indeed, ROS enhances LOX-1 and LOX-1 enhances ROS. This positive feedback has a multitude of benefits particularly under low levels of stress. Dandapat *et al.* (56) showed that small concentrations of ox-LDL (<5 μg protein/ml) promote capillary tube formation by inducing low levels of ROS release. This process involves LOX-1-mediated activation of NADPH oxidase-MAPKs-NF-κB pathway. The evidence for the role of proposed pathway in ox-LDL-mediated capillary tube formation came from the use of specific inhibitors of NADPH oxidase, p38, and p44/42 mitogen-activated protein kinase (MAPKs), as well as the use of gp91^{phox} NADPH oxidase knockdown experiment. However, if ROS generation is excessive, ROS-LOX-1 axis can induce cell injury.

The NADPH oxidase system is the most potent ROS-generating system in vascular endothelial cells. ROS induce the transcription of LOX-1, and LOX-1 enhances ROS generation. LOX-1 is key step in the activation of NADPH oxidase-MAPKs-NF-κB pathway (53). The key post-translational modifications involved in oxidase activation are the phosphorylation of p47^{phox}, p67^{phox}, and GTPase Rac. This relationship between NADPH oxidase and LOX-1 was investigated by Hu *et al.* (108, 111). They provided evidence that p47^{phox}, p22^{phox}, gp91^{phox}, and Nox-4 subunits of

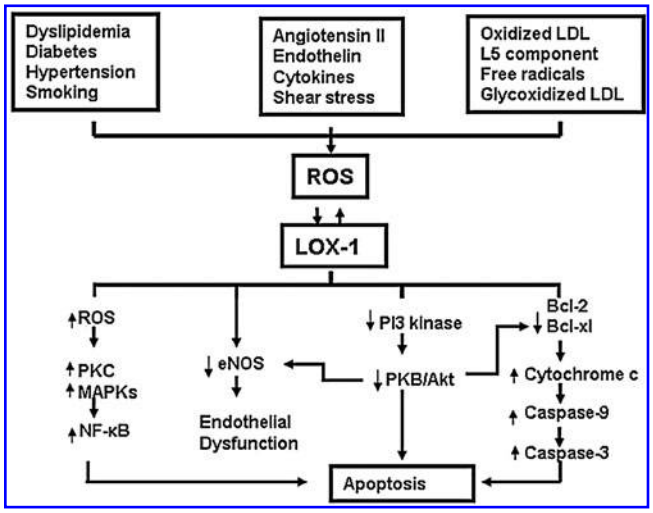

FIG. 2. Interaction between reactive oxygen species (ROS), LOX-1, and apoptosis. ROS are induced in response to a host of disease states and mediators. The L5 component of low-density lipoprotein (LDL) is an electronegative component of LDL abundant in dyslipidemic but not in normolipidemic human plasma. ROS induce expression of LOX-1; LOX-1 activation in turn induces release of ROS, suggesting a positive feedback loop between oxidative stress and LOX-1 expression. LOX-1 activation then leads to apoptosis of endothelial cells (ECs). Adapted from Li and Mehta (150).

NADPH oxidase are markedly increased in the LDL receptor (LDLR) knockout (KO) mice. The upregulation of NADPH oxidase subunits was reduced by LOX-1 deletion in the LDLR KO mice. It is noteworthy that the expression of all four subunits of NADPH oxidase was lower in the LOX-1 KO mice, indicating that LOX-1 deletion reduces the basal expression of NADPH oxidase. During ischemia-reperfusion, both $p22^{phox}$ and $p47^{phox}$ subunits of NADPH oxidase were increased in both wild-type and LOX-1 KO mice (*vs.* sham ischemia-reperfusion mice), but the LOX-1 KO mice had a smaller increase. Taken together, these data suggest that ROS serve as the pivotal "signal bridge" between the NADPH oxidase and LOX-1 (Fig. 2). Further studies are required to confirm the integral role for NADPH oxidase in modulating LOX-1 expression.

C. ROS, LOX-1, and transcription factors

ROS serve as common intracellular messengers of the activation of the redox-sensitive transcription factor, nuclear factor-kappa B (NF-κB). Although other transcription factors may also be involved in the transcriptional regulation of LOX-1, most studies have emphasized an important role of NF-κB in a variety of disease states, including atherogenesis, myocardial ischemia, hypertension, and tumorigenesis (51, 64, 83, 85, 108, 128). The discussion here will focus on the regulation of LOX-1 expression and activity. It is now known that the self-amplifying positive feedback loop between ROS and LOX-1 involves NF-κB expression. This interaction may be associated with cell-survival, motility, and cell activation as well as cell death.

We and others (53, 56, 87, 159) have shown that H_2O_2 is an activator of NF-κB in endothelial cells, and that overexpression of catalase blocks NF-κB activation induced by TNFα, in which H_2O_2-induced NF-κB activation can occur without degradation of IκBα (122). Further, Nox2 transcription is dependent on NF-κB; two potential *cis*-acting elements in the murine Nox2 promoter control NF-κB-dependent regulation (4).

The transcriptional mechanism for ox-LDL-induced human LOX-1 gene expression by "promoter bashing" was studied by Chen *et al.* (33), who identified that the promoter region between nt-1494 and −1599 is required for ox-LDL-induced LOX-1 promoter activation in human coronary artery endothelial cells (HCAECs). Finer mutational analysis and electrophoretic mobility shift assays strongly indicated that the Oct-1 binding site within this region plays a critical role in human LOX-1 promoter transactivation in response to ox-LDL. These authors (34) also examined the mechanism of Ang II–induced human LOX-1 promoter activation using the same methodology. As will be discussed later, Ang II is a powerful stimulus for LOX-1 gene upregulation. Interestingly, Chen and colleagues found that the promoter region between nt-2131 and −2247, which includes an active NF-κB binding site, is required for Ang II–induced human LOX-1 promoter transactivation (34).

D. Protein kinases in LOX-1 activation and signaling events promoted by LOX-1

Li *et al.* identified an important role of protein kinase C plays in LOX-1-induced intracellular signaling (148). LOX-1 *via* downstream signaling involving protein kinase C mediates the expression of CD40 and CD40 ligand in endothelial cells in response to ox-LDL. These findings indicated that ox-LDL through LOX-1 triggers CD40 signaling pathway that activates inflammatory response in endothelial cells. Other intracellular protein kinases, such as p42/44MAPK, also known as extracellular signal-regulated kinase (ERK1/2), and p38MAPK play a critical signaling pathway in LOX-1-mediated gene expression of monocyte chemoattractant protein-1 (MCP-1) and other adhesion molecules that subsequently lead to enhanced monocyte adhesion to activated endothelial cells. Related studies showed that the activation of these redox-sensitive transcription factors is mediated *via* different signaling pathways depending on the stimulus. Aside from MAPKs, c-Jun N-terminal kinase (JNK), and p21-activated kinase (PAK), other kinases such as Src kinases, phosphoi-

Table 1. The Signaling Events Promoted by LOX-1 Dependent on Oxidized Low-Density Lipoprotein

Signaling events promoted by LOX-1	*Abbreviations*	*Reference*
p38 mitogen-activated protein kinase	p38 MAPK	Chen, *et al.*[32] *Atherosclerosis* 184: 295–301 Hu, *et al.*[110] *Hypertension* 50: 952–957
p42/44 mitogen-activated protein kinase	p42/44 MAPK	Chen, *et al.*[32] *Atherosclerosis* 184: 295–301 Hu, *et al.*[107] *Hypertension* 50: 952–957
Phosphoinositide 3-kinase	PI3K/Akt	Lu, *et al.*[172] *Circ Res* 104: 619–627
c-Jun NH2-terminal kinase	JNK	Takabe, *et al.*[256] *Arterioscler Thromb Vasc Biol* 30: 436–441
Protein kinase C	PKC	Li, *et al.*[148] *Arterioscler Thromb Vasc Biol* 23: 816–821
p21-activated kinase	PAK	Hsu, *et al.*[105] *J Biol Chem* 276: 28719–28730
Protein tyrosine kinase	PTK	Li *et al.*[151] *Am J Physiol* 275: H568–H576
Angiotensin II type 1 receptor	AT1R	Kang, *et al.*[126] *Physiol Genomics* 42: 42–54 Kang, *et al.*[125] *J Cardiovasc Pharmacol* 55: 176–183
Peroxisome proliferator-activated receptor gamma	PPARγ	Mehta, *et al.*[185] *Arterioscler Thromb Vasc Biol* 23: 2203–2208
Lectin-like oxidized low-density lipoprotein receptor-1	LOX-1	Hu, *et al.*[108] *Cardiovasc Res* 76: 292–302
Nicotinamide adenine dinucleotide phosphate-oxidase	NADPH oxidase	Hu, *et al.*[108] *Cardiovasc Res* 76: 292–302 Hu, *et al.*[111] *Cardiovasc Res* 79: 287–293

nositide 3-kinase (PI3K)/Akt, and protein tyrosine kinase (PTK) may also play a role in this process. The most acknowledged signaling events promoted by LOX-1 are shown in the Table 1.

E. LOX-1 and apoptosis

Several lines of evidence suggest that ox-LDL results in cell injury. Recent studies from our laboratory and other groups have shown that ox-LDL can induce apoptosis in a variety of cell types, including HCAECs, smooth muscle cells, and macrophages (35, 144, 150, 172). The ox-LDL-induced apoptosis involves downregulation of antiapoptotic proteins c-IAP-1and Bcl-2, (156) release of cytochrome *c* and Smac, activation of pro-apoptotic signals caspase-9 and caspase-3, and finally induction of apoptosis. On the other hand, ox-LDL does not affect Fas, c-FLIP, or caspase-8 pathway in HCAECs. These observations provide specificity into the causative role of LOX-1 in ox-LDL-mediated endothelial injury (Fig. 1). The concentrations of ox-LDL required to induce apoptosis are usually $>20\,\mu$g protein/ml; these concentrations are quite high and seen only in patients with acute coronary syndromes (74, 243, 267). Low physiologic concentrations ($<5\,\mu$g protein/ml) do not cause significant apoptosis, and are associated with cell proliferation. This dose-dependant effect of ox-LDL on cell survival/death will be discussed in greater detail later in this article.

Recently, Takabe *et al.* (256) found that ox-LDL-induced JNK activation regulates mitochondrial redox status and manganese superoxide dismutase (MnSOD) protein degradation *via* JNK-dependent ubiquitination, leading to endothelial cell apoptosis. Ox-LDL-induced caspase-3 activity was attenuated by JNK inhibition, and enhanced by MnSOD inhibition using small interfering RNA. Further, overexpression of MnSOD abrogated ox-LDL-induced caspase-3 activities (256). The involvement of LOX-1 in this process is not known.

F. LOX-1 and endothelial cell proliferation

As discussed above, LOX-1 is key step in the activation of NADPH oxidase- MAPK-NF-κB pathway. MAPKs constitute a large kinase network that regulates physiologic processes such as cell growth and differentiation, and apoptosis. Three MAPK pathways have been characterized: the ERK pathway, the JNK stress-activated protein kinase, and the p38 MAPK pathway. In general, ERK1/2 signaling promotes cell survival under mild oxidative stress, whereas JNK and p38 MAPK induce apoptosis by involving direct phosphorylation of antiapoptotic Bcl-2, transactivation of transcription factor-activator protein-1 (AP-1), and stabilization of p53 (18, 37, 150, 152, 162). p38 MAPK also acts as a pro-inflammatory signal (66, 256) (Fig. 3).

Signal transduction *via* PI3K plays an important role in regulating cell proliferation, survival, and motility. A moderate level of ROS activates PI3K signaling and promotes cell survival. PI3K/protein kinase B (also known as Akt) transduces the signal for cell survival mainly through phosphorylation of target molecules by Akt. This results in inactivation of pro-apoptotic proteins and activation of transcription factors that target the expression of antiapoptotic proteins.

Dandapat *et al.* (56) showed that small concentrations of ox-LDL ($<5\,\mu$g protein/ml) promote capillary tube formation by inducing low levels of ROS release *via* LOX-1-mediated activation of NADPH oxidase-MAPKs-NFκB pathway. They found that the small concentrations of ox-LDL that are probably physiologic led to LOX-1 transcription also induced NADPH oxidase (both $gp91^{phox}$ and $p47^{phox}$ subunits), activated MAPK (both p38 and p44/42 components), and NF-κB p65, resulting in capillary formation from HCAECs. The evidence for the role of proposed pathway in ox-LDL-mediated capillary tube formation came from the use of specific inhibitors of NADPH oxidase, p38 MAPK, and p44/42 MAPK, as well as the use of $gp91^{phox}$ NADPH oxidase knockdown experiment. The NADPH oxidase inhibitor apocynin and

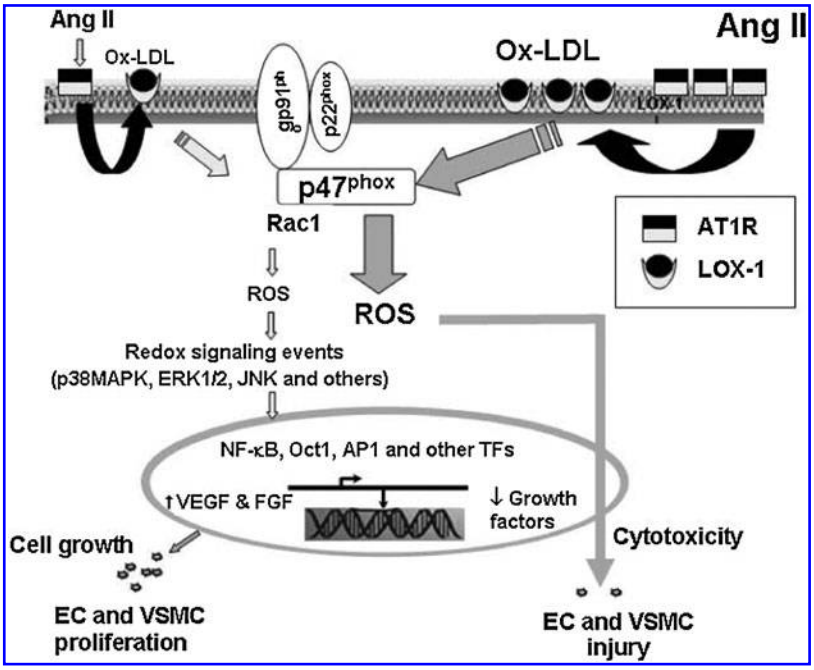

FIG. 3. Interaction between oxidized-LDL (ox-LDL) and angiotensin II (Ang II) on cell biology. ox-LDL at low concentrations induces LOX-1 expression, and resultant activation of NADPH oxidase and MAPKs followed by translocation of redox-sensitive transcription factor nuclear factor-kappa B (NF-κB). This subsequently induces VEGF transcription, which contributes to EC and smooth muscle cell (SMC) proliferation. At high concentrations, ox-LDL induces LOX-1 overexpression and generation of large amounts of ROS, which inhibit cell growth or induce direct cytotoxicity [adapted from Dandapat *et al.* (56)]. Ox-LDL *via* LOX-1 induces transcription and activation of angiotensin II type 1 receptor (AT1R), which in turn upregulates the expression of LOX-1. This positive feedback loop between dyslipidemia and renin-angiotensin system leads to the genesis of Ang II–induced hypertension and subsequent cardiac remodeling, particularly in patients with dyslipidemia.

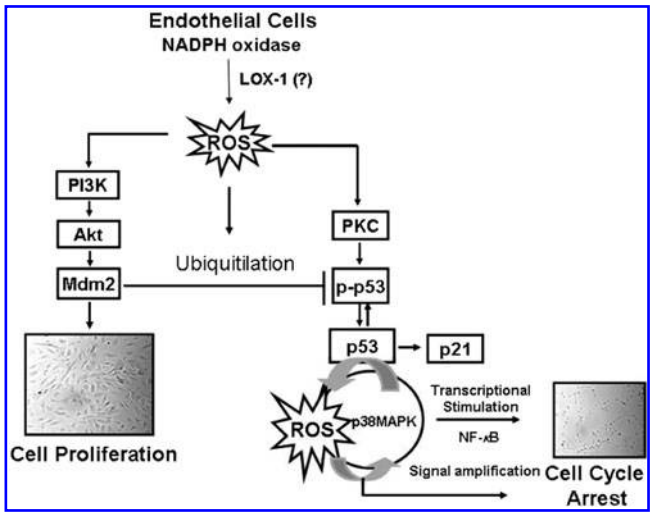

FIG. 4. A hypothesized model of a close interaction between ROS generation and expression of cell cycle regulatory proteins. Generation of ROS in small amounts (*left*) enhances murine double minute2 (Mdm2) expression mainly *via* phosphatidylinositol 3-kinase (PI3k/Akt) activation. Activation of PI3K/Akt pathway also results in phosphorylation and stabilization of Mdm2. Accumulation of Mdm2 represses p53 activity by p53 ubiquitylation and degradation. This results in cell proliferation. ROS in high concentration (*right*) activates, phosphorylates, and amplifies p53 expression. Large amounts of ROS in a positive feedback fashion with p53- result in activation of p38 mitogen-activated protein kinase (p38 MAPK) and transcriptional stimulation of NF-κB resulting in cell cycle arrest and apoptosis as end-result.

siRNA gp91^{phox} blocked the downstream signaling of MAPKs-NFκB pathway. The p38 MAPK inhibitor SB203580 and the p44/42 MAPK inhibitor U0126 blocked NF-κB expression (Fig. 2).

G. Cell cycle regulatory proteins and cell survival

Endothelial cells undergo significant change in response to changes in hemodynamic forces to maintain homeostasis and an anticoagulant smooth surface. Depending on the milieu, the endothelium can undergo angiogenesis (proliferation) or remodeling (apoptosis) during state of ischemia and oxidative stress. In this process, the state of NADPH oxidase activation serves a very major role, particularly as a regulator of cell cycle. A variety of signaling proteins are involved in the activation of ROS-MAPKs-NF-κB pathway (Fig. 4).

It is now well established that cyclins play a positive role in cell cycle transition *via* their ability to associate with and activate their cognate cyclin-dependent kinases (CDKs). A novel CDK-interacting protein, p21 (also designated WAF1/CIP1), has been identified in cyclin A, cyclin D1, cyclin E, and Cdk2 immunoprecipitates. p21$^{WAF1/CIP1}$, a potent inhibitor of CDKs, has been implicated in the control of G1 to S phase transition in mammals. It regulates cell cycle progression and inhibits apoptosis of endothelial cells in a concentration-dependent manner working in a coordinated manner with a series of regulatory molecules such as p53. Low levels of p21^{cip1} have been shown to be necessary to prevent endothelial cells from undergoing apoptosis, whereas high concentrations of p21^{cip1} lead to cell cycle arrest (162, 196, 214).

Both p21$^{WAF1/cip1}$ and p53 have been reported to be redox sensitive. In fact, p53-dependent upregulation of proline oxidase and downregulation of MnSOD results in the generation of ROS that in turn activates p53 in a redox-regulatory loop *via* p38 MAPK (16, 256). Suppression of MnSOD by p53-activation is an alternative pathway to oxidative stress. This p53/ROS cross-talk appears important in the regulation of cellular redox balance. p53 activation coordinates many cellular stress responses by regulating genes involved in DNA repair, cell cycle arrest, and apoptosis. Following stress stimuli, p53 is activated through a variety of post-translational modifications, including phosphorylation on serine 15 (88, 196, 198, 206).

More candidates (18, 242, 250) have been added to the list of p53-induced pro-oxidant genes, which include BAX, PUMA, and p66^{Shc}. Suppression of the antioxidant gene MnSOD by p53 activation or overexpression is an alternative way to increase cellular ROS, conferring oxidative stress. This p53/ROS cross-talk is important to regulate cellular redox balance. In the absence of strong stress stimuli, p53 is expressed at low level, which leads to upregulation of antioxidant genes. However, in cells with strong damage, the p53 pro-oxidant function is activated and p53 target genes involved in apoptosis are expressed. In fact, the p53 pathway uses the G_1/S and G_2/M checkpoint mechanisms to arrest cell cycle progression and thus prevent propagation of DNA damage, whereas cells attempt to repair it. However, if the damage is too severe, activation of the p53 pathway results in apoptotic cell death as the ultimate means of preventing possible malignant transformation of the damaged cells. Since the cell cycle proteins depend at least in part on ROS generation, it is likely that LOX-1 activation is an important intermediary step (Fig. 3).

H. Role of LOX-1 in atherogenesis

Most cardiovascular risk factors, such as smoking, diabetes mellitus, dyslipidemia, and hypertension, induce oxidative stress in the vessel wall. As LDL-cholesterol traverses the subendothelial space, it becomes oxidized in response to oxidative stress, resulting in the formation of ox-LDL. A number of clinical and epidemiological studies suggest that ROS oxidize lipids and that the oxidatively modified LDL is a more potent pro-atherosclerotic mediator than the native unmodified LDL (193). The suggestion is based on the observations that high plasma levels of ox-LDL are present in patients with atherosclerosis and metabolic disorders, and in atherosclerosis-prone apolipoprotein E-null mice (115a, 142, 215). Ox-LDL is found in atherosclerotic lesions, and one of its many pro-atherogenic properties is its cytotoxicity toward all cells in the vessel wall.

In general, increased production of ROS may affect all four fundamental mechanisms that contribute to atherogenesis: oxidation of LDL, endothelial cell dysfunction, VSMC growth, and monocyte migration and their transformation into foam cells. As the process of atherogenesis proceeds, inflammatory cells and other constituents of the atherosclerotic plaque release large amounts of ROS, which further facilitate atherogenesis.

The term "endothelial dysfunction" has been used to refer to several pathological conditions, including altered antico-

agulant and anti-inflammatory properties of the endothelium, impaired modulation of vascular growth, and deregulation of vascular remodeling. However, an important characteristic of endothelial dysfunction is impaired synthesis, release, and activity of endothelium-derived nitric oxide (NO). Studies from several laboratories have demonstrated that endothelial NO inhibits a number of steps involved in atherogenesis (54, 184, 260). For example, endothelium-derived NO inhibits endothelial activation (characterized by a pro-adhesive cell surface), platelet aggregation, and VSMC proliferation and endothelium–leukocyte interactions. Inactivation of NO by superoxide anions that are generated in large amounts in all atherosclerosis risk factors limits the bioavailability of NO and leads to nitrate tolerance, vasoconstriction, and hypertension as well as atherogenesis. Ox-LDL and other mediators of atherosclerosis such as Ang II, by increasing ROS generation and LOX-1 upregulation, induce endothelial dysfunction.

Proliferation of VSMCs is a characteristic feature of atherosclerosis. Indeed ROS have been shown to stimulate VSMC growth. The increase in VSMC growth by ROS results as a result of the increased generation of a variety of growth factors, such as fibroblast growth factor (28, 29), insulin-like growth factor-1, and epidermal growth factor and expression of their receptors (121). There is increasing evidence that ROS, *via* NADPH oxidase activation, play a critical role in Ang II–induced VSMC proliferation and hypertrophy. It is well known that ROS can also induce VSMC death by either apoptosis or necrosis, but this process, which occurs in the final stage of atherosclerosis, requires large amounts of ROS. Further, ox-LDL enhances the formation of matrix metalloproteinases (MMPs) in vascular endothelial cells and fibroblasts without significant effect on tissue inhibitors of metalloproteinase (TIMPs). This may well be the basis of the rupture of the soft atherosclerotic plaque leading to acute coronary syndromes (111, 114, 251). In addition, ox-LDL upregulates the expression of its own receptor LOX-1 on endothelial cells and other SRs, such as class A macrophage SR type I/II(SR-A I/II), class B macrophage SR type I (SR-BI), CD36, CD68, and macrophage receptor with a collagenous structure (MARCO) on macrophages/monocytes. The increased expression of these receptors is responsible for the uptake of ox-LDL and the formation of foam cells, which is another hall-mark of atherosclerosis.

It is increasingly being recognized that atherosclerosis is a chronic inflammatory disease whose pathogenesis involves disturbed lipoprotein metabolism, the formation of pro-inflammatory lipid peroxidation products, and the host immune response. Ox-LDL activates inflammatory and immune response cells and facilitates the release of a number of growth factors from monocytes/macrophages. Increased adhesion of monocytes to endothelial cells has been linked to the development and progression of atherosclerosis in humans. At least one study has shown that elevated concentrations of glucose induce monocyte adhesion to endothelial cells, and this effect is mediated by increased production of ROS (164). A number of studies have shown that ROS upregulate the expression of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), MCP-1, P-selectin, L-selectin, E-selectin, and platelet endothelial cell adhesion molecule-1 in vascular endothelial cells (30, 33, 152, 237). Expression of these molecules is critical in the adhesion of monocytes to endothelial cells. Ox-LDL also triggers the CD40/CD40L signaling pathway that activates the inflammatory reactions. Other studies have shown that induction in arterial wall of heme-oxygenase, which scavenges ROS, may lead to the attenuation of monocyte adhesion, and may represent a regulatory mechanism to counter the effects of ROS (45, 47).

Palinski and Witztum (219) showed that atherosclerotic lesions contain a wide variety of lipid peroxidation products, which in turn can be recognized by specific innate and adaptive immune responses. LDL is oxidized, generating various oxidation-specific neoepitopes, such as malondialdehyde-modified (MDA-modified) LDL (MDA-LDL) or the phosphorylcholine headgroup of oxidized phospholipids. These epitopes are recognized by both adaptive T cell-dependent and innate T cell-independent type 2 immune responses. These authors also showed that immunization of mice with MDA-LDL induces a T cell-dependent response and atheroprotection (215, 216). It was reported that interleukin (IL)-5 links adaptive and natural immunity specific to epitopes of ox-LDL and protects mice from atherosclerosis, in part by stimulating the expansion of atheroprotective natural IgM specific for ox-LDL (15). Their experiments left no doubt that ox-LDL was indeed generated *in vivo* in the artery wall and atherosclerotic lesions. They further showed that autoantibodies to such epitopes were present in humans and were related to the extent of atherosclerosis. Navab and Berliner (210, 211) provided evidence that specific pro-inflammatory oxidized phospholipids that result from the oxidation of LDL phospholipids containing arachidonic acid are recognized by the innate immune system in animals and humans. These oxidized phospholipids are largely generated by potent oxidants produced by the lipoxygenase and myeloperoxidase pathways. Indeed, their observation that ox-LDL was immunogenic and that the ensuing immune responses were sufficiently important to modulate atherogenesis contributed significantly to the growing awareness of the importance of immune responses in the pathogenesis of atherosclerosis.

Almost all studies have shown adverse effect of ox-LDL, including cell apoptosis and death. Of note, the concentration of ox-LDL used in these studies has ranged from 10 to 100 μg protein/ml, concentrations that are at least 1-log, and may be 2-logs, higher than those seen in normal human sera. These high concentrations of ox-LDL ($\geq 10\mu$g protein/ml) induce profound activation of endothelial cells followed by their death. It is interesting that the pathways leading to ox-LDL-induced cell injury appear to be the same that leads to cell growth observed in response to small concentrations of ox-LDL. The only difference seems to be the generation of large amounts of ROS when high concentrations of ox-LDL are used. One of the manifestations of endothelial cell growth appears to be angiogenesis, which is induced by small amounts of ox-LDL (29) and Ang II (110)-mediated *via* activation of LOX-1.

The process of angiogenesis in atherosclerosis has important clinical consequences. Angiogenesis seems to have both beneficial and deleterious effects in atherosclerosis and its consequences. Progressive angiogenesis in a primary atherosclerotic lesion may cause plaque expansion and plaque vulnerability, and enhance the risk of significant clinical disease by promoting intravascular thrombosis. The development of an atherosclerotic lesion involves three distinct pathoanatomic stages. In the initiation stage, injury to cell function

occurs to such a degree that overall tissue structure sustains specific alterations. As shown in animal models of atherogenesis, this initial stage is already associated with formation of vasavasorum in areas of subsequent atherosclerotic plaque development. The overall appearance of these vasavasorum (or neovasculature) is fairly disorganized and similar to the vascular network surrounding a cancerous lesion. In the progression stage, there is extension of adventitial neovasculature to the media and eventually into the enlarging plaque, whereby the composition of the neovasculature is reduced from a complete blood vessel with a smooth muscle cell layer to a ring of capillary endothelial cells. The main lumen contributes only up to 30% to the neovasculature of the atherosclerotic plaque. The degree of angiogenesis is independent of the size of the plaque. Eventually, most advanced atherosclerotic lesions are fairly well neovascularized, especially the so-called vulnerable plaque regions. Further, the aggressive neovascularization process seems to characterize the advanced inflamed biologically "active" plaque. In line with this view, neovasculature is encountered in 50% of atherectomy samples from patients with acute coronary syndrome. Neovascularization is five times more prevalent in unstable plaques than in stable atherosclerotic plaques. Carotid endarterectomy sample-based studies have noted a highly positive association between plaque neovasculature density and symptomatology, and both these parameters are closely related to intraplaque hemorrhage and rupture. With regard to the complication stage of atherosclerosis, the neovasculature density is highest in the ruptured atherosclerotic plaques, and microvasculature count at the plaque base is independently associated with plaque rupture.

Macrophage accumulation and apoptosis in atherosclerotic lesions promote the formation of necrotic core, a characteristic feature of advanced atherosclerotic plaques. It appears that the intracellular ROS formation plays a critical role in ox-LDL-induced macrophage death, but the source of these intracellular ROS has not been well defined. In a number of studies (144, 145, 176, 283), Nox4 was overexpressed in monocytes and mature macrophages, and shown to be localized to the endoplasmic reticulum and to which foci within the nucleus. Nox4 colocalizes with $p22^{phox}$, and both proteins seem to be upregulated in response to ox-LDL, whereas Nox2/$gp91^{phox}$ levels remain unchanged. Induction of Nox4, intracellular ROS formation and macrophage cytotoxicity induced by ox-LDL could be blocked by MEK1/2 inhibitors, but not by inhibitors of p38-MAPK, JNK, or Janus-activated kinase-2 (JAK2). Selective knockdown of Nox4 RNA inhibited both intracellular ROS production and macrophage cytotoxicity induced by ox-LDL, whereas Nox4 overexpression enhanced both ox-LDL-stimulated ROS formation and macrophage death. Thus, Nox4 may be a key factor in ox-LDL-mediated atherogenesis.

These studies suggest that LOX-1 mediates many of the effects of ox-LDL, that is, endothelial cell growth, dysfunction, activation, and adhesion of monocytes/macrophages; all critical features of atherosclerosis. Ang II, a critical player in atherogenesis, *via* its type 1 receptor (angiotensin II type 1 receptor [AT1R]) activation upregulates the expression of LOX-1 mRNA, and ox-LDL *via* LOX-1 upregulates the expression of AT1Rs (149, 152–154, 157–159, 250). Li *et al.* (157) showed that Ang II and ox-LDL interact synergistically to induce ox-LDL uptake. Activation of both AT1 and LOX-1 receptors *via* redox signaling leads to cell dysfunction culminating in endothelial apoptosis and injury, monocyte adhesion and activation, and eventually atherosclerosis. These observations provide strong evidence that LOX-1 is a key modulator of the development of Ang II–induced hypertension and subsequent cardiac remodeling. Figure 4 summarizes many of the similar effects of ox-LDL, mediated by LOX-1 activation, and Ang II, mediated by AT1R activation. The mutually facilitatory cross-talk between LOX-1 and AT1Rs may explain the coexistence of multiple risk factors in the same patient and the increase in atherosclerosis risk with the presence of multiple risk factors.

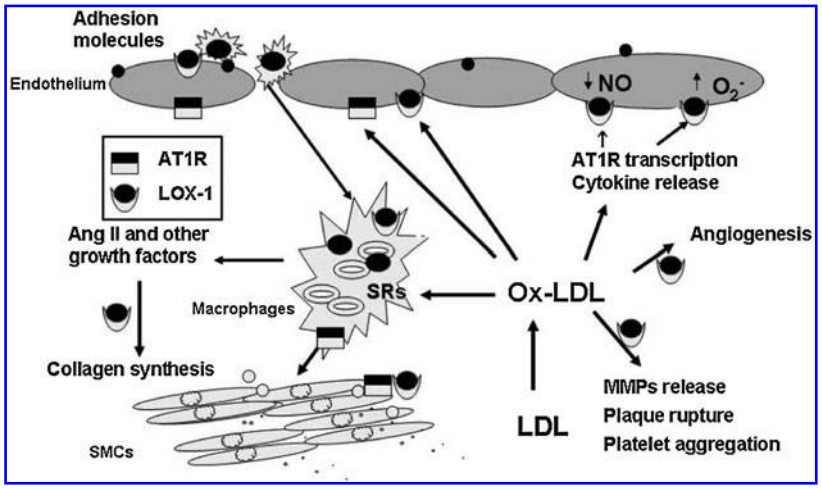

FIG. 5. Interactions between Ang II and ox-LDL in relation to atherogenesis. Ox-LDL *via* LOX-1 upregulates AT1R expression and Ang II transcriptionally upregulates the expression of LOX-1. Activation of both AT1R and LOX-1 *via* redox signaling leads to cell dysfunction culminating in apoptosis and cell injury, monocyte adhesion, and activation, critical steps in atherogenesis. It is interesting that the pathway leading to ox-LDL-induced cell injury appears to be the same that lead to angiogenic response when ECs are exposed to small concentrations of ox-LDL. Notably, LOX-1 also plays a critical role in the expression of growth factors, such as Ang II, PDGF, and heparin-binding epidermal growth factor-like protein. The secretion of these growth factors is associated with the migration and proliferation of smooth muscle cells and fibroblasts, leading to the progression of atherosclerosis. LOX-1 functions as a scavenger receptor (SR), which binds and internalizes ox-LDL, resulting in the transformation of SMCs and monocyte/macrophages into foam cells. In addition, LOX-1 has also been identified on platelets, and plays a role in platelet activation, aggregation, and thrombus formation as well as in matrix metalloproteinases (MMPs) release, which is the basis of fibrous plaque rupture.

I. Evidence for the pathogenic role of LOX-1 in atherogenesis from gene deletion studies

Li *et al.* (153) and Mehta *et al.* (190) have provided the conclusive evidence of the role of LOX-1 in atherogenesis (Fig. 5). In studies in LOX-1-null mice (190), it was shown that the binding of ox-LDL to aortic endothelium was reduced and endothelium-dependent vasorelaxation was preserved after treatment with ox-LDL. In the LDLR-null mouse model of atherosclerosis, the extent of atherosclerosis after 18 weeks of high fat diet was reduced by almost 50% after LOX-1 deletion. Expression of redox-sensitive transcription factor NF-κB and the inflammatory marker CD68 was also attenuated in the LDLR/LOX-1 double KO mice. On the other hand, anti-inflammatory cytokine IL-10 expression and SOD activity as well as endothelial NO synthase (eNOS) expression were preserved in the double KO mice (195). In the LDLR KO mice, collagen deposition was also significantly reduced with LOX-1 abrogation (111, 190).

In others studies in a model of Ang II–induced hypertension in the C57BL/6 mice, Hu *et al.* (112) showed that LOX-1 abrogation diminishes the development of Ang II–mediated hypertension. Perhaps more importantly, LOX-1 deletion reduced inflammation, cardiac hypertrophy and signals for collagen formation. Many of the signals for cardiac remodeling are similar to those seen in vascular remodeling seen in atherosclerosis (264).

III. ROS, LOX-1, and Myocardial Ischemia

Myocardial injury during ischemia-reperfusion is a complex situation, which involves several biochemical, cellular, and molecular alterations. Bolli *et al.* (17) provided direct *in vivo* evidence that oxygen radicals play an important role in the pathogenesis of myocardial stunning after transient ischemia, and implicated $^{-}$OH as a primary culprit. A coronary occlusion as short as 5 min is sufficient to cause a measurable burst of free radical production upon reperfusion; recurrent 5 min periods of occlusion followed by 10 min periods of reperfusion cycles result in recurrent bursts of free radical generation, which was shown to be associated with the generation of a mixture of carbon-centered spin-trapped radicals (most likely by-products of lipid peroxidation) and tocopheroxyl radicals (17). Mehta *et al.* (188) showed that a brief period of ischemia and reperfusion in anesthetized dogs was associated with loss of coronary flow reserve, accumulation of neutrophils in the reperfused regions, and loss of myocardial contractility; these phenomena could be ameliorated with administration of SOD before reperfusion.

Ischemia-reperfusion, with anoxia or hypoxia in the arterioles and capillaries, induces macrophages, endothelial cells, and other immune cells to generate highly injurious ROS.

ROS cause the release of pro-inflammatory cytokine, and ROS react with NO to produce reactive nitrogen species such as peroxynitrite that are highly reactive and destructive radicals. As mentioned earlier, oxidant load upregulates NADPH oxidases, followed by stimulation of redox-sensitive transcription factors, such as NF-κB. This initiates the translation and prolonged expression of cell-surface adhesion molecules such as leukocyte adhesion molecule CD18, endothelial ICAM-1 and P-selectin, and a release of cytokines to further promote the recruitment of neutrophils to the heart after ischemia-reperfusion (61, 104). This further propagates ischemia initiates by arterial occlusion. In addition, accumulating neutrophils become activated and enhance tissue injury by releasing proteolytic enzymes such as MMPs.

A. LOX-1 expression during myocardial ischemia-reperfusion

ROS are released during reperfusion and induce peroxidation of lipid bilayer of cell membrane, injure endothelial cells, and promote migration and accumulation of inflammatory cells in the ischemic-reperfused areas (149). Inhibition of ROS has been shown to reduce the phenomenon of reperfusion injury (17) and improve cardiac dysfunction (156, 287) in several animal models. ROS oxidize lipids, and result in increased ox-LDL levels. Although not consistently observed, a number of studies have shown that administration of ROS scavengers before reperfusion can limit the so-called reperfusion injury (135, 170).

Li *et al.* (156) showed that the expression of LOX-1 was markedly increased in rats subjected to ischemia-reperfusion, whereas ischemia alone was not enough to increase the expression of LOX-1. Positive immunoreactivity for LOX-1 was identified mainly in the endocardial and the subendocardial regions of the reperfused myocardium. The upregulation of LOX-1 contributes to reperfusion injury as evident from the data on the use of LOX-1 antibody, which decreased infarct size and improved cardiac function. Administration of a specific binding antibody to LOX-1 also reduced ischemia-reperfusion-mediated apoptosis and lipid peroxidation. Administration of the LOX-1 antibody before ischemia also attenuated the upregulation of LOX-1.

Other studies showed that LOX-1 is involved in the genesis of oxidant stress and inflammation during myocardial ischemia-reperfusion (155) (Figs. 8–11). Work from Mehta and Sawamura laboratories showed that LOX-1 can act as an adhesion molecule for inflammatory cells (103, 184).

It is now amply evident that a host of mediators are expressed during ischemia-reperfusion, including cytokines and Ang II, which account for oxidative stress mostly by activating NADPH oxidase system. The intense oxidant stress, particularly in the infarct-prone region (area at risk), induces upregulation of genes, such as fibronectin, osteopontin, collagen, and MMPs, soon after ischemia. Enhanced expression of fibronectin and osteopontin resulting in collagen formation leads to myocardial diastolic dysfunction. Ischemia along with cytokines and Ang II also triggers fibroblast growth. Chen *et al.* (39) showed that insertion of LOX-1 plasmids in cardiac fibroblasts that are naturally low expressers of LOX-1 alters the biology of fibroblasts to pro-inflammatory phenotype. Further oxidized-LDL treatment enhances collagen formation in fibroblasts that can be blocked by a LOX-1 antibody (39, 41). These observations collectively suggest that LOX-1 may be an important player in myocardial ischemia-reperfusion injury not only by inducing oxidative stress, but also by inducing signals for collagen in the ischemic tissues. Our findings in LOX-1 KO mice revealed that taking away LOX-1 indeed limits early cardiac remodeling signal after ischemia-reperfusion (108) and improve cardiac diastolic function.

Some other studies using on altering cellular oxidation-reduction (redox) equilibrium reduction are noteworthy. As mentioned earlier, oxidant state is an important modulator of

various cellular functions. Thioredoxin (Trx) is an important protein that maintains the cellular redox status, and the oxidation state of Trx can influence the overall redox equilibrium of a cell. It was originally identified in *Escherichia coli* as a hydrogen donor for ribonucleotide reductase, the essential enzyme providing deoxyribonucleotide for DNA replication. The Trx system includes Trx, Trx reductase, and peroxiredoxins, and uses NADPH as a source of reducing equivalents. Trx is a low-molecular-mass protein (12 kDa) with cytoplasmic, membrane, extracellular, and mitochondrial distribution. Multiple forms of Trx have been identified, including cytosolic Trx1 and mitochondrial Trx2. A pair of cysteines within a highly conserved, active site sequence can be oxidized to form a disulfide bond, which is then reduced by Trx reductase.

Trx is an efficient protein disulfide reductase. Besides being an antioxidant itself, Trx also is an important regulator of the expression of other antioxidant genes, such as MnSOD, and is known to modulate the activation of redox-responsive transcription factors, such as NF-κB. Hypoxia induces oxidation of Trx, and this oxidation is potentiated in the presence of 6-aminonicotinamide, an inhibitor of glucose-6-phosphate dehydrogenase. Muniyappa *et al.* (206) found that Trx redox state is modulated in hypoxia independent of ROS and is a critical determinant of cell cycle regulation.

A recent study showed that redox regulation by Trx plays a crucial role in signal transduction and cytoprotection against ROS inside the heart (58). Turoczi *et al.* (269) indicated the loss of Trx-1 from the ischemic-reperfused myocardium. Thus, Trx may well be an important component of the cellular defense against cardiac injury (57). In studies from Das group (206, 232), human Trx attenuated hypoxia/reoxygenation injury in murine endothelial cells under thiol-free conditions, suggesting Trx-induced protection of myocardium through a novel redox-signaling pathway. In other studies (58), resveratrol was shown to provide cardioprotection by maintaining intracellular redox environments, and Trx-2 is likely to play a role in switching ischemia-reperfusion-induced death signal into survival signal.

B. Role of LOX-1 in thrombosis and acute coronary syndromes

Myocardial ischemia-reperfusion represents a clinically relevant problem associated with thrombolysis, percutaneous coronary interventions, and coronary bypass surgery. Injury to the myocardium during ischemia-reperfusion includes cardiac contractile dysfunction, arrhythmias, loss of flow reserve, and irreversible myocyte damage (67, 184). Thrombosis is usually the event that leads to myocardial ischemia and stroke, and platelets are the usual initiators in this process. Platelets have been shown to internalize ox-LDL, resulting in diminished eNOS activity in platelets and enhanced platelet aggregation (45, 213). Although platelets express a modest amount of LOX-1, LOX-1 antibody appears to decrease arterial thrombus formation in the rats as well as in humans, suggesting a contributory role of LOX-1 in ox-LDL-mediated platelet activation and thrombosis (43, 180a).

There is a significant body of evidence pointing to the detrimental role of ox-LDL in large amounts in patients with acute coronary syndromes and hyperlipidemia. The plasma levels of ox-LDL are markedly elevated in patients with acute coronary syndromes, and ox-LDL is present in the atherosclerotic tissues of rupture-prone segments (67, 213). Ox-LDL levels are elevated in patients with acute coronary syndromes (67, 94), as well as in those with heart failure (268), and may even be used as a prognosis indicator in these patients.

Hayashida *et al.* (94) also showed that sLOX-1 levels are elevated in patients with acute coronary syndromes, and could be used to differentiate with unstable coronary disease from those with stable coronary disease. This is important since the native LDL levels do not differentiate these patients.

An association of polymorphisms in the human ox-LDL receptor (OLR1) gene and myocardial infarction susceptibility has been reported. Tatsuguchi *et al.* (259) for the first time examined the significance of LOX-1 polymorphism in coronary artery disease, and identified a single-nucleotide polymorphism (SNP) in the human OLR1 gene in patients with acute myocardial infarction. They found that OLR1 or a neighboring gene linked with G501C SNP is important for the incidence of myocardial infarction. Mango *et al.* (177) linked intronic SNPs to myocardial infarction. They also showed that SNPs in OLR-1 gene regulate the expression of a new functional splicing isoform of the OLR1 gene, LOXIN, which lacks exon 5. Macrophages from subjects carrying the nonrisk disease haplotype at the *OLR1* gene have an increased expression of LOXIN at mRNA and protein level, which results in a significant reduction of apoptosis in response to ox-LDL. Expression of LOXIN in different cell types results in loss of surface staining, indicating that truncation of the C-terminal portion of the protein has a profound effect on its cellular trafficking. Further, the pro-apoptotic effect of LOX-1 receptor in cell culture is specifically rescued by the coexpression of LOXIN in a dose-dependent manner. The demonstration that increasing levels of LOXIN protect cells from LOX-1-induced apoptosis sets a ground work for developing therapeutic approaches for prevention of plaque instability (177). The identification and characterization of the new splice variant of the *OLR1* gene suggest that this variant have a functional role on plaque instability and pathogenesis of myocardial infarction, and may provide the basis for the rationale to develop a therapeutic approach directed at LOX-1/LOXIN (184).

IV. ROS, LOX-1, and Hypertension

The renin-angiotensin system and its effector hormone, Ang II, have well-known endocrine properties that contribute to hypertension and cardiac remodeling. Previous studies have shown that AT1R activation stimulates the expression of LOX-1 (34, 154), and LOX-1 activation, in turn, upregulates AT1R expression. Activation of both AT1R and LOX-1 induces a state of oxidative stress (184).

Although LOX-1 expression is minimal in the aorta from normal rats, Nagase *et al.* (209) reported that its expression was markedly upregulated in spontaneously hypertensive rats, suggesting a correlation between LOX-1 and hypertension. This concept is supported by the *in vitro* observations from our laboratory that Ang II upregulates LOX-1 gene expression and ox-LDL upregulates AT1R expression in cultured HCAECs (154, 157) and angiotensin-converting enzyme (ACE) inhibitors and AT1R blockers decrease LOX-1 expression (203). All these findings suggest that LOX-1 contributes

to the pathogenesis of hypertension, which is induced by the activation of renin-angiotensin system.

To examine this postulate, Hu *et al.* (112) infused LOX-1 KO and wild-type mice with Ang II (50 ng/min) or norepinephrine (100 ng/min) for 4 weeks. Systolic blood pressure exhibited a progressive increase during the infusion period, reaching a peak value on day 14 and remaining at plateau through day 28 in the wild-type mice and LOX-1 KO mice. The rise in blood pressure was much less in the LOX-1 KO mice compared with that in wild-type mice, despite the fact that basal systolic blood pressure was similar. In contrast to the effect of Ang II, norepinephrine infusion caused a similar rise in blood pressure in wild-type and LOX-1 KO mice for the duration of the study. Thus, LOX-1 deletion resulted in a selective attenuation of blood pressure in response to Ang II (112). Moreover, these authors (112) showed in this study that interruption of the positive feedback loop between Ang II and LOX-1 might reduce the genesis of Ang II–induced cardiac remodeling (Fig. 11).

Ang II has well-known endocrine properties that contribute to renal fibrosis (13). As discussed earlier, at least part of biological effects of Ang II may be mediated *via* LOX-1 expression and activation. LOX-1 is upregulated in the kidneys of salt-loaded Dahl salt-sensitive rats and chronic renal failure rats and parallels glomerulosclerotic changes and renal dysfunction, suggesting a possible link between LOX-1 and the progression to glomerulosclerosis, renal fibrosis, and renal failure (209). Hu *et al.* (113) showed that LOX-1 plays a key role in the development of Ang II–induced renal damage. In this study, Ang II infusion for 4 weeks increased indices of renal injury (glomerulosclerosis, arteriolar sclerosis, tubulointerstitial damage, and renal collagen accumulation) in the wild-type mice, and this phenomenon was significantly attenuated in the LOX-1 KO mice. Along with the morphologic evidence of renal injury, renal function (blood urea nitrogen and creatinine) decreased in the wild-type mice, and the deterioration of renal function was significantly less in the LOX-1 KO mice. The reduction in collagen formation was accompanied by a decrease in connective tissue growth factor mRNA, AT1R expression, and phosphorylation of p38 and p44/42 MAPKs. Overall, LOX-1 deficiency reduced Ang II–induced hypertension and subsequent renal injury *via* interruption of the positive feedback loop between Ang II and LOX-1.

Further, ACE inhibitors, AT1R blocker, and aldosterone blocker are efficacious in the treatment of hypertension, and their beneficial effects, including prevention of cardiovascular and renal disease and mortality, have been established in large clinical trials (262, 263). Kobayashi *et al.* (134) suggested that these effects may be due not only to decreased synthesis of Ang II, but also to the ability of these drugs to prevent the breakdown of the potent vasodilator bradykinin-eNOS and oxidative stress-LOX-1 pathway (133, 134).

As discussed earlier, LOX-1 expression and activation participates, at lest in part, in the genesis of the diminished endothelium-dependent vasorelaxation and induction of hypertension. It has been observed that LOX-1 deletion results in the maintenance of endothelial continuity and eNOS expression in the LOX-1 KO mice. Preservation of endothelial vasodilator function by LOX-1 deletion may also be the basis of reduced hypertensive response to Ang II and reduced cardiac and renal remodeling in hypertensive animals.

V. ROS and LOX-1: Role of Different AT1R

Both *in vivo* and vitro studies have suggested an interaction between the renin-angiotensin system and hypercholesterolemia (109, 157). Dyslipidemia upregulates AT1R and Ang II in turn upregulates LOX-1 and facilitates uptake of ox-LDL into cells. However, the role of angiotensin II type 2 receptor (AT2R) in this interplay is unknown.

A. AT1R and AT2R

There are many receptors for Ang II in different tissues, but it appears that AT1R and AT2R mediate most of functions of Ang II in physiological or pathological conditions (70, 274). Both receptors belong to a seven transmembrane receptor superfamily coupled with G-protein, and play their role *via* the activation of G protein-mediated signaling pathway (25, 120, 274). AT1R and AT2R only share about 34% amino-acid sequence homology, but they have similar affinity for Ang II and function collaboratively. AT1R can be selectively blocked by biphenylimidazoles, such as losartan, and AT2R can be blocked by tetrahydroimidazopyridines, such as PD123319 (19).

The *AT1R* gene resides on the chromosome 3, and is expressed abundantly in many adult tissues. AT1R mediates most of the physiological actions of renin-angiotensin system such as vasoconstriction, SMC hypertrophy, SMC proliferation, endothelial apoptosis, matrix synthesis and accumulation, pro-oxidative and pro-fibrotic effects, water and sodium intake, renal sodium retention, vasopressin, aldosterone release, hypertension, atherosclerosis, and myocardial remodeling (68, 120). In general, AT1R plays a much more robust role in the adult animal than AT2R. The AT2R is more abundant in the embryonic tissue than AT1R. The pro-oxidative effect of AT1R plays an important role in the Ang II–mediated pathological processes. In cardiomyocytes, Ang II causes the activation of NOX2, $p22^{phox}$, $p40^{phox}$, $p47^{phox}$, $p67^{phox}$, and the small G protein Rac1 *via* AT1R activation, and promotes SMC hypertrophy and perivascular fibrosis, which can be attenuated by losartan (278, 279). In vascular cells, AT1R-induced ROS generation leads to the dysfunction of endothelial cells and vascular SMCs.

The *AT2R* gene has been mapped to chromosome 10, and its cDNA encodes a 363-amino acid (68). AT2R is thought to be associated with the development of fetal organs (98). It expresses highly only in the fetal tissues and decreases very soon after birth (276). In adults, AT2R exists at very low level in vascular tissues such as aorta and coronary arteries. AT2R, however, is upregulated in pathological conditions, such as myocardial infarction and ischemia-reperfusion injury (19, 72).

Although much work has been done on the biology of AT2R, its precise physiological function is not clear. Currently, it is known whether AT2R has functions in mediating vasodilation, and reduction in cellular apoptosis, migration, hypertrophic, proliferation, and fibrosis, and thus it may have a salutary role in hypertension and atherogenesis (19, 120). AT2R activation is believed to oppose AT1R actions, but sometimes, it also has synergistic effects with AT1R. It is now evident that AT2R activation promotes cell apoptosis in several cell types such as fibroblasts, neurons, SMCs, endothelial cells, and some cancer cells (160, 257). However, the pro-apoptotic effect of AT2R in cardiomyocytes remains controversial. For example, Qi *et al.* (229) reported that overexpression of AT2R in neonatal cardiomyocytes through

recombinant adenovirus transduction can significantly increase cell apoptosis (229). Other studies, however, showed that the increased AT2R expression does not affect cardiomyocyte apoptosis (205). Kong and Rabkin (136) even suggest that Ang II does not have a significant effect on cardiomyocyte apoptosis. These variable effects probably reflect different types of cell lines from different kinds of animals of different ages used in these studies. The methods used in these studies to increase AT2R expression were also different.

B. Cross-talk between LOX-1 and AT1R

Morawietz *et al.* (203) reported that Ang II increases the expression of the LOX-1 gene and the uptake of ox-LDL in Ang II–treated human umbilical vein endothelial cells. Similar responses were also observed in the HCAECs in our laboratory, and in monocytes, macrophages, and human VSMCs by other groups (109, 130, 131, 157). Work in our laboratory showed that the Ang II response ranged in a dose-dependent manner from 10^{-12} to 10^{-6} mol/l, and could be completely blocked by losartan, but not by PD123319 (157, 185). This suggests that the effect of Ang II is mediated by AT1R, and not AT2R, activation. A recent study showed that AT1R expression could be upregulated by the induction ox-LDL and that the expression of AT1R was enhanced together with the expression of LOX-1 (128). This suggests that AT1R upregulation in response to ox-LDL may be mediated by the activation of LOX-1 (127, 159) (Figs. 2 and 5).

There is increasing evidence that ROS plays an important role in the cross-talk between LOX-1 and AT1R. This process is in an NADPH oxidase-dependent manner and is mediated by ROS generation (110, 127, 236). It has been known that Ang II induces oxidative stress and ROS generation *via* AT1R transcription. Inhibition of the NADPH oxidase activity attenuates ROS generation, AT1R and LOX-1 expression. The upregulation of LOX-1 also stimulates ROS generation, and then enhances AT1R expression and activity. Work done in our laboratory has shown that AT1R and LOX-1 inhibition attenuate Ang II–mediated oxidant stress and the expression of NADPH oxidase ($p40^{phox}$ and $gp91^{phox}$ subunits) (127). Work from our laboratory has shown that low concentrations of ox-LDL and Ang II induce capillary formation from endothelial cell through ROS-dependent pathway, and this effect can be inhibited by apocynin (NADPH oxidase inhibitor) (56, 110). Besides NADPH oxidase and ROS, there are other factors that influence the cross-talk between AT1R and LOX-1. For example, a recent study showed that ACE2 plays an important role in regulating the expression of LOX-1 and AT1R. The overexpression of ACE2 in the abdominal aorta significantly lowers the expression of LOX-1 and AT1R at the same time (286); nonetheless, these authors believe that the final role of ACE2 on the expression of LOX-1 and AT1R is mediated through regulation of ROS. This information, along with previous observations, strongly suggest that NADPH oxidase and ROS are an important signal bridge in the cross-talk between AT1R and LOX-1.

C. Cross-talk between LOX-1 and AT2R

Recent studies in our laboratory show that AT2R overexpression inhibits LOX-1 expression, thereby reducing atherosclerosis (109). In this study, AT2R was overexpressed in homozygous LDLR- KO mice by using recombinant adeno-associated virus type-2 (AAV) carrying AT2R cDNA (AAV/AT2R) transduction. LOX-1 expression was dramatically increased in the LDLR KO mice, but not in AAV/AT2R animals. Atherogenesis in the aorta was also reduced in the AAV/AT2R-treated animals by ~50% compared with the LDLR KO animals. This work suggested that there may be a cross-talk between LOX-1 and AT2R. Li *et al.* (157), however, showed in cultured endothelial cells that the inhibition of AT2R using PD123319 does not affect LOX-1 expression. Watanabe *et al.* (276) also indicated that the increase of LOX-1 expression and its activity by ox-LDL stimulation does not affect AT2R expression. Thus, the evidence for a cross-talk between LOX-1 and AT2R is controversial.

VI. ROS and LOX-1: Fibrosis in Atherosclerotic Arteries and Ischemic and Hypertensive Hearts

Cardiomyofibroblasts differentiate into fibroblasts, proliferate in the ischemic heart, and become activated to form collagen as well as release MMPs (165). Because of being a source of both collagen forming and degrading enzymes, fibroblasts are involved in cardiac remodeling (38). Under physiologic conditions, there is a balance between collagen production and degradation. However, this balance could be disrupted in several pathologic conditions. For example, anoxia/reoxygenation stimulates the expression of both collagen type I and MMP-1 (40), whereas Ang II enhances the expression of collagen type I, but inhibits the expression of MMP-1(38). Ang II regulation of collagen type I expression in cardiac fibroblasts can be modulated by peroxisome proliferator-activated receptor gamma (PPARγ) ligand pioglitazone. (38) Overall, a balance between collagen synthesis and degradation determines plaque stability and myocardial stiffness.

Collagen deposition in the hearts is clinically relevant since patients whose hearts show pronounced collagen deposition have worse prognosis than those who have less or no significant collagen deposition (217).

Ang II, which is present in significant amounts in the ischemic hearts, is a potent stimulus for fibroblast proliferation (38). The effects of Ang II are mediated by AT1R activation since AT1R blockers significantly reduce fibroblast proliferation and generation of collagen as well as MMPs (38). Ang II enhances ROS generation *via* activation of NADPH oxidase, $p22^{phox}$ and $p67^{phox}$ subunits followed by activation of p38 and p44/42 MAPKs. This is followed by NF-κB and AP-1 translocation into the nucleus (194). These appear to be key steps in the formation of collagen as well as MMPs.

Chen and Mehta (36) showed that both α-tocopherol and γ-tocopherol reduced Ang II–induced oxidative stress, measured as carboxy-H_2-2′,7′-dichlorodihydrofluorescein diacetate uptake, in cardiac fibroblasts. Both α-tocopherol and γ-tocopherol also reduced Ang II–induced collagen formation and MMP activity. In other experiments, these authors showed that the therapeutically achieved concentrations of pravastatin and pioglitazone exerted a modest inhibitory effect on oxidative stress and subsequent oxidative stress-mediated signaling pathway, as well as procollagen-1 synthesis in cardiac fibroblasts. Importantly, the combination of pravastatin and pioglitazone, each in low concentration, exerted a potent inhibitory effect on oxidative stress and related signaling pathways, leading to reduced procollagen-1

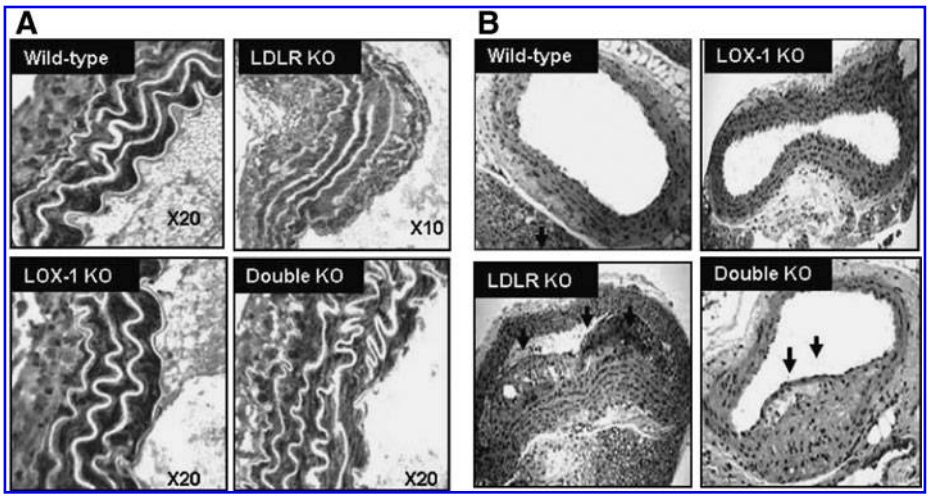

FIG. 6. Evidence for the critical role of LOX-1 in the atherogenesis. (A) Vascular SMC proliferation in LDLR knockout (KO) mice on high-fat diet, a well-known model of atherosclerosis. Note that LOX-1 deletion in the LDLR KO (double KO) mice fed high-fat diet reduces SMC proliferation and migration. **(B)** Intima thickness is significantly reduced in the LOX-1 KO mice (*vs.* wild-type mice) and double KO mice (*vs.* LDLR KO mice).

expression. The effects of the combination of pravastatin and pioglitazone were qualitatively similar to those of α-tocopherol and γ-tocopherol. This is a clinically relevant observation since the statin group of drugs has been shown to modulate myocardial stiffness in models of myocardial ischemia (95).

In recent studies in our laboratory (108), we have established that wild-type mouse hearts exhibit signals of collagen formation, such as fibronectin and osteopontin, as early as after 1 h of ischemia. This phenomenon of collagen deposition continues during chronic ischemia, and mouse hearts exposed to 4 weeks of chronic ischemia exhibit large scars. This is associated with oxidant load and evidence of inflammation at least in the early stages of ischemia (Lu *et al.*, unpublished data). Hearts from LOX-1 KO mice exhibited much less oxidant load, inflammation, and scar formation. Naturally, this translated into improved cardiac hemodynamics as assessed by dP/dt (developed pressure over time) in the left ventricle and improved cardiac ejection fraction (Fig. 10).

The role of LOX-1 in cardiac fibroblast biology became apparent from experiments in which the isolated fibroblasts were transfected with LOX-1 cDNA and then treated with ox-LDL (41). LOX-1-transfected cells showed markedly increased uptake of dil-labeled ox-LDL (*vs.* plasmid alone-treated cells). Further, LOX-1-transfected cells showed intense oxidant load and marked increase in the formation of collagen. It is of note that LOX-1 expression in fibroblast is minimal to absent; however, it is possible that in the *in vivo* setting, particularly during the state of anoxia, myofibroblasts transform selectively into fibroblasts and inherit LOX-1 from adjacent cardiomyocytes and begin to form large amounts of collagen. Although initially it was thought that atherosclerotic tissues have small to modest amounts of fibrous tissue, recent experimental studies show extensive dispersion of collagen throughout the atherosclerotic regions where it serves as cement holding the proliferating smooth muscle cells and macrophages together. Clinical studies have also shown that the major constituent of the atherosclerotic plaque is fibrous tissue. Statin and PPARγ ligands, particularly in combination, reduce the burden of fibrosis and result in reduction of the size of the plaque (49). In experimental atherosclerosis in the LDLR KO mice, deletion of LOX-1 resulted in a marked reduction in collagen (111). The collagen accumulation data were corroborated with procollagen-1 measurements. Expression of osteopontin and fibronectin, which was increased in the wild-type mice, was reduced in the LOX-1 KO mice. The enhanced expression of NADPH oxidase (p47 ^{phox}, p22 ^{phox}, gp91 ^{phox}, and Nox-4 subunits) in the wild-type mice was not seen in mice with LOX-1 deletion. Further, phosphorylation of Akt-1 and eNOS and expression of heme-oxygenase-1 were found to be reduced in the wild-type mice, but not in the mice with LOX-1 deletion. These observations provide compelling evidence that LOX-1 plays an important role in enhanced collagen deposition in atherosclerotic regions *via* pro-oxidant signals (Fig. 7). After vascular injury, especially in patients with hypertension, diabetes, or dyslipidemia, the intense

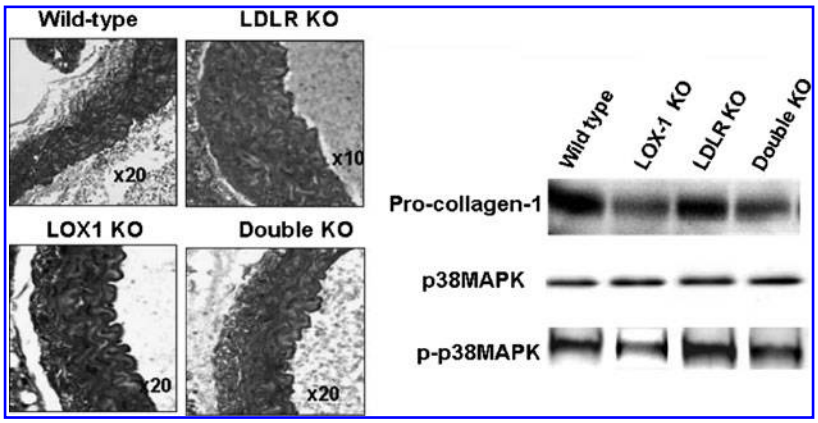

FIG. 7. Collagen accumulation in atherosclerosis. *Lighter shade* indicates extensive collagen deposition on the atherosclerotic artery in the LDLR KO mice. Note that LOX-1 deletion in the LDLR KO mice (double KO mice) reduces collagen deposition. The *right panel* shows reduction in procollagne-1 and p-p38MAPK signals by LOX-1 deletion (western data).

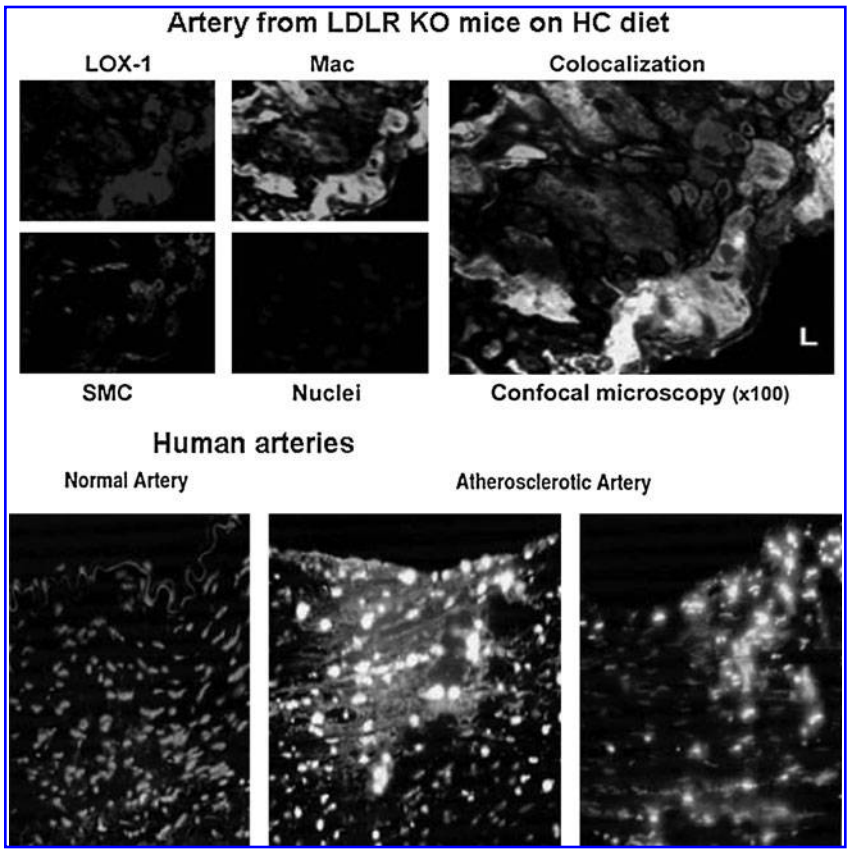

FIG. 8. LOX-1 in atherogenesis. Fluorescent immunohistochemistry shows localization of LOX-1 primarily in macrophages, but also in SMCs of LDLR KO mice in high cholesterol diet (*upper panel*). Also note that atherosclerotic human arteries show extensive apoptosis, mostly in the subintmal region (*lower panel*).

oxidant load leads to LOX-1 expression culminating in VSMC proliferation and vascular restenosis (Fig. 12). This may well be a model of restenosis following percutaneous vascular interventions such as balloon angioplasty, atherectomy, or stent placements.

VII. Genomics of LOX-1 Deletion

Kang *et al.* (125) studied the genome-wide alterations in wild-type and LOX-1 KO mouse hearts, and provided the first set of data on gene expression profiling. The LOX-1 KO mice showed upregulation of several genes, including *Ddx3y* and *Eif2s3*. Ang II infusion enhanced expression of genes known to be associated with cardiac remodeling, such as *Agt*, *Ace*, *Timp4*, *Fstl*, and *Tnfrst12a*, as well as oxidant stress-related genes *Gnaq*, *Sos1*, and *Rac1*. *Ddx3y* exhibits growth suppressor function, which may reflect a link between development of hypertension and cardiac remodeling. *Eif2s3y* was named from eukaryotic translation initiation factor 2, subunit 3, structural gene Y-linked. *Eif-2* encoded by *Eif2s3y* functions in the early steps of protein synthesis by forming a ternary complex with GTP and initiator tRNA. *Camk2a* and *Txnl4* were the most downregulated genes, and both relate to cardiac hypertrophy. *Camk2a* is inhibited by Rad overexpression.

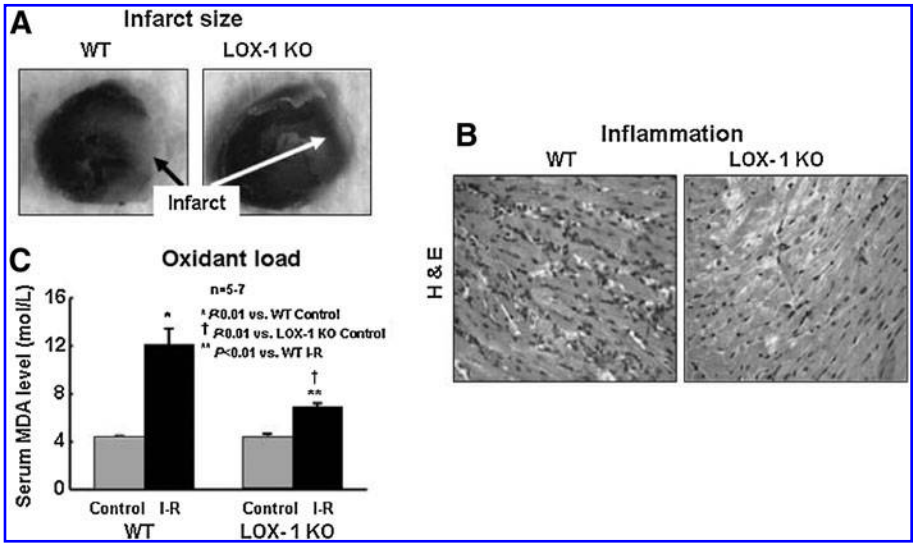

FIG. 9. Oxidant stress (ROS release), LOX-1, and myocardial ischemia. A short period of myocardial ischemia (1 h of occlusion and 1 h of reperfusion of the left coronary artery) induces extensive infarct **(A**, *right***)**, accumulation of inflammatory cells **(B,** *right***)**, and high levels of MDA in serum **(C)**. All these changes are attenuated in the LOX-1 KO mice subjected to same degree of myocardial ischemia and reperfusion. **(A**, *left*; **B**, *left*; **C)**.

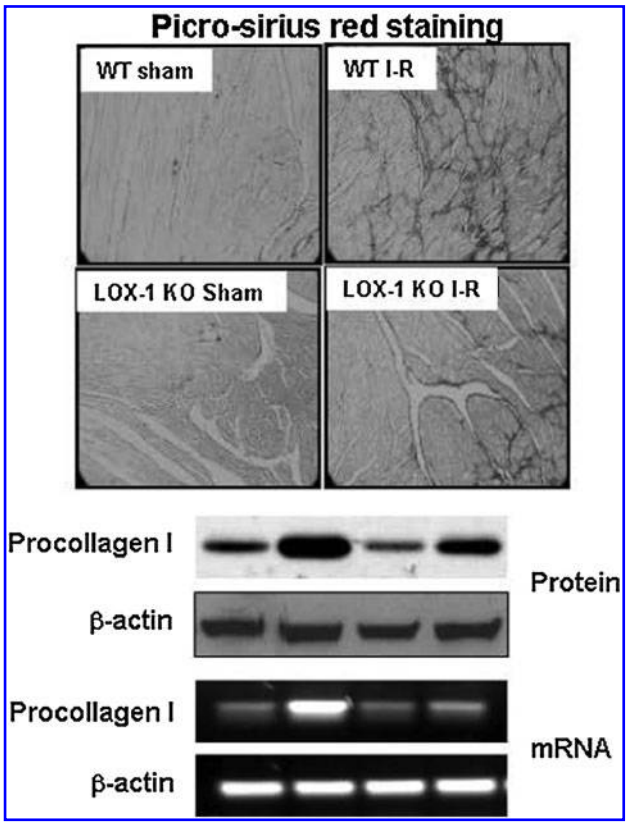

FIG. 10. Oxidant stress (ROS release), LOX-1 and collagen accumulation after myocardial ischemia. A short period of myocardial ischemia (1 h of occlusion and 1 h of reperfusion of the left coronary artery) induces extensive signal for collagen expression and deposition as seen in Masson's trichrome- and Picrosirius-stained myocardial sections. There is a marked increase in procollagen-1 mRNA and protein in the wild-type (WT) mice subjected to ischemia and reperfusion. All these changes are attenuated in the LOX-1 KO mice subjected to the same degree of myocardial ischemia and reperfusion.

Chang *et al.* (27) demonstrated that Rad-deficient mice are more susceptible to cardiac hypertrophy. Kashiwase *et al.* (128) showed that *Camk2* mediates cardiomyocyte hypertrophy *via Ask1* (or *Map3k5*) and NF-κB activation. It is possible that *Txnl4* downregulation protects LOX-1 KO mice from hypertension and subsequent cardiac hypertrophy. Hu *et al.* (112) showed that LOX-1 deletion attenuated the rise in blood pressure and subsequent cardiac remodeling in response to Ang II. Importantly, ROS generation, NADPH oxidase expression, and phosphorylation of p38 and p44/42 MAPKs were much less pronounced in the LOX-1 KO mice given Ang II. These alterations in biochemical and structural abnormalities were associated with preservation of cardiac hemodynamics in the LOX-1 KO mice. Indeed, cardiac fibroblasts from LOX-1 KO mice revealed attenuated pro-fibrotic response upon treatment with Ang II.

VIII. Evidence for Oxidant Stress in Humans with Atherosclerosis

A host of studies have shown evidence for increased oxidant stress in all risk factors for atherosclerosis, including diabetes mellitus, hypertension, dyslipidemia, smoking, hyperhomocysteinemia, and obesity (220, 221, 254, 266). This evidence has been subject of several reviews, and will not be discussed here in detail for space constraints. Here, we will mention some of the studies that indicate increased oxidant load in these patients:

1. There is increased oxidation of LDL-cholesterol in subjects with subclinical or clinical atherosclerosis.
2. Serum ox-LDL levels are elevated in patients with ischemic heart disease, with higher levels in patients with acute coronary syndromes than in those with stable coronary artery disease. This suggests that the acute oxidant stress may be the basis of acute coronary syndromes. Attempts have been made to correlate plasma ox-LDL levels with prognosis. Although this correlation was apparent in some studies (102), others (280) failed to show a similar correlation. This may reflect vagaries in the measurement of ox-LDL in plasma or serum. Further, a host of drugs taken by patients may affect oxidation of LDL-cholesterol.
3. Plasma levels of antibody to ox-LDL have been shown to be elevated in some, but not in all studies (31). Again, the discrepancy may reflect differences in methodologies that have used different epitopes of the ox-LDL to measure the antibody in plasma.
4. A host of studies (73, 91, 244, 258) have shown reduction in endothelium-dependent vasorelaxation in patients with coronary atherosclerosis. Some investigators (26) have shown that offspring of patients with coronary atherosclerosis also exhibit reduced endothelium-dependent vasorelaxation. The endothelium-dependent vasorelaxation is believed to reflect synthesis and/or release or activity of endothelium-derived NO. The reduced generation or activity NO moiety is believed to be due to increased oxidant load.
5. The loss of endothelium-dependent vasorelaxation correlates with plasma ox-LDL levels in patients with coronary atherosclerosis and/or hypercholesterolemia.
6. The attenuation of endothelium-dependent vasorelaxation is observed in almost all coronary risk factors, and generally correlates with the number of coronary risk factors (167, 226).
7. Kume's group (94, 139) has also shown that plasma levels of sLOX-1 are elevated in patients with acute coronary syndromes, but not in patients with stable angina. The elevated levels of sLOX-1 were a significant predictor of acute coronary syndromes than the native LDL-cholesterol levels. The sLOX-1 levels had greater predictor value that did C-reactive protein.
8. In keeping with the injurious effects of ROS on endothelial cells, plasma levels of endothelial remnants, which are referred to as microparticles and may act as pro-thrombotic molecules, are elevated in coronary risk factors (191).
9. Oxidant species activate platelets and inflammatory cells and facilitate formation of platelet-neutrophil/monocyte conjugates, and the levels of these conjugates are increased in circulation in patients with unstable angina (223).

IX. Therapeutic Implications of Oxidant Load and Its Inhibition

It is abundantly clear that there is oxidant load in all stages of atherosclerosis, from its initiation to plaque rupture

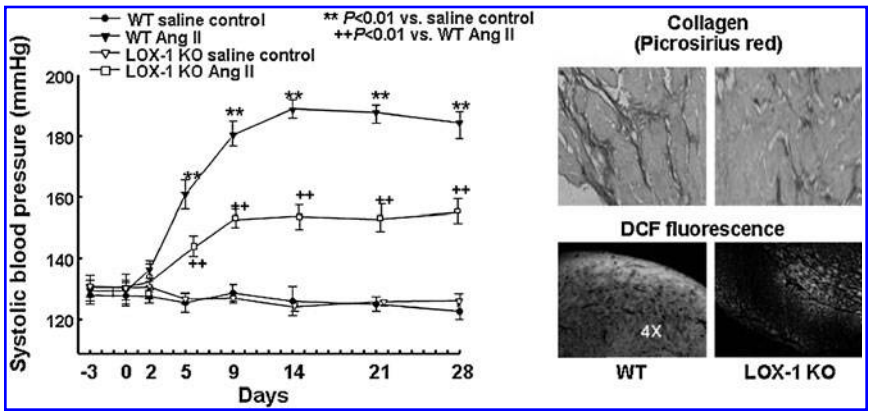

FIG. 11. Oxidant stress (ROS release), LOX-1, and hypertension. Ang II infusion in WT mice results in sustained hypertension, collagen deposition in the heart (Picrosirius staining), probably secondary to intense oxidant load (Dichloro-fluorescin [DCF] fluorescence). LOX-1 KO mice given the same dose of Ang II shows attenuated rise in blood pressure, collagen deposition, and DCF fluorescence.

resulting in acute coronary syndromes, stroke, and renal artery occlusion. The evidence for oxidant load comes from studies in animal models of atherosclerosis as well as in humans. On the basis of this strong evidence, a large number of studies have been performed in animals with atherosclerosis and its complications, such as myocardial ischemia, and in humans with common disease states associated with atherosclerosis.

A. Experimental studies

1. Antioxidants and lifespan. Gerschman *et al.* (80) postulated that oxygen-free radicals are the common molecular mechanism of oxygen and radiation toxicity. Subsequently, Harman (90) theorized that free radicals produce random and cumulative cellular injury leading to tissue and organ aging.

Bahadorani *et al.* (6) examined the effect of vitamin A (retinol), vitamin C (ascorbic acid), and vitamin E (α-tocopherol) on *Drosophila melanogaster* lifespan under different oxidative stress conditions. Among the vitamins tested, α-tocopherol but not vitamin A or vitamin C, prolonged average and maximum lifespan for wild-type flies under hyperoxia. Vitamin A supplementation extended lifespan of SOD1-deficient flies under normoxia, and ascorbic acid supplementation extended lifespan of wild-type flies under normoxia. Melov *et al.* (195) increased the natural antioxidant systems of *Caenorhabditis elegans* with small synthetic SOD/catalase mimetics. Treatment of wild-type worms with EUK-134, an catalase/SOD mimetic (7), increased their mean lifespan by a mean of 44 percent, and treatment of prematurely aging worms resulted in normalization of their lifespan (a 67 percent increase).

In our unpublished studies (Ayyadevara *et al.*, 2010), we have noted a similar prolongation of lifespan in *C. elegans* subjected to growth medium enriched with vitamin E. We observed that enrichment of growth medium with aspirin resulted in a similar prolongation of lifespan of *C. elegans* (median lifespan: control, 14.5 days; aspirin 0.5 m*M*, 19.4 days; aspirin 1 m*M*, 18.8 days; aspirin 2 m*M*, 18.4 days). In these studies, aspirin reduced H_2O_2 generation, as measured by Dichloro-fluorescin fluorescence. Aspirin also enhanced the levels of SOD1, catalase2, and jnk-1 expression (Fig. 13).

However, the results from the use of a wide variety of antioxidants on rodent lifespan have not been consistent. For example, Morley and Trainor (204) reported that vitamin E at 400 mg/kg throughout mouse life had no effect on median lifespan, whereas Blackett and Hall (16) using 2500 mg/kg reported an increase in rat median lifespan but not in maximal lifespan. On the other hand, Reckelhoff *et al.* (233) administered 5000 mg/kg of vitamin E from 52 to 88 weeks of rat age and observed an attenuation of the decline of aging-related renal function. Navarro *et al.* (212) showed that male mice given

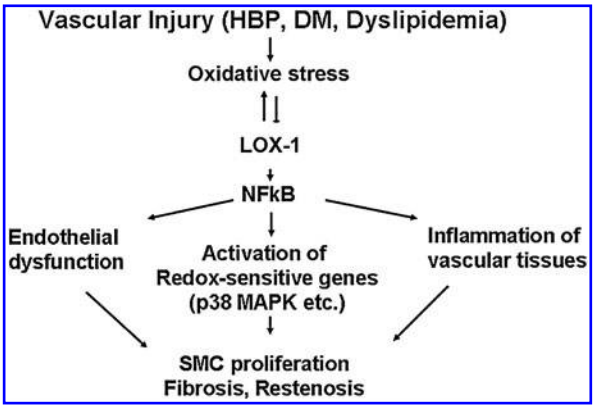

FIG. 12. Vascular injury, oxidant stress, LOX-1, and vascular restenosis. Vascular injury as seen in hypertension, diabetes, and dyslipidemia induces oxidant stress, LOX-1 transcription, activation of redox-sensitive genes, inflammation, and SMC proliferation. This is a theoretical model of vascular restenosis after vascular injury.

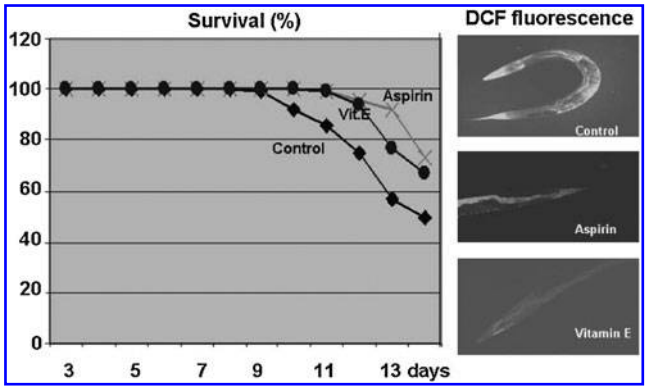

FIG. 13. Vitamin E (Vit. E), a chain-breaking antioxidant, and aspirin prolong survival in *Caenorhabditis elegans*. Both vitamin E and aspirin decrease ROS formation as indicated by DCF fluorescence. Hypothesized pathways of ox-LDL-mediated cell death/survival.

vitamin E (5.0 g tocopherol acetate/kg of food) from 28 weeks of age showed a 40% increase in median lifespan, whereas female mice exhibited only 14% increase in median lifespan.

2. Antioxidants and vascular injury. Vascular injury results in the generation of large amounts of ROS; hence, antioxidants have been utilized in repair and restoration of vascular function following the vascular injurious process. Muscoli *et al.* (207) investigated superoxide generation and its role in neointima formation in a balloon injury rat carotid artery model. After 14 days of injury, there was a significant restenosis with smooth muscle cell proliferation and neointima formation that was associated with an enhanced expression of LOX-1, nitrotyrosine (the footprint of peroxynitrite) staining, and lipid peroxidation. Pretreatment of rats with M40401 [a manganese(II) complex with a bis(cyclo-hexylpyridine-substituted) macrocyclic ligand], a synthetic SOD mimetic that is a selective scavenger of superoxide (0.5–10 mg/kg i.p. daily), reduced neointima formation, nitrotyrosine staining, and LOX-1 expression. They concluded that removal of superoxide formation occurring in injured arteries by M40401 reduced both neointima formation and LOX-1 expression. Along this line of thought, Hinagata *et al.* (97) showed that the expression of LOX-1 was increased within 24 h after the balloon injury and peaked at day 7. LOX-1 expression was observed predominantly in medial smooth muscle cells until day 3, and then shifted to predominantly intimal smooth muscle cells. At day 14, the expression was concentrated in the regenerated endothelial cells. To examine the contributory role of LOX-1 in the growth of intimal smooth muscle cells, rats were treated with anti-LOX-1 antibody intravenously every 3 days after balloon injury. Anti-LOX-1 antibody administration suppressed intimal hyperplasia, oxidative stress, and leukocyte infiltration. These findings suggest the importance of ROS generation and LOX-1 expression in the pathogenesis of neointimal formation in conjunction with oxidative stress and smooth muscle cell proliferation.

3. Antioxidants and atherogenesis. In models of atherosclerosis, use of antioxidants has resulted in a wide variation in results. Chen *et al.* (42) showed that dietary supplementation with vitamin E, an HMG CoA reductase inhibitor lovastatin, or a calcium channel blocker amlodipine each reduced atherosclerosis in New Zealand white rabbits fed a high cholesterol diet. Each of the therapies decreased oxidation of LDL-cholesterol and preserved antioxidant activity. However, some other studies (75, 76) have not shown a consistent salutary effect of vitamin E in atherogenesis.

In contradiction to the variable effects of antioxidants, a number of studies have shown salutary effects of Ang II AT1R inhibitors as well as ACE inhibitors in animal models of atherogenesis (48, 163). It is of note that AT1R activation is thought to be a potent stimulus for ROS generation in atherosclerotic lesions (71). Along this line of thought, HMG CoA reductase inhibitors (statins) have been shown to reduce atherogenesis in a number of animal models (179, 277). These agents besides lowering LDL-cholesterol inhibit inflammation, reduce the expression of LOX-1 in endothelial cells and platelets at transcriptional levels, and enhance eNOS expression and activity (20, 32, 175, 218).

Importantly, there is evidence for strong cross-talk between renin-angiotensin system (mediated *via* AT1R) and dyslipidemia (mediated *via* LOX-1). Chen *et al.* (32) showed that simultaneous blockade of AT1R by candesartan and reduction of LDL-cholesterol with rosuvastatin reduced the redox state, pro-inflammatory p38MAPK activation and LOX-1 expression resulting in a marked reduction in atherogenesis in ApoE KO mice fed a high cholesterol diet.

Resveratrol, a compound in red wine, is a phytopolyphenol compound with multiple effects, including inhibition of oxidative stress/ROS generation, inflammation, oxidative modification of LDLs, and platelet aggregation (69). This agent has been shown to reduce the initiation and progression of atherosclerosis, and to impede the progression of atherosclerosis (79, 202, 289). This agent has lately gained much recognition in the lay press because of its potential to exert a multitude of salutary effects in a variety of disease states, presumably because of its antioxidant effects.

4. Antioxidants and myocardial ischemic injury. There has been an immense interest in the reduction of ischemic injury to the heart, brain, kidney, and other organs ever since it became apparent that transient ischemia is associated with release of large amounts of ROS. As reviewed by Powers *et al.* (227), there is growing body of evidence that mitochondrial injury plays a major role in ischemia-induced injury, because mitochondria seem to be the final arbitrators of ischemia-induced cell death and determine whether the tissue will die from necrosis or apoptosis. Ischemia-reperfusion injury may have particular relevance in surgical situations when the coronary blood flow is transiently stopped (188). Similarly, percutaneous coronary angioplasty of the coronary and other circulatory beds is associated with reperfusion injury. A number of studies in different animal models have been conducted; unfortunately, results of most animal studies on the use of antioxidants have been discordant (117, 192, 238).

Nonetheless, we have learnt that controlled gradual reperfusion has significant beneficial effect on the extent of tissue injury compared with abrupt restoration of blood flow (224). Further, it appears that part of the protective effect of preconditioning is mediated by release of ROS (92, 189); hence, use of antioxidants may ameliorate the protein induced by ROS.

5. Antioxidants in hypertension and diabetes. Hypertension and diabetes (129), two of the most important risk factors in atherogenesis, are associated with increased oxidant load (81, 92, 189). Oxidant stress may be the cause of hypertension and diabetes as well as the result of the disease state. Oxidative stress stemming from Ang II–mediated NADPH oxidase activation may well be the basis of vascular dysfunction seen in these disease states. As discussed earlier, dyslipidemia seen in diabetes has the potential to activate AT1R expression. Some recent studies suggest that alterations in genetic background can modulate the expression of different components of NADPH oxidase. Polymorphisms have been identified in the promoter of the $p22^{phox}$ gene, an essential subunit of NADPH oxidase, which can influence the activity of this enzyme. These polymorphisms may be the basis of genetic susceptibility to oxidative stress in hypertension (92).

Some experimental studies have shown that of oxidative stress caused by renin-angiotensin system activation is the basis of hypertension (92). Along this line of thought, we examined Ang II–induced hypertension in mice. Ang

II–induced hypertension was attenuated in LOX-1 KO mice. ROS generation, NADPH oxidase expression, and phosphorylation of p38 and p44/42 MAPKs were also much less pronounced in the LOX-1 KO mice given Ang II. These observations provide strong evidence that ROS generation plays an important role in the genesis of Ang II–mediated hypertension, and that LOX-1 is a key modulator of the development of hypertension (113).

B. Human studies

For reason of space limitation and to focus on the physiology of ROS and LOX-1, these trials will not be discussed here. It is suffice to say that although some trials have yielded positive data, a large number of studies have not shown benefit of oral antioxidants in patients with atherosclerosis-related disease states (118, 169). The reasons for lack of efficacy are discussed elsewhere (200).

X. Conclusions on the Role of ROS and LOX-1 in Atherosclerosis and Related Disorders

The extensive series of experiments described herein suggest that ox-LDL mediates it adverse effects on blood vessels, resulting in atherosclerosis, primarily *via* activation of LOX-1. The upregulation of LOX-1 by ox-LDL appears to be the key to endothelial dysfunction (and apoptosis), SMC growth and migration, monocyte transformation into macrophages, and finally platelet aggregation—seen in atherogenesis. Vascular and cardiac remodeling in stress states also involves LOX-1 upregulation and activation. Perhaps very importantly, the stimulus for angiogenesis, a hall-mark of late stage atherosclerosis and cardiac remodeling, is also LOX-1. Accordingly, LOX-1 blockade or deletion strategies may have a role in clinical diseases states characterized by vascular remodeling and tissue dysfunction in pro-oxidant states like diabetes, hypertension, smoking, and dyslipidemia.

XI. ROS, LOX-1, and Tumorigenesis

A. Commonalities between atherogenesis and tumorigenesis

There are numerous parallels between atherogenesis and tumorigenesis, especially in relation to causative metabolic derangements. For example, obese subjects have not only increased serum levels of LDL-cholesterol and triglycerides, but more importantly a state of increased oxidative stress leading to conversion of LDL into ox-LDL (225). This process of LDL oxidation plays a key role in the development of atherosclerosis in both obese and lean patients; perhaps, same is true in the development of certain cancers. There is a positive correlation between increased serum ox-LDL levels and an increased risk of colon, breast and ovarian cancer (82, 180, 225). Clinical and epidemiological studies have reported robust linkage between cancer development and lipid metabolic disorders. For example, patients found to have metabolic syndrome, inflammatory diseases, or autoimmune conditions show increased incidence and aggressiveness of tumor formation (82, 180, 225, 261). In a prospectively studied group of 900,000 U.S. adults, obesity was associated with substantially increased risk for a wide range of cancers, including esophagus, colon, rectum, liver, gallbladder, pancreas, kidney, non-Hodgkin's lymphoma, multiple myeloma, stomach, prostate, breast, uterus, cervix, and ovary cancers (24). Moreover, childhood obesity translates into the increased risk for breast, ovarian, endometrial, colon, and renal cancers (77). Conversely, many drugs such as metformin, sulindac, tocilizumab, simvastatin, and cerulenin used for treatment of diabetes and metabolic disorders inhibit tumor growth (100, 106, 143, 236).

B. Involvement of LOX-1 in tumorigenesis: proof of concept

Recently, Hirsch *et al.* (99) compared the cancer gene signature to microarray transcriptional profiling studies from individuals with disease states, and found a significant overlap between cancer gene signature and expression signatures found in obesity (147), atherosclerosis (249), and metabolic syndrome (46). Specifically, they identified 24 common central nodes, including inflammation-related nodes such as interferon (IFN)-γ, IL-1β, IL-6, and NF-κB, in these patients, suggesting that inflammatory processes are important for both cancer and metabolic diseases. Insulin and LDL-cholesterol appear as central nodes in cancer gene networks, suggesting the importance of protein and lipid metabolism in cellular transformation.

Interestingly, upregulation of LOX-1 and other genes involved in lipid metabolism featured prominently in transformation transcriptomes of two isogenic cell types, including normal mammary epithelial cells MCF10a and primary fibroblasts. LOX-1 activated NF-κB-dependent pro-inflammatory and hypoxia signaling and inhibition of LOX-1 reduced the level of the inflammatory genes (*e.g.*, IL-1β, IL-6, and IL-8) and hypoxia-regulated genes (hypoxia-inducible factor-1 [HIF-1α], vascular endothelial growth factor [VEGF], and carbonic anhydrase 9 [CA9]), as well as activation of the transcription factor NF-κB through inhibition of IkBα phosphorylation. Further, inhibition of TNFα blocked the activation of HIF-1α, VEGF, and CA9, the downstream targets of LOX-1 (Fig. 14). In addition, simvastatin, a lipid lowering drug that inhibits LOX-1 expression in endothelial cells, strongly inhibited the cellular transformation in a manner associated with inhibition of NF-κB activity, but it did not affect growth of nontransformed MCF10A cells. Treatment of MCF-10A cells with a low concentration of ox-LDL induced cellular transformation and colony formation in NF-κB-dependent manner, with 40%–50% transformation after 72 h and 85% transformation after 120 h. Taken together, these observations imply that LOX-1 regulates the inflammatory and hypoxia responses during transformation in the MCF-10A model and is essential for maintaining the transformed state in cancer cell lines of diverse developmental origin. These findings also suggest that LOX-1 can serve as a link between cancers and a variety of metabolic diseases.

C. LOX-1 KO transcriptome in relation to cancer

We have analyzed microarray data from LOX-1 KO and wild-type mice for genes involved in cellular transformation. Twenty-five out of 238 genes upregulated during transformation were inhibited by more than 20% in tissues of LOX-1 deficient mice (Fig. 14). The majority of LOX-1 sensitive genes contained NF-κB binding site in their promoters in the proximity (500 nt) of transcriptional start site. Further search revealed broad inhibition of NF-κB target genes outside of the

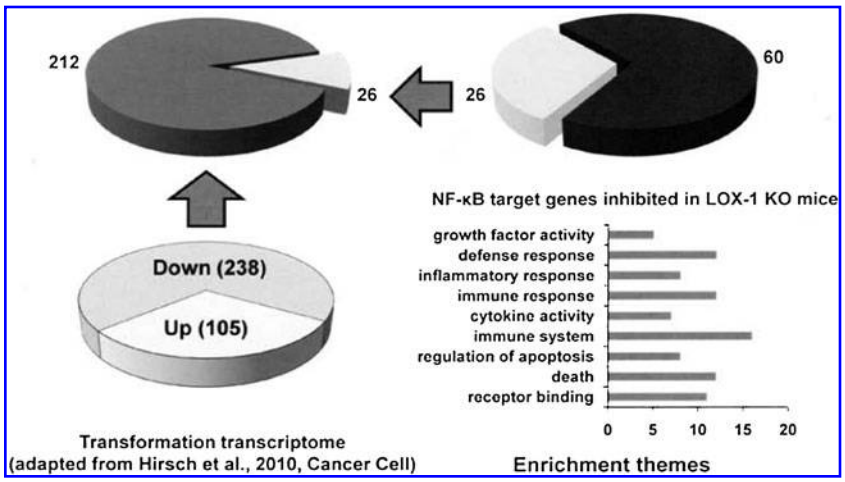

FIG. 14. Genomics of LOX-1 deletion in relation to transformation transcriptome. The transformation transcriptome identified by Hirsch *et al.* (99) includes 238 upregulated genes (*left*). Among them, 26 genes are inhibited In LOX-1 KO mice by more than 20% and, with one exception, all of these genes are regulated by NF-κB transcription factor (*right*). Overall, LOX-1 deletion causes a broad inhibition of NF-κB target genes with prevailing enrichment themes concerning inflammatory and immune responses as well as regulation of apoptosis.

transformation-associated gene pool with the prevailing enrichment themes of apoptosis, proliferation, wound healing, and defense. In addition and independently from overt NF-κB transcriptional control, abrogation of LOX-1 caused profound inhibition of rate-limiting enzymes involved in lipogenesis, including ATP-citrate lyase (−1.7-fold), acetyl-coenzyme A carboxylase alpha (~1.9-fold), fatty acid synthase (−5.9-fold), stearoyl-CoA desaturase 1 (−5-fold), and ELOVL family member 6, elongation of long chain fatty acids (−3-fold). The active *de novo* synthesis of saturated fatty acids actively occurs only in the liver and adipose tissue and is essentially nonexistent in other tissues (141). In contrast, many cancers, including prostate and breast cancer (10, 183, 282), rely almost exclusively on *de novo* synthesis regardless of nutritional availability. The expression levels of FASN positively correlate with poor cancer prognosis in a range of tumors (3, 245, 247), its genomic amplification is a common occurrence in some cancers (245), and its overexpression promotes transformation of epithelial cells (197). Similarly, overexpression of SCD1 has been observed in several types of cancers, including mammary cancer (138, 161, 171). Upregulation of SCD1 is associated with transformation (223) and its knock-down, in addition to reduction of MUFA synthesis, results in decrease in cell proliferation, a loss of anchorage-independent growth and impaired apoptosis (240). Based on the role of LOX-1 in dyslipidemia signal transduction, its upstream position in relation to NF-κB and its role as a regulator of *de novo* lipogenesis, we suggest that LOX-1, in addition to its established angiogenic effect at small concentrations, may have at least two independent mechanisms of pro-oncogenic action based on activation of NF-κB signaling pathway, and a novel function of LOX-1 as a potent regulator of lipogenesis.

D. Potential mechanisms of LOX-1 action as a pro-oncogene

1. Ox-LDL/LOX-1 and ROS production. Generation of ROS is an inevitable consequence of aerobic metabolism and an intrinsic part of intracellular redox signaling involved in regulation of many processes, including proliferation, differentiation, and defense. ROS come from many sources, including mitochondria, plasma membrane NADPH oxidase, cytochrome P450, NO synthase, xanthine oxidases, and peroxisomal and endoplasmic reticular oxidases. In general, mitochondria are considered to be a primary source of ROS in the cell, although the hierarchy of contributors may vary depending on the cell type. For example, NADPH oxidase is a major producer of ROS in endothelial cells (1). Similarly, various NADP oxidase subunits are highly expressed in epithelial cells from different organs, including cornea, trachea, thyroids, colon, and kidney (26a, 59, 79a, 215, 241).

As species with mutagenic properties, ROS have been implicated in transformation and cancer development (168). A considerable fraction of ROS in cancer cells is contributed by NADPH oxidase, as overexpression of its various subunits has been reported for many cancers (123). Apart from general genotoxicity, the oncogenic effects of ROS originating from NADPH oxidase appear to be based on transformation-specific mechanisms. Overexpression of NADPH oxidase is mitogenic (231) and is tightly linked to a transformation potential of mutated Ras proteins (123). Nox1 transcription is renin-angiotensin system dependent (201), and renin-angiotensin system-mediated transformation is accompanied by overproduction of superoxide, whereas its prevention, in turn, inhibits proliferation of renin-angiotensin system-transformed cells and cancer cell lines (5, 116, 201). Increased NOX1 activity alone also appears to be oncogenic as its overexpression in NIH3T3 cells resulted in anchorage-independent growth and increased tumorigenic potential in immunodeficient mice (252). In absence of Ras mutation, the oncogenic potential of Nox1 may be linked to inactivation of p53 *via* inhibition of SIRT1-mediated p53 Lys382 acetylation (228). Similar effects are reported for Nox4 localizing to mitochondria, which, upon overexpression, induced tumorigenic transformation of normal breast epithelial cells (86).

Ox-LDL stimulates membrane assembly of $p22^{phox}$, $p47^{phox}$, gp91, Rac1, and ROS production in endothelial cells resulting in multi-fold increase in oxidative DNA damage, which is abrogated by inactivation of LOX-1 (146, 265). Similar and also LOX-1-mediated effects are observed in the process of platelet–endothelial interaction accompanied by significant increase in superoxide production (52). NADPH oxidase appears to be a sole source for ox-LDL/LOX-1 generated ROS, as the decrease in O_2^- production was observed only with inhibition of NADPH oxidase, but not cyclooxygenase, eNOS, xanthine oxidase, or mitochondrial nicotinamide adenine dinucleotide dehydrogenase. Activation and translocation of NF-κB to the nucleus is an early (within

minutes) consequence of ox-LDL–LOX-1 interaction (53, 181), resulting from ROS-mediated downregulation and phosphorylation of IKBα (181).

Apart from interaction with ox-LDL, LOX-1 functions as an intermediary for other NADPH oxidase activators. It has been shown that ox-LDL upregulates not only LOX-1, but AT1R as well (152). Ang II itself stimulates ox-LDL uptake (157) and thus recruits the ox-LDL/LOX-1 signaling arm, which is critically involved in Ang II–stimulated ROS production and angiogenesis (110). TNFα upregulates LOX-1 in endothelial and epithelial cells (166, 199), and ramps up Nox4-mediated ROS production (11). Similarly, TGFβ1 increases Nox4-dependent production of ROS in endothelial cells (115). It is of note that while reducing overall ox-LDL uptake by monocytes *via* CD36 class B SR, TGFβ1 upregulates LOX-1 (65) as well as the LOX-1 mediated signaling.

2. LOX-1 and angiogenesis. Angiogenesis, the process of new blood vessel formation from the pre-existing vessels, plays an important role in both tumor growth and arteriosclerosis. VEGF, one of the major angiogenesis factor, is induced in growing tumors and stimulates endothelial cell proliferation and migration primarily through the VEGF receptor type 2 (VEGFR2, Flk1/KDR). Many stimuli, including hypoxia, growth factors, cytokines, and oxidative stress, can increase VEGF expression in tumor cells, which is correlated with increased microvessel counts, progression to a growing tumor, and metastasis (2, 198). In response to tumor hypoxia, many angiogenesis-related genes, including VEGF and erythropoietin, are upregulated by HIF-1, which is a heterodimeric transcription factor composed of HIF-1α and HIF-1β subunits. HIF-1 activates the transcription of many genes involved in multiple aspects of tumor growth, including angiogenesis, cell survival, and invasion. High levels of HIF-1 expression have been observed in many cancers, and a hypoxic microenvironment results in significant reprogramming of the gene expression profile in the invasive tumor cells (12, 132). Sensitivity to hypoxia is also an important determinant for multiple stages of carcinogenesis.

It is recognized that low-concentration ox-LDL may paradoxically protect normal endothelial cells against apoptosis provoked by high-concentration ox-LDL. As described earlier, studies in our laboratory (56, 110) have established that the small pro-angiogenic concentrations of ox-LDL and Ang II that led to LOX-1 expression also induced NADPH oxidase (both $gp91^{phox}$ and $p47^{phox}$ subunits), activated MAPK (both p38 and p44/42 components) and NF-κB p65, and resulted in VEGF expression. Along the same line of thought, components of ox-LDL may stimulate cancer cell proliferation and mutagenesis *in vitro*. In contrast, high concentrations of ox-LDL may arrest cancer cell growth, activate p53-dependent apoptosis, and initiate autophagy. Zabirnyk *et al.* (284) have investigated the dual role of ox-LDL in cancer cells, and showed that ox-LDL can activate both apoptosis and autophagy in cancer cells. Apoptosis definitely leads to cellular death, whereas autophagy is activated as a pro-survival mechanism. The key metabolic enzyme of proline degradation, proline oxidase, is regulated by ox-LDL and it plays a regulatory role in ox-LDL-mediated pro-survival autophagy. This mechanism is based on one of the chemical components of ox-LDL, 7-ketocholesterol, through PPARγ. These authors also showed that the effect of proline oxidase on autophagic machinery is executed through the generation of superoxide and subsequent regulation of beclin-1. In brief, autophagy, specifically beclin-1 transcription, is regulated by proline oxidase-dependent superoxide through ox-LDL-derived 7-ketocholesterol involving PPARγ. This mechanism provides sustained cellular survival under the conditions of ox-LDL-mediated stress. These concepts are summarized in Figure 6.

3. LOX-1, adhesion molecules and tumor dissemination. Apart from LOX-1-mediated downstream events, LOX-1 itself displays adhesive properties. It has been shown that monocytes actively attach to a LOX-1-coated surface (12) and to normally adhesion-negative CHO cells and fibroblasts transfected with LOX-1 expression vector (41, 93). In *in vivo* studies, neutralization of LOX-1 with a binding antibody rescued rats from lethal dose of endotoxin and, at lower doses, prevented leukocyte infiltration and reduced number of rolling leukocytes (103). The upregulation of LOX-1 in response to dyslipidemia is, perhaps, one of the earliest events that translate into deterioration of antiadhesive status of intima due to metabolic syndrome. In addition to ox-LDL, many other stimuli, including TNFα, hyperglycemia, and endotoxin, upregulate LOX-1 (184), which, in turn, enhance the expression of key molecules such as ICAM, VCAM, P-selectin, and MCP-1 that are necessary for transendothelial migration. It is possible that dyslipidemia and subsequent LOX-1 expression/activation and ROS release are the basis of adhesion of cancerous cells to the intima and their transmigration to sub-endothelial layers by these mechanisms.

The escape of detached cancer cells into the blood stream and their spread to distant organs is facilitated by aberrant morphology of and reduced cell–cell contacts in the tumor. The endothelium in vessels supplying the tumor is disorganized, and exhibits irregular spacing, abnormal basement membrane and increased leakiness (96). High permeability and primitive structure of vessels often leads to their collapse and does not efficiently solve a problem of oxygen supply; therefore, a state of hypoxia (and hypoxia-driven signaling) is a constant feature of growing tumors. Also, the expression of a key component of adherens junctions, E-cadherin, is invariably negatively associated with increasing aggressiveness of epithelial tumors and poor prognosis (9, 60). The weakening of adhesion between cancer cells confers multiple benefits including reduction of contact inhibition, greater availability of oxygen *via* passive diffusion, higher motility, and, therefore, greater potential for dissemination.

Extravasation of tumor cells occurs in areas with morphologically and functionally intact vasculature and, therefore, is mechanistically very similar to the process described for leukocyte migration. It has been reported that tethering of breast cancer cells to endothelium is mediated by E-selectin binding to the CD44 variant 4 (285). It has also been shown that upregulation of LOX-1 by TNFα promotes both adhesion and transendothelial migration of MDA-MB-231 breast cancer cells (166). Among known ligands for LOX-1, phosphatidylserine is a potential counterpart responsible for the adhesive interaction of endothelium and cancer cells. LOX-1 has been shown to cause platelet aggregation through calcium-dependent binding to phosphatidylserine (180a, 256). On the other hand, cancer cells display overabundance of phosphatidylserine on their outer membranes (270). Similarly to transendothelial migration of leukocytes, attachment of breast

cancer cells (often producing VEGF) to the endothelium results in dissociation of VE-cadherin/catenin complex within few minutes (21). The proposed chain of signaling events includes Src-dependent phosphorylation of the guanine exchange factor Vav2, which through Rac causes increase in ROS production and serine 665 phosphorylation of VE-cadherin followed by association with β-arrestin2 and endocytosis. In addition to Rac, other Rho GTPases, including RhoA and RhoC, are shown to be required for tumor cell transmigration (275).

Although data on transendothelial migration of cancer cells in relation to ox-LDL/LOX-1 are scarce, the available information on mechanisms of LOX-1-assisted monocyte transmigration allows for plausible models for LOX-1 involvement in cancer cells extravasation. Ox-LDL and other stimuli activate LOX-1 in both monocytes and endothelial cells (63), and recent studies, including our own unpublished data, indicate that ox-LDL evokes similar response from epithelial cells of different origins. After activation of LOX-1 several other adhesion molecules providing machinery for a proper positioning of monocytes in relation to inter-endothelial junctions are upregulated in endothelial cells and monocytes (288). In the absence of direct evidence, similar transcriptional signature for LOX-1 upregulation can be suggested for cancer cells as well. For example, one of the key factors involved in both monocyte and cancer cell migration is MCP-1 (62), which is a transcriptional target of LOX-1 (149). In *in vitro* studies, invasiveness of prostate cancer cells correlated with induced expression of MCP-1 in bone marrow endothelial cells and was suppressed in presence of MCP-1 antibody (84, 174). Similarly, TNFα—a powerful stimulator of LOX-1 expression in both endothelial and epithelial cells—enhanced transendothelial migration of myeloma cells *via* increased production of MCP-1 (119).

4. LOX-1 and immune surveillance. LOX-1 emerges as an important component of innate and adaptive immunity responsible for key steps of antigen presentation on the surface of antigen-presenting cells (APCs). After endocytosis by APCs, exogenous antigens are presented on MNC class II molecules, whereas endogenous antigens are loaded onto MHC class I molecules for priming of, respectively, CD^{4+} and CD^{8+} T-cells. Among APCs, dendritic cells (DCs) are unique in a sense that they can communicate with both CD^{4+} and CD^{8+} T-cells (8). The initiation of immune response by DCs to a large degree depends on cross-presentation based on recognition of antigen-bearing carrier molecules by receptors binding followed by internalization and processing of antigens. The mechanism of cross-presentation and the receptors involved in this process are a focus of ongoing research interest.

The ligand specificity of LOX-1 is rather broad and includes bacteria and their components, apoptotic cells, modified ox-LDL, C-reactive protein, anionic phospholipids, activated leucocytes, platelets, and diverse members of heat-shock proteins (78, 89, 180a, 182, 216, 219, 246). Heat shock proteins (HSPs) enter extracellular compartment as a result of either active secretory process or cellular damage caused by apoptosis or necrosis and are capable of eliciting signaling events in immune system either directly or as recognizable vehicles of antigens. The primary function of HSPs is maintenance of proper protein tertiary structure and prevention of protein aggregation. Additionally, HSPs serve as a powerful danger signal activating innate immune response. Exposure to many pathogens results in rapid production of large quantities of HSPs followed, depending on the context, by either stimulatory or inhibitory effects on immunity (178, 272). Secreted or released from damaged cells HSPs are capable of binding with various antigens and, *via* interaction with APC receptors, are ultimately responsible for their cross-presentation.

LOX-1 has been shown to interact and internalize a variety of members from HSP60, HSP70, and HSP90 families of the proteins. HSP60 is located in mitochondrial matrix (230) and is released into extracellular compartment in response to an assortment of cytotoxic exposures (23). In a recent study by Xie *et al.* (281), LOX-1 was identified as a primary receptor for Hsp60. Compared to cells transfected with empty vector or Dectin-1 construct, CHO-LOX-1 transgenic cells demonstrated avid and saturable binding of HSP60 to LOX-1 that persisted after internalization and was inhibited by LOX-1 antibody. Further, binding of HSP60 to bone marrow-derived DCs was partially dependent on LOX-1 availability as well. Delivery and cross-presentation of $OVA_{230\text{-}359}$ fused to Hsp60 was confirmed by elevated secretion of IL-2 by T-cell hybridoma B3Z, which was partially blocked by LOX-1 antibody. Members of HSP70 family are often overexpressed in tumor cells as a protection against apoptosis and are further upregulated in response to antitumor therapy. HSP70-based antitumor therapy has shown significant promise (22, 50, 173). The range of candidate receptors for HSP70-tumor antigen complexes in APCs has been extensively studied, and the functions associated with surface binding and internalization have been assigned primarily to SRs, including LOX-1, SREC-1, and FEEL-1 (264). In this study, HSP70-peptide complexes from human tumor cells selectively bound to CHO cells overexpressing either of these receptors and were consequently internalized. Endoplasmic reticulum bound Gp96 (235), also known as tumor rejection antigen, belongs to a HSP90 family and is commonly overexpressed in tumors (234). It has been shown to cross-prime CD^{8+} T-cells (14) and DCs (271). Recently, it has been demonstrated that efficiency of cross-presentation of peptides shaperoned by gp94 on APCs depends on interactions with LOX-1 and CD91 receptors (182), which are inhibited in a dose-dependent manner by respective alternative ligands ox-LDL and α2-macroglobulin.

Interestingly, LOX-1 appears to be a direct transcriptional target for IFN as it is significantly upregulated specifically in IFN-α conditioned DCs, but not in DCs treated with Il-4 (222). It also follows from our microarray analysis (222; M. Khaidakov, unpublished data) that stimulation by IFN of some genes reported in the Parlato study may be LOX-1 mediated. In LOX-1 KO transcriptome, the classical IFN target genes such as ISG20, and transcription factors IRF2 and IRF7 were significantly inhibited, which suggests an upstream position for LOX-1 in this signaling sequence.

Thus, the accumulated body of evidence suggests significant dependence of HSPs-mediated cross-presentation of antigens on APCs on the interaction of LOX-1 with HSP-peptide complexes. This emphasizes the bi-directional effects of LOX-1 that can potentially affect tumor development. Also, since the efficiency of HSP-LOX-1 interaction is shown to be compromised by the excess of competing LOX-1 ligand ox-LDL (182), it may have obvious epidemiological implications. For

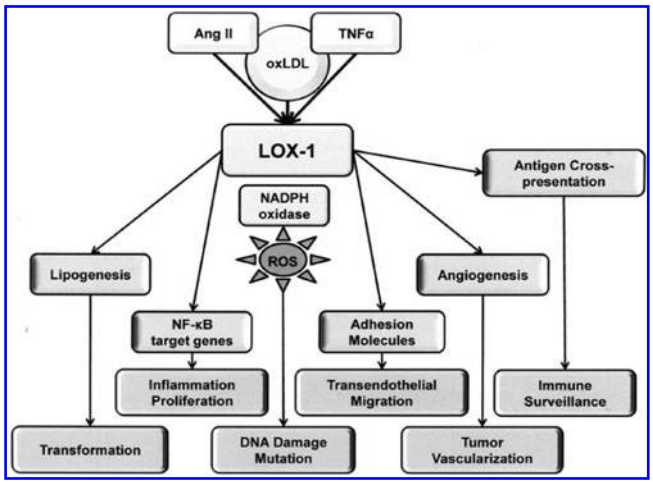

FIG. 15. Potential mechanisms through which ROS and LOX-1 may be involved in tumorigenesis. LOX-1 is upregulated by a number of factors, including ox-LDL, hormones, and cytokines. Activation of LOX-1 triggers complex cell type-specific reactions with both pro- and antioncogenic connotations. Majority of proposed mechanisms are prooncogenic and include activation of NF-κB signaling pathway (inflammation, proliferation, and inhibition of apoptosis), increase in adhesive properties on endothelium conducive to more efficient transendothelial migration of cancer cells, acquired ability for *de novo* lipogenesis, which is essential for cellular transformation, and promotion of angiogenesis. At the same time, LOX-1 receptor is an important element of antigen cross-presentation mechanism responsible for internalization of heat shock protein-tumor peptides by antigen-presenting cells and, therefore, is essential for efficient immune surveillance.

example, the elevated levels of circulating ox-LDL observed in metabolic syndrome may compromise efficiency of immune response to tumor antigens (due to reduced presentation on APCs) and be partially accountable for higher susceptibility to cancers found in obese subjects.

Figure 15 summarizes the multiple mechanisms by which LOX-1 overexpression and activation (in response to ROS) may lead to tumor cell growth and dissemination

XII. Conclusions on the Role of ROS and LOX-1 in Tumorigenesis

LOX-1 is involved in a number of processes that can either promote or attenuate transformation and tumorigenesis. On the one hand, LOX-1 is an upstream regulator of NF-κB activity and, therefore, under certain conditions (*e.g.*, obesity associated with increased levels of ox-LDL), may contribute to the development of inflammation which increases probability of cancer. Similar effects may be produced by its suggested role in stimulation of *de novo* lipogenesis, as a switch to internal synthesis of unsaturated fatty acids is one of the prerequisites for efficient transformation. As an adhesive molecule, LOX-1 can interact with phophatidylserine expressed on the membrane of tumor cells, and thus facilitate their transendothelial migration. Finally, as pro-angiogenic factor, LOX-1 can promote tumor growth *via* more efficient neovascularization. On the other hand, a key role of LOX-1 in antigen cross-presentation implies that its activation may improve immune surveillance.

Lipid metabolism genes are important for transformation of cells and are upregulated in cancer tissues. As in atherosclerosis, ox-LDL and its receptor LOX-1 activate the inflammatory pathway through NF-κB, leading to cell transformation. LOX-1 is important for maintaining the transformed state in developmentally diverse cancer cell lines and for tumor growth, suggesting a molecular connection between cancer and atherosclerosis. Pathophysiologic effects of ox-LDL in atherosclerosis are firmly established. The lowering of plasma ox-LDL results in a marked retardation of atherosclerosis progression. However, atherosclerosis has been an insufficient model for carcinogenesis. In spite of accumulating pro-carcinogenic epidemiologic data, there are no studies on the direct effect of ox-LDL on cancer development.

Clearly, additional work is needed to elucidate ox-LDL/LOX-1-dependent metabolic signaling responsible for the effects of ox-LDL on carcinogenic processes. These studies should be directed at (i) definition of the role of each Nox subtype and its regulatory role in tumor angiogenesis; (ii) determination of the activation mechanisms of NADPH oxidase by various angiogenesis factors; and (iii) identification of the molecular targets of oxidase-derived ROS in signaling pathways involved in angiogenic switch in various cancer cells. The development of specific inhibitors of NADPH oxidases and redox signaling components (kinase, phosphatase, transcription factors, and genes) as well as understanding the mechanism by which dietary antioxidants inhibit angiogenesis could provide useful therapeutic strategies for treatment of various angiogenesis-dependent pathologic states such as cancer. (iv) Oxidant signaling events, transcription factor, and gene expression activated by ROS that lead to tumor development, progression, invasion, metastasis, and therapeutic resistance.

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Abbreviations Used

AAV = adeno-associated virus type-2
ACE = angiotensin-converting enzyme
Ang II = angiotensin II
AP-1 = activator protein-1
APCs = antigen-presenting cells
AT1R = angiotensin II type 1 receptor
AT2R = angiotensin II type 2 receptor
CA9 = carbonic anhydrase 9
CD = cytoplasmic domain
CDK = cyclin-dependent kinases
CTLLD = C-type lectin-like ligand binding domain
DCF = Dichloro-fluorescin
DCs = dendritic cells
ECs = endothelial cells
eNOS = endothelial nitric oxide synthase
ERK1/2 = extracellular signal-regulated kinase 1/2
HCAECs = human coronary artery endothelial cells
HIF-1 = hypoxia-inducible factor-1
HSP = heat shock protein
ICAM-1 = intercellular adhesion molecule-1
IFN-γ = interferon-gamma
IL-1β = interleukin-1beta
JAK2 = Janus-activated kinase-2
JNK = c-Jun N-terminal kinase
KO = knockout
LDL = low-density lipoproteins
LDLR = low-density lipoprotein receptor
LOX-1 = lectin-like oxidized low-density lipoprotein receptor-1
MCP-1 = monocyte chemoattractant protein-1
Mdm2 = murine double minute2
MMPs = matrix metalloproteinases
MnSOD = manganese superoxide dismutase
ND = neck domain
NF-κB = nuclear factor-kappa B
NO = nitric oxide
ox-LDL = oxidized low-density lipoprotein
p38 MAPK = p38 mitogen-activated protein kinase
p42/44 MAPK = p42/44 mitogen-activated protein kinase
PI3K/Akt = phosphoinositide 3-kinase
PPARγ = peroxisome proliferator-activated receptor gamma
ROS = reactive oxygen species
SNP = single-nucleotide polymorphism
SRs = scavenger receptors
TD = transmembrane domain
TIMPs = tissue inhibitors of metalloproteinases
TNFα = tumor necrosis factor-α
Trx = thioredoxin
VCAM-1 = vascular cell adhesion molecule-1
VEGF = vascular endothelial growth factor
VSMCs = vascular smooth muscle cells
WT = wild type

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