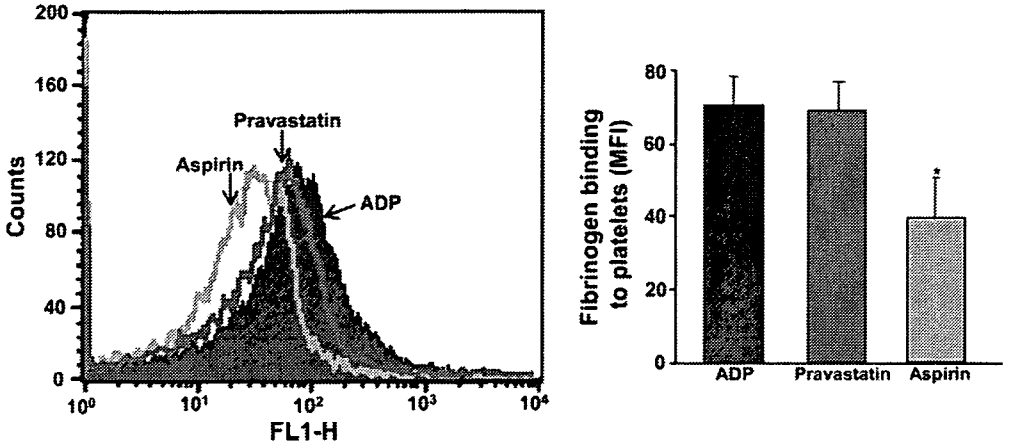

Fig. 7. Alexa Fluor 488-conjugated fibrinogen binding to platelets was examined by flow cytometry. Platelets were initially treated with 10 mM aspirin or 10 μM pravastatin, and then 10 μM ADP was added together with 200 μM Alexa Fluor 488-fibrinogen, and the mixture was incubated at 37°C for 15 min. Left, inhibition with aspirin of Alexa Fluor 488-conjugated fibrinogen to 10 μM ADP-stimulated platelets; pravastatin has a minimal effect in a representative experiment. Right, bar graphs of MFI from three independent experiments. *, $p < 0.05$ versus ADP alone.

study, we addressed the question of whether LOX-1 on platelets plays a role in platelet aggregation.

LOX-1 is known to mediate binding to ox-LDL, which itself is a product of oxidative stress. The role of oxidative stress in thrombosis has become appreciated recently (Krötz et al., 2004). ROS released by endothelial cells and immune cells (neutrophils, monocytes, and macrophages) and by platelets may play an important role in thrombus formation. Here, we provide another possible contribution of LOX-1 in modifying platelet aggregation. LOX-1 is an important component of integrin-mediated platelet binding to fibrinogen, which is the ultimate step in thrombosis.

ADP stimulation activates platelet integrins, mainly $\alpha_{IIb}\beta_3$ and to a lesser extent $\alpha_2\beta_1$, through a process called inside-out signaling (Li et al., 2003c; Abrams, 2005). This signaling pathway, common in all other integrins, induces conformational changes of these important adhesion molecules (Takagi et al., 2002; Vinogradova et al., 2002). In the present study, we show that LOX-1 affects the inside-out signaling of platelet integrins by inhibiting protein kinase C activation. To confirm the specificity of this reaction, we used RGD-peptide to inhibit integrin binding to fibrinogen (Basani et al., 2001; Xiong et al., 2002), and we were able to show similar response. Protein kinase C has been reported to be involved in inside-out signaling (Abrams, 2005; Han et al., 2006; Yacoub et al., 2006). It is likely that antibody engagement of LOX-1 on platelet triggers other signaling events that interfere with this process. The downstream signaling pathway of LOX-1 is still largely unknown. However, it is clear that antibody engagement may induce clustering of LOX-1 receptors and activate its downstream signaling cascades, or that it may block them (Li et al., 2003a,b). Further studies are warranted to clarify this issue.

We also show that aspirin and pravastatin reduce LOX-1 expression. This finding correlates with reduction of ROS generation and enhanced NO release. It is noteworthy that aspirin and pravastatin had synergistic effects on these measurements. Because oxidative stress stimulates LOX-1 expression (Mehta et al., 2006) and LOX-1 expression itself induces oxidative stress, it is conceivable that the reduction in LOX-1 expression by aspirin and pravastatin is either the basis and/or a consequence of reduction in oxidative stress.

In a previous study in endothelial cells (Mehta et al., 2004), aspirin in a dose- and time-dependent manner was shown to reduce ox-LDL-mediated LOX-1 expression. The effect of aspirin was thought to be the effect of salicylate moiety, because treatment of endothelial cells with salicylate, but not indomethacin, resulted in the suppression of LOX-1 expression, an effect similar to that of aspirin. It is noteworthy that both aspirin and its component salicylate decreased superoxide anion generation. Other studies in endothelial cells have shown that the HMG CoA reductase inhibitors simvastatin and atorvastatin reduce LOX-1 expression, up-regulate protein kinase B activity, and reduce ^{125}I-ox-LDL uptake in endothelial cells (Mehta et al., 2001). This was thought to result in an increase in endothelial nitric-oxide synthase expression and activity.

It is possible that inhibition of platelet aggregation by aspirin reflects oxidative stress reduction. Oxidative stress has clearly been shown to enhance platelet aggregation and thrombosis (Krötz et al., 2004). Platelets produce ROS because they have NAD(P)H-oxidase activity (Seno et al., 2001; Krötz et al., 2002). ROS affect thrombosis by increasing platelet recruitment into growing thrombus (Krötz et al., 2002). It is noteworthy that we did not see inhibition of platelet aggregation by pravastatin, even though ROS was significantly reduced. However, this does not necessarily mean that pravastatin does not have antithrombotic inhibition. It is possible that by reducing ROS level, pravastatin reduces platelet recruitment into the growing thrombus. In support of this hypothesis are the observations from our laboratory that another HMG CoA reductase inhibitor, atorvastatin, reduced the weight of thrombus in the rat aorta exposed to oxidative stress (Gaddam et al., 2002). Furthermore, there is evidence that regulation of integrins may be affected by redox condition owing to the modifications of its disulfide bonds that may affect its affinity through conformational changes (Lahav et al., 2002; Walsh et al., 2004). Again, we observed that aspirin, but not pravastatin, by reducing ROS, inhibited integrin-mediated binding of platelet to fibrinogen. It is still unclear whether the redox state that can affect integrin disulfide bonds can be replicated in our fibrinogen binding assay, or for that matter, whether the redox state that alters disulfide bonds occurs in vivo. It is conceivable that ROS produced during oxidative stress may increase platelet LOX-1 expression. Clues in favor of this assumption

are emerging from preliminary observations on the effects of direct ROS generation by the Fe(III)-ascorbate reaction (Ciuffi et al., 1999) on thrombus formation in carotid arteries of wild-type and LOX-1-deficient mice.

In hypercholesterolemic patients, atorvastatin has been reported to reduce LOX-1 expression on platelets (Puccetti et al., 2005b). The level of expression of LOX-1 was measured by flow cytometry with a different clone of monoclonal antibody (JM90) than the antibody we used in this study. Both monoclonal antibodies are humanized, but most probably they recognize different epitopes of LOX-1 (Puccetti et al., 2005b). Even though the data of Puccetti et al. (2005b) were presented as percentage of LOX-1-positive platelets rather than MFI, this supports our findings of LOX-1 expression on platelets. This study and other studies show that platelet activation with thrombin, as opposed to ADP, also up-regulates expression levels of LOX-1 on the surface of platelets (Bruni et al., 2005; Puccetti et al., 2005a,b). Moreover, statins reduce the expression levels of P-selectin (Bruni et al., 2005; Puccetti et al., 2005a). This finding has been suggested to explain the antiplatelet effect of statins. Interestingly, in agreement with our study is the report that the effect of statins on down-regulation of LOX-1 on platelets occurs much earlier than the reduction in serum LDL-cholesterol level. This suggests that the antiplatelet effects of statins are independent of serum cholesterol effects. It is not clear whether HMG-CoA reductase is fully functional in platelets. In our system, this confounding factor was excluded. It is unlikely that the effect of pravastatin seen in the present study could have been mediated by the HMG-CoA inhibitory effect, because the LOX-1 down-regulation effect was observed almost immediately.

In conclusion, we propose that platelet LOX-1 plays an important role in ADP-induced platelet integrin binding to fibrinogen via interaction with protein kinase C and that LOX-1 thereby participates in inside-out signaling of platelet integrins (Fig. 8). Our study also shows that aspirin and pravastatin can reduce LOX-1 expression level associated with reduction in ROS production by platelets. These data suggest the importance of LOX-1 in platelet aggregation. Furthermore, they elucidate its role in the mechanism of antiplatelet effect of combination of aspirin and pravastatin. More importantly, we also demonstrate the synergistic effect of aspirin and pravastatin as antiplatelet agents.

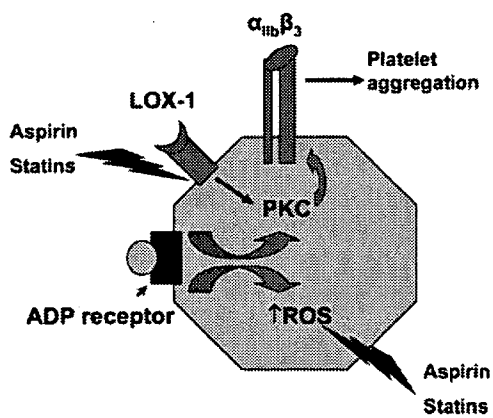

Fig. 8. ADP stimulation through ADP receptor activates inside-out signaling pathway of platelet integrins, mainly $\alpha_{IIb}\beta_3$, which involves PKC. LOX-1 blocking with anti-LOX-1 monoclonal antibody inhibits integrin activation by inhibiting PKC activation. Aspirin and pravastatin reduce both expression of LOX-1 and ROS.

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Lectin-like oxidized LDL receptor-1 as extracellular chaperone receptor: Its versatile functions and human diseases

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Abstract

Well-known coronary risk factors such as hyperlipidemia, hypertension, smoking, and diabetes are reported to induce the oxidative stress. Under the oxidative stress, low-density lipoprotein (LDL) is oxidatively modified in the vasculature, and formed oxidized LDL induces endothelial dysfunction, expression of adhesion molecules and apoptosis of vascular smooth muscle cells. It has become evident that these cellular responses induced by oxidized LDL are mediated by lectin-like oxidized LDL receptor-1 (LOX-1). LOX-1 was originally identified from cultured aortic endothelial cells as a receptor for oxidized LDL; however, recent investigations revealed that LOX-1 has diverse roles in the host-defense system and inflammatory responses, and it is involved in the pathogenesis of various diseases such as atherosclerosis-based cardiovascular diseases and septic shock. Beside oxidized LDL, LOX-1 recognizes multiple ligands including apoptotic cells, platelets, advanced glycation end products, bacteria, and heat shock proteins (HSPs). The HSPs function as a chaperone to affect protein folding of newly synthesized or denatured proteins. There are accumulating evidences that the HSPs released into the extracellular space have potent biological activities and it may work as a kind of cytokines. It is demonstrated that LOX-1 works as a receptor for HSP70, since it has high affinity for HSP70. The interaction of LOX-1 with HSP70 is involved in the cross-presentation of antigen. Given the potent and wide variety of biological activities, more understanding their interaction provides potential therapeutic strategy for various human diseases.

Keywords: Oxidative stress; Oxidized LDL; Heat shock protein

1. Identification of LOX-1 and its versatile functions

According to the response to injury theory, the endothelial injury associated with dysfunction is a very first step of atherosclerosis. Possible causes of endothelial dysfunction leading to atherosclerosis include hypertension, diabetes, oxidative stress, hemodynamic forces such as high shear stress, some pathogens, or oxidized LDL. Clinical investigations have demonstrated that well-known coronary risk factors, including diabetes, hyperlipidemia, hypertension, obesity and smoking, are associated with oxidative stress. Under the oxidative stress, LDL particles, which were trapped in the vessel wall, are oxidatively modified. Formed oxidized LDL is extensively accumulated into macrophages, resulting in the formation of foam cells. The treatment of endothelial cells with oxidized LDL induces pro-inflammatory responses. Oxidized LDL induces the production of wide variety of inflammatory cytokines or chemokines by vascular cells. Oxidized LDL induces the adhesion of blood cells to endothelium and vascular inflammation, which are essential in pathogenesis of atherosclerosis-based cardiovascular diseases. Therefore, the identification of signaling pathway induced by oxidized LDL and the clarification of detailed mechanisms is important for the understanding the pathogenesis of atherosclerosis-based cardiovascular diseases. In 1997, the lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) was cloned from a DNA library of bovine aortic endothelial cells employing expression cloning as the receptor for

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oxidized LDL on endothelial cells [1]. It is now evident that the atherogenic properties of oxidized LDL are mediated mainly via LOX-1. The activation of LOX-1 by oxidized LDL induces the up-regulation of monocyte chemotatic factor (MCP)-1, intercellular adhesion molecule (ICAM)-1, and vascular cell adhesion molecule (VCAM)-1, and the release of reactive oxygen species via NADPH oxidase [2,3]. LOX-1 expression is up-regulated in various pathological conditions affecting vasculature including hypertension, diabetes, hyperlipidemia and atherosclerosis [4,5].

LOX-1 is a type II membrane glycoprotein with a molecular weight of 50 kDa, as shown in Fig. 1. LOX-1 exists as a disulfide-linked homodimer on the cell surface and the C-type lectin-like domain acts as an oxidized LDL binding domain [6]. A single homodimer of LOX-1 displays a lower affinity for oxidized LDL; however, chemical cross linking experiments demonstrate that LOX-1 assembles as a three homodimeric hexamer at the cell surface, and the clustering of LOX-1 on the cell surface results in the specific high affinity binding to oxidized LDL as shown in Fig. 2 [7].

To investigate the role of LOX-1 in endothelial dysfunction and atherogenesis *in vivo*, we generated mice overexpressing LOX-1 (LOXtg) in C57BL/6 and apolipoproteinE-null mice (apoEKO) backgrounds [8]. In the LOXtg mice, the expression of the transgene was prominent in coronary vessels and cardiomyocytes. The immunohistochemical analysis of LOXtg/apoEKO mice revealed that both oxidized LDL and 8-hydroxy-deoxyguanosine accumulated in the coronary arteries. Thus, LOX-1 expression in the coronary arteries was closely associated with the oxidative stress in the LOXtg/apoEKO mice. Furthermore, the expression of ICAM-1 and VCAM-1, as well as the number of macrophages around blood vessels, were significantly increased in LOXtg/apoEKO mice compared to control littermates. Furthermore, the LOXtg/apoEKO mice displayed accelerated coronary atherosclerosis. Interestingly, the lipid deposition was frequently associated with atherosclerotic plaques in the LOXtg/apoEKO mice, as shown in Fig. 3. Compared to the apoEKO mice, the LOXtg/apoEKO mice showed an approximately 10-fold increase in the atherosclerotic plaque area after they were maintained 3-weeks on a high-fat diet. Thus, LOX-1 plays a critical role in the pathogenesis of atherosclerosis-based cardiovascular diseases.

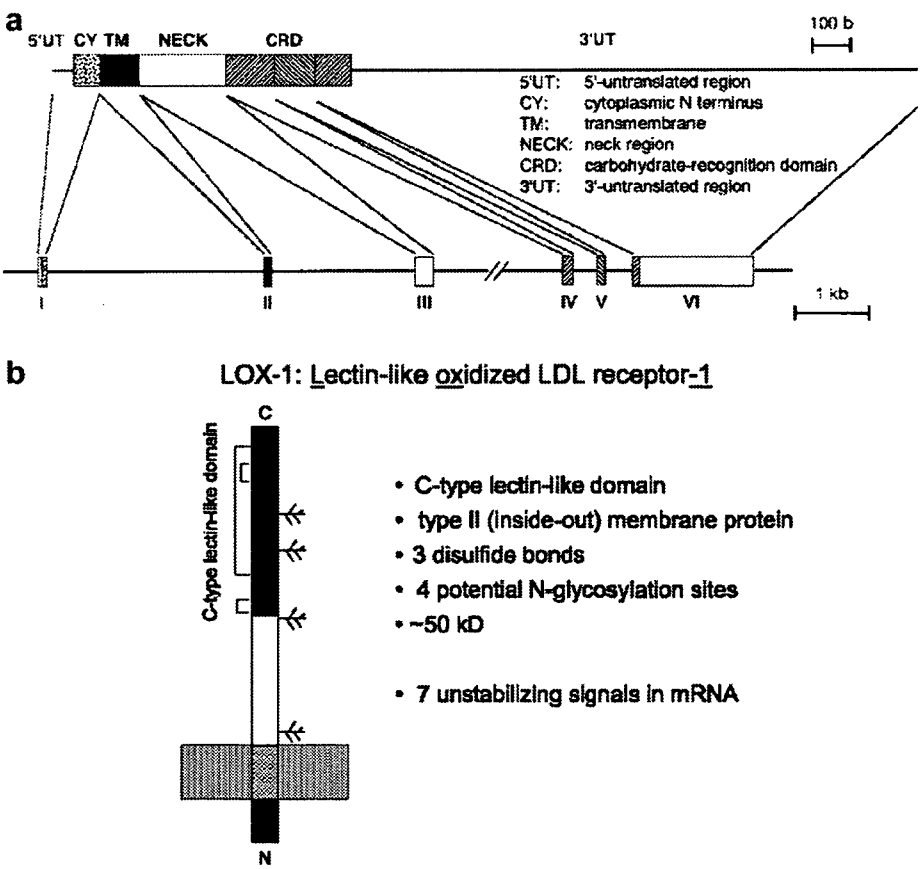

Fig. 1. (a) Structural organization of the human LOX-1 gene and mRNA. The scheme indicates the domain structure of the LOX-1 protein encoded by LOX-1 mRNA. The translated region is indicated by boxes: dotted box, cytoplasmic region; black box, transmembrane domain; open box, neck region; and striped box, CRD domain. The lower scheme indicates the exon–intron organization of the human LOX-1 gene. Exons are indicated by boxes numbered I–VI; introns, and 5′- and 3′-flanking sequences are indicated by lines. (b) Schema indicating the structure of the LOX-1 protein. Cited from Ref. [6].

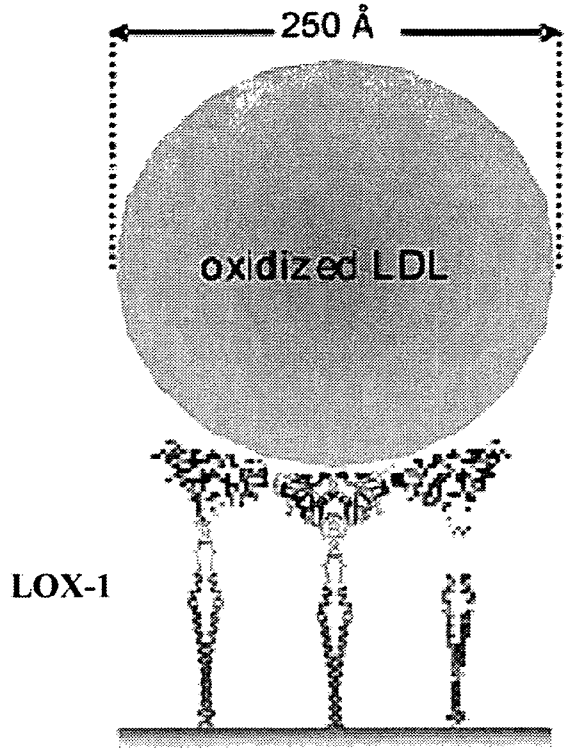

Fig. 2. The schema showing the binding of oxidized LDL to LOX-1. Cited from Ref. [7].

LOX-1 is highly homologous in the lectin-like domain of NKR-P1, which is essential for the activation of natural killer cells and is a member of the natural killer cell receptor family discovered in this group of C-type lectin-like receptors. This observation suggests the possibility that LOX-1 could recognize various ligands other than oxidized LDL, and that it plays some role in the host-defense system. In fact, recent progress in research on LOX-1 reveals that this hypothesis is true. LOX-1 recognizes not only oxidized LDL but also acidic phospholipids, apoptotic cells, damaged cells, platelets, bacteria, and advanced glycation end products (AGEs). Thus, besides playing a significant role in atherogenesis, LOX-1 is an important member in the host-defense system and is involved in inflammatory responses [9,10].

2. LOX-1 and inflammatory responses and host-defense system

The significance of LOX-1 in the inflammatory responses was demonstrated by animal models of septic shock. In a rat model of endotoxemia, injection of a high dose of endotoxin into rats induced leucopenia and death of the animals [11]. Pretreatment of anti-LOX-1 antibody reduced the degree of leucopenia and rescued the animals, whereas control IgG had no effect. Furthermore, in a model of endotoxin-induced uveitis, anti-LOX-1 antibody significantly suppressed leukocytes infiltration and protein exudation. In situ videomicroscopic analysis revealed that this LOX-1 blocking antibody reduced the number of rolling leukocytes and increased the velocity of rolling. These findings suggest that LOX-1 function as a vascular tethering ligand. Thus, LOX-1 is a key molecule in the inflammatory process as an adhesion molecule for leukocyte recruitment.

Furthermore, it has become evident that LOX-1 is critical for host-defense system and tumor immunity via the

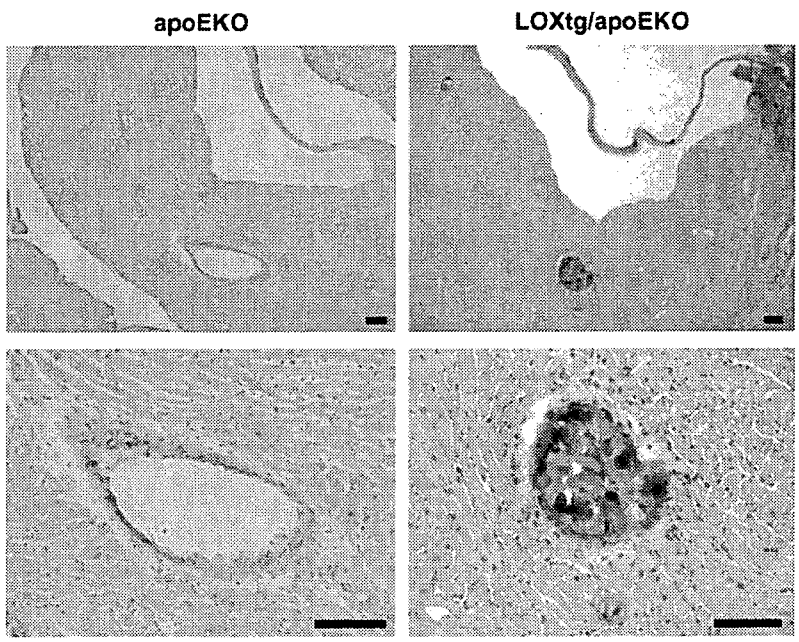

Fig. 3. Association of lipid deposition with atherosclerotic lesion. This figure is an oil-red O staining of the heart section of the apoE knockout mice and LOX-1 transgenic/apoE knockout mice who were fed a high-fat diet for 3 weeks. Small coronary arteries of the LOX-1 transgenic/apoE knockout mice show atherosclerotic changes with lipid deposition. Scale bars = 100 μm. Cited from Ref. [8].

interaction with heat shock proteins (HSPs). It is reported that the HSPs bind to LOX-1 in endothelial cells, epithelial cells, and antigen-presenting cells (APC) [12,13]. Considering important roles of the HSPs in host-defense system or stress response, the clarification of their interaction of with LOX-1 provides an important insight into the understanding pathogenesis of various inflammatory diseases.

3. HSPs and human diseases

HSPs work as chaperone to affect protein folding of newly synthesized or denatured proteins. The family of HSPs is organized by molecular sizes and functional classes, and it is composed of the HSP100, hsp90, Hsp70, Hsp60, Hsp40, and small heat shock protein families. The HSPs are expressed both constitutively and under stressful conditions, and their expression of HSPs is dynamically regulated. In addition to heat shock, various physiological and pathophysiological stimuli induce a marked increase in the HSPs as the result of such stress as ischemia, oxidative stress, hypoxia, reperfusion, glucose deprivation, or exposure to toxin. The principle role of HSPs is cytoprotection against such stress conditions.

The dysregulation of HSPs is likely associated with various diseases, including cardiovascular diseases, neurodegenerative disorders, and inflammation diseases. It is reported that some HSPs are dynamically up-regulated in atherosclerotic vessels. For example, Xu et al. demonstrated that the intensity of HSP60 expression correlated positively with the severity of atherosclerosis [14]. Furthermore, it is reported that the stress-inducible form of HSP70 was correlated with the development of Apo E-deficient mice [15]. Recently, it is reported that comparative two-dimensional electrophoretic analysis on carotid atherectomy specimens revealed that the expression of HSP27 is increased in the normal-appearing vessels compared with the plaque core [16]. This result suggests that the up-regulated HSP27 was involved in the early stage of atherosclerosis. It is well known that under the ischemic conditions, the up-regulated HSPs exert cytoprotective effects. Indeed, it is reported that the gene transfer HSP70 could preserve ventricular function under the ischemic-reperfusion injury [17]. Conversely, the deletion of the HSP70 gene deteriorates the ischemic area in cerebral infarction in mice [18]. The aggregates of misfolded proteins are observed in a number of neurodegenerative disorders, including Huntington disease, Parkinson disease, and Alzheimer disease. Various investigations demonstrated that the overexpression of HSPs suppressed the formation of aggregates in these pathological conditions. Thus, the intracellular HSPs play a pivotal role in the protection against these diseases.

The HSPs are traditionally regarded as intracellular protein; however, besides the intracellular role, there are reports indicating that some HSP acts as an intercellular signaling molecule since they activate the intracellular signaling system. There are evidences that elevated levels of antibodies against HSP70 in patients with autoimmune diseases [19]. This finding indicates that some HSPs exists in the extracellular milieu. Interestingly, the serum levels of HSP27 correlate with HSP70 and C-reactive protein [16]. Thus, the serum levels of HSPs might be associated with the disease activity or the state of inflammation. Very recently, it is reported that circulating HSP60 in the blood may be a determinant of endothelial dysfunction [20]. However, their cause–result relationships is obscure, that is, it is still unknown whether the increased levels of HSPs in extracellular spaces lead to the disease, or the releases of HSPs from the damaged or injured cells in the active pathological conditions results in the increased levels in the blood stream.

4. Extracellular HSPs and their receptors

These clinical investigations above mentioned rise to the question whether HSPs in the extracellular milieu work as a cytokine and function as a local modulator. If so, the specific receptor for HSPs should exist. Indeed, it is shown that HSP70 selectively bind to some cell types, including natural killer cells dendritic cells, macrophages, peripheral blood cells. Previous investigations reported that HSPs induced a wide variety of inflammatory responses such as production of pro-inflammatory cytokines and via CD14/Toll like receptor (TLR) 2 or 4 [21]. Furthermore, the HSPs exogenously administered possess the activities of HSP. From this viewpoint, chaperokine is referred as such activity of the HSPs, whereas there is some debate with regard to the cytokine activity of extracellular HSPs. It is pointed out that some of these cytokine-effects may be due to contamination of lipopolysaccharide. Nonetheless, there are accumulating evidences that HSPs work in the extracellular spaces. The 'work place' of HSPs is not only intracellular, but also the extracellular space.

So far, several receptors have been proposed as HSP receptors including Toll-like receptor 1 and 4 with their cofactor CD14, CD36, low-density lipoprotein-related protein CD91, SRA, and LOX-1. Thus, several scavenger receptors may work as a HSP receptor. Calderwood et al. examined the relative binding affinity of exogenous HSPs to various receptors, including LOX-1, TLR2, TLR4, and LRP/CD91 [12]. Among these receptors, LOX-1 has the highest affinity to HSP70. Furthermore, they demonstrated that HSP70 binds to human umbilical endothelial cells (HUVEC). However, the LOX-1 blocking antibody did not suppress the HSP70 to HUVEC, whereas this antibody blocked the binding of HSP70 to LOX-1-expressing CHO cells. Thus, LOX-1 is one of potent receptors for the HSP; however, other undermined receptor should be involved in the interaction of HSP to endothelial cells.

5. LOX-1 as receptor for HSP and cross-presentation of antigen

The antigen-presenting cells such as dendritic cells plays a crucial role at the interface between innate and adaptive

immunity. LOX-1 is expressed on dendritic cells and involved in antigen cross-presentation [13]. Cross-presentation is a process by which some exogenous molecules such as HSP are endocytosed by APC via specific receptors, gain access to the MHC class I pathway, and stimulate CD8+ cytotoxic T cells. Cell lysis following injury or infection releases a number of intracellular protein including HSPs into extracellular space. Under certain situations, the HSPs released into extracellular environment form a complex with intracellular peptides. In case of tumor, the HSP-intracellular peptide complexes are recognized by component of immune system, which leads to antitumor immunity. Delneste reported that targeting tumor antigen to LOX-1 *in vivo* induces the development of a protective antitumor CD8+ T cell response [13]. Thus, LOX-1 might be one of targets for cancer immunotherapy.

6. Conclusion

LOX-1 was originally identified as a receptor of oxidized LDL in the endothelial cells. However, progress in the research of LOX-1 has revealed its diverse functions. Among wide variety of biological activities, LOX-1 functions as a receptor of the HSPs. Considering the potent and wide variety of extracellular HSPs, more understanding the interaction of LOX-1 and HSPs provides a novel potential therapeutic strategy for various human diseases.

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LOX-1 deletion alters signals of myocardial remodeling immediately after ischemia–reperfusion

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Abstract

Objective: Chronic ischemia is associated with alterations in genes that result in myocardial remodeling. An important biochemical basis of cardiac remodeling is generation of reactive oxygen species (ROS). A few studies have suggested that acute ischemia triggers signals for remodeling. We examined the hypothesis that targeted deletion of lectin-like oxidized-LDL receptor (LOX-1) may inhibit signals related to cardiac remodeling.

Methods and results: We generated LOX-1 knockout (KO) mice on C57BL/6 (wild-type mice) background, and subjected wild-type and KO mice to ischemia–reperfusion (I–R). The wild-type mice developed a marked reduction in left ventricular systolic pressure and $\pm dp/dt_{max}$ and an increase in left ventricular end-diastolic pressure following I–R, and this change was much less in the LOX-1 KO mice, indicating preservation of left ventricular function with LOX-1 deletion. There was evidence for marked oxidative stress (NADPH oxidase expression, malondialdehyde and 8-isoprostane) following I–R in the wild-type mice, much less so in the LOX-1 KO mice ($P<0.01$). In concert, collagen deposition (Masson's trichrome and Picro-sirius red staining) increased dramatically in the wild-type mice, but only half as much in the LOX-1 KO mice ($P<0.01$). Collagen staining data was corroborated with procollagen-I expression. Further, fibronectin and osteopontin expression increased in the wild-type mice, but to a much smaller extent in the LOX-1 KO mice ($P<0.01$).

Conclusions: These findings provide compelling evidence that LOX-1 is a key modulator of cardiac remodeling which starts immediately following I–R.

Keywords: Remodeling; Ischemia; Reperfusion; NADPH oxidase; Extracellular matrix

1. Introduction

Heart failure is often the end result of cardiovascular disease states, such as myocardial ischemia [1]. Heart failure is characterized by abundant accumulation of extracellular matrix (ECM) proteins in the extracellular space. Among the ECM proteins, collagens constitute up to 85% [2,3]. Collagen type I is usually present in the form of thick fibres

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with a high tensile strength, and is considered a major determinant of myocardial stiffness [3,4]. Fibroblasts are the major source of collagen-I in the myocardium [3,5]. Proliferation of cardiac fibroblasts and deposition of collagen are directly associated with both systolic and diastolic, especially the latter, heart failure [6].

Collagen accumulation in the heart depends not only on its production, but also on its degradation by proteinases, such as matrix metalloproteinase (MMP-2, MMP-3 and MMP-9) [3]. Experimental studies suggest that reactive oxygen species (ROS) released in the early stages of ischemia–reperfusion (I–R) play a major role in the activation of MMPs, and that NADPH oxidase activation, a major source of ROS in the ischemic heart, is a key event in this process [7,8]. Release of cytokines and activation of renin–angiotensin system resulting in the formation of angiotensin II (Ang II) during acute ischemia are also associated with release of ROS and changes in collagens and MMPs [9,10].

Many investigators have highlighted the importance of matricellular protein osteopontin as a key mediator in the cardiovascular system, specifically in vascular remodeling, vascular calcification and left ventricular remodeling [11–13]. Recently, it has been shown that an osteopontin-NADPH oxidase signaling cascade promotes MMP-9 activation [14].

LOX-1 is a lectin-like oxidized-LDL receptor [15]. In previous studies, we showed that LOX-1 is involved in the genesis of oxidant stress and inflammation during myocardial I–R [16,17]. LOX-1 can also act as an adhesion molecule for inflammatory cells [18]. In other studies, we 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 [19]. Further oxidized-LDL treatment enhances collagen formation in fibroblasts that can be blocked by a LOX-1 antibody. These observations collectively suggest that LOX-1 may be an important player in myocardial I–R injury not only by inducing oxidative stress, but also by inducing signals for collagen and MMPs in the ischemic tissues.

We hypothesized that LOX-1 deletion would, by altering the major mediator of myocardial I–R injury, i.e. oxidant stress, improve cardiac diastolic function during ischemia–reperfusion. In addition, it would block or reduce the signal for myocardial remodeling process. Our findings in LOX-1 knockout (KO) mice reveal that "taking away" LOX-1 indeed limits early cardiac remodeling signal following I–R, and this effect is mediated by inhibition of NADPH oxidase.

2. Methods

C57BL/6 mice (also referred to as wild-type mice) were originally obtained from Jackson Laboratories. The homozygous LOX-1 KO mice were developed as described recently [20], and backcrossed 8 times with C57BL/6 strain to replace the genetic background. C57BL/6 and homozygous LOX-1 KO (on C57BL/6 background) mice were bred by brother-sister mating and housed in the breeding colony at University of Arkansas for Medical Sciences, Little Rock, Arkansas. This investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Male mice were utilized in the present studies at 8–10 weeks of age. All experimental procedures were performed in accordance with protocols approved by the Institutional Animal Care and Usage Committee.

2.1. PCR analysis of LOX-1 expression

Wild-type and LOX-1 KO mice were killed with CO_2 anesthesia, and blood, aorta and heart tissues were collected. Genomic DNA was obtained using DNAzol® Reagents (Invitrogen). PCR analysis of genomic DNA was done with the primer pair for deleted portion of LOX-1 gene: 5′-ggccaaccatggctatgggagaatgg-3′ and 5′-cagcgaacacagctccgtcttgaagg-3′, and for neomycin resistant gene: 5′-cgtttccgctgt caccgg-3′ and 5′-caacgctatgtcctgatagcggtcc-3′. 30 cycles of PCR were performed at 94 °C for 40 s, 60 °C for 1 min and 72 °C for 1 min. PCR-amplified products were visualized by ultraviolet light following electrophoresis in 1.5% agarose gel containing ethidium bromide.

2.2. Immunofluorescence staining

The expression of LOX-1 protein and the uptake of ox-LDL were analyzed using immunofluorescence staining. The cryothin sections of aorta were treated with Cy3-labeled anti-mouse LOX-1 monoclonal antibody (1 μg/ml, TS58). Thoracic aortas were incubated for 12 hours at 37 °C in DMEM/10% FCS containing 10 μg/ml DiI-labeled oxidized LDL which was prepared as described previously [21], then washed with PBS three times and snap frozen. To confirm the presence of endothelium, immunostaining with anti-von Willebrand factor, biotinylated anti-CD31 and avidin-FITC was performed, and subjected to observation under a laser confocal microscope.

2.3. Myocardial ischemia–reperfusion protocol

Animals were anesthetized with sodium pentobarbital (60 mg/kg, IP). Anesthesia was maintained via supplemental doses of sodium pentobarbital (30 mg/kg, IP) as needed. Mice were mechanically ventilated with room air using a Harvard respirator (model 683). The respirator's tidal volume was set at 1.4 ml/min and the rate at 110 strokes/min. Electrocardiographic leads were connected to the chest and limbs for continuous monitoring throughout the experiment.

After equilibration period of 10 min, a left thoracotomy was performed in the fourth intercostal space and the pericardium opened to expose the heart. A 6-0 silk suture was passed around the left coronary artery at a point two thirds of the way between its origin near the pulmonary conus and the cardiac apex and a snare was formed by passing both ends of the suture through a piece of polyethylene tubing. Occlusion of the

coronary artery, by clamping the snare against the surface of the heart, caused an area of epicardial cyanosis with regional hypokinesis and ECG changes. Reperfusion was achieved by releasing the snare and was confirmed by conspicuous hyperemic blushing of the previously ischemic myocardium and gradual resolution of the changes in the ECG signal. Another group of animals underwent the same procedure but without ligation of the coronary artery.

The chest wall was approximated and covered with Parafilm wax paper to prevent desiccation. Anesthetized mice were subjected to 60 min of coronary artery occlusion followed by 60 min of reperfusion, except the sham groups.

2.4. Assessment of left ventricular hemodynamics

To assess the hemodynamic status, a 1.4-Fr Millar (SPR-671) pressure transducer catheter was inserted through the right carotid artery into the left ventricle (LV); the position of the catheter was confirmed by typical wave form. Hemodynamic measurements were recorded at baseline and during reperfusion. Analog inputs from the pressure transducer were amplified using a Bridge amplifier and digitized with a PowerLab data-acquisition system (AD Instruments). All parameters were calculated from an average of 30 consecutive beats at each time point. Subsequent off-line evaluations provided LV systolic pressure (LVSP) and LV end-diastolic pressure (LVEDP). The first derivatives of the pressure over time ($\pm dp/dt_{max}$) were calculated from LV pressure tracings as a marker of systolic and diastolic ventricular function, respectively.

Table 1
Primer sequences used for RT-PCR

mRNA	Forward primer	Reverse primer	Annealing temperature (°C)	Size (bp)
$p22^{phox}$	5′-tgg cct gat tct cat cac tgg-3′	5′-ggg aca act cca cag aaa ctc-3′	55	580
$p47^{phox}$	5′-tcc cca gcc agc, act, atg-3′	5′-cag aga tga ccg tgg caa c-3′	56	462
Procollagen-I	5′-tgc ttg cag taa ctt cgt gcc-3′	5′-tgg acc acg ggg acc tgc agg-3′	58	387
MMP-2	5′-tct gcg ggt tct ctg cgt cc-3′	5′-cac ggt ttc agg gtc cag g-3′	55	363
MMP-3	5′-tct tcc ggt cct gct gtg gc-3′	5′-gaa tcc aca ctc tgt ctt ggc-3′	59	389
MMP-9	5′-tcg gct gca gct ctg ctg cc-3′	5′-tca tcg atc atg tct cgc gg-3′	55	376
Fibronectin	5′-ccg gtg gct gtc agt cag a-3′	5′-gtc tca atg gtg gtc tcc tcc-3′	57	368
Osteopontin	5′-tgt ttg gca ttg cct cct cc-3′	5′-cat cga ctg tag gga cga ttg g-3′	56	391
β-actin	5′-ttc tac aat gag gct gcg ttg-3′	5′-cac tgt gtt ggc ata gag gtc-3′	55	560

2.5. Determination of infarct size

At the end of reperfusion, the left coronary artery was re-occluded in the same location as before, and 1 ml 1% Evans blue (Sigma) was injected into the LV cavity and was allowed to perfuse the non-ischemic portions of the heart. The entire heart was excised, weighed, rinsed of excess blue dye, trimmed of right ventricular and atrial tissue, and sliced into 1-mm-thick sections from the apex to base. The slices were incubated in 1% triphenyl tetrazolium chloride (TTC, Sigma) at 37 °C for 15 min to stain the viable myocardium brick red. The slices were then fixed in a 10% formalin solution for 24 h. Each slice was imaged with computer-assisted planimetry (NIH Image J 1.34s) by an observer blinded to sample identity and following parameters was analyzed: (1) area at risk (AAR) as a percent of the left ventricle (LV) (AAR/LV), (2) the infarct area (IA) as a percent of AAR (IA /AAR).

2.6. Determination of oxidant stress in the left ventricular tissues

At the end of reperfusion, entire LV was homogenized in ice-cold 20 mM phosphate buffer. Malondialdehyde (MDA) was measured in the LV homogenate spectrophotometrically and expressed as nmol MDA/g. We also measured 8-iso-prostane, a nonenzymatic metabolite of ROS by enzyme immunoassay (EIA) in the LV homogenates as described previously [22]. The tissue 8-isoprostane level was expressed as ng/g.

2.7. Quantitative analysis of collagen positive area

Paraffin-embedded heart tissues were cut into 5 to 6 sections each 5 μm thick, and the sections were stained with Masson's trichrome and Picro-sirius red. The images were captured by digital imaging system and analyzed with Image pro software (Media Cybernetics). Area positive for collagen was recorded for each section and averaged for each mouse. Data were obtained from at least 3 mice in each group.

2.8. Expression analysis

At the end of reperfusion, entire LV was isolated for the expression analysis of LOX-1, NADPH $p22^{phox}$, NADPH $p47^{phox}$, procollagen-I, MMPs, osteopontin and fibronectin using standard methodologies of RT-PCR and Western blot [9]. Total RNA from the entire LV was extracted using Trizol (Invitrogen). The PCR primers and conditions employed are shown in Table 1. Densities of protein and mRNA bands relative to β-actin were analyzed.

Real-time RT-PCR analysis for the expression of NADPH oxidase ($p22^{phox}$ and $p47^{phox}$ subunits) was performed as described previously [23]. GAPDH was used as a standard control. The primers used are as follows. GAPDH: forward, 5′-AACTTTGGCATTGTGGAAGG-3′;

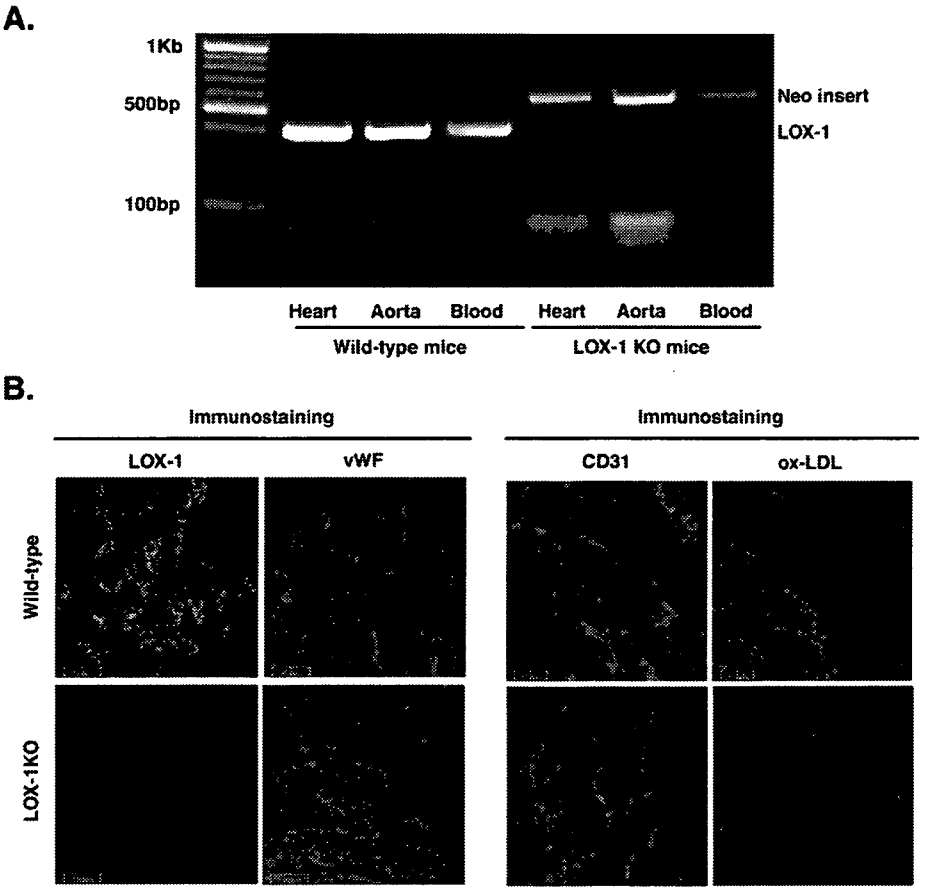

Fig. 1. Evidence for the deletion of LOX-1. (A) PCR analysis of LOX-1 in blood, aorta and heart; (B) Immunofluorescence staining of LOX-1 protein and ox-LDL uptake in aorta.

reverse, 5′-ACACATTGGGGGTAGGAACA-3′. $p22^{phox}$: forward 5′-GCCAACGA GCAGGCGCTGGC-3′; reverse, 5′-CTCGAGGGTATTCCAGCAG-3; $p47^{phox}$: forward, 5-GTGTACATGTTCCTGGTTAAG-3′; reverse, 5′-ATG-GAACTCGTAGATCTCG-3′.

2.9. Statistical analysis

Data are expressed as means±SE. The between-group difference in the infarct size was evaluated by unpaired *t*-test. All hemodynamic values were analyzed with a two-way ANOVA with repeated measures. All other data were analyzed by a two-way ANOVA with a Bonferroni post-hoc test. A $P<0.05$ was considered significant.

3. Results

3.1. Confirmation of LOX-1 deletion

In the genomic DNA from wild-type mice, we amplified a 403 bp LOX-1 fragment by PCR. This fragment was absent in the LOX-1 KO mice tissues. Instead, a neomycin-specific PCR fragment of 564 bp was detected. A representative experiment is shown in the Fig. 1 A. Further, LOX-1 expression was not detected in the vascular tissues of LOX-1 KO mice, although its presence in the endothelium of wild-type mice was confirmed by immunostaining. The endothelial integrity was confirmed by simultaneous staining with von Willebrand factor (Fig. 1 B left). Further, the uptake of ox-LDL in endothelium was undetectable in the LOX-1 KO mice, but clearly seen in the wild-type mice (Fig. 1 B right). These data confirm the genotype of wild-type and LOX-1 KO mice.

3.2. Ischemia–reperfusion induces expression of LOX-1

In keeping with previous studies [16,17], expression of LOX-1 was increased during I–R in the hearts of wild-type mice. The LOX-1 KO mice, as expected, did not show LOX-1 protein at baseline or during I–R (Fig. 2 A).

3.3. Targeted deletion of LOX-1 limits myocardial injury and preserves myocardial function

The basal values of indices of cardiac function (HR, LVSP, LVEDP and $\pm dP/dt_{max}$) were similar in the wild-type and LOX-1 KO mice. In both groups of mice, sham I–R caused no significant change in cardiac function (Table 2).

Following I–R, there was a marked decrease in HR, LVSP, and $\pm dp/dt_{max}$, and a significant increase in LVEDP in the wild-type mice. Reduction in $-dp/dt_{max}$ and increase in

Table 2
Effect of 60-min ischemia followed by 60-min reperfusion on hemodynamic parameters in wild-type and LOX-1 KO mice

	Baseline	Reperfusion time (min)			
		15	30	45	60
Left ventricular systolic pressure (mmHg)					
WT Sham	125±13	130±12	126±11	122±7	123±10
WT I–R	121±8	88±7**	79±10**	59±9**	47±14**
LOX-1 KO Sham	128±8	129±9	127±6	129±7	128±9
LOX-1 KO I–R	124±11	111±9	93±9#+	95±7#+	94±9#+
Left ventricular end-diastolic pressure (mmHg)					
WT Sham	2.2±0.2	2.2±0.4	2.1±0.5	2.2±0.3	2.3±0.5
WT I–R	2.4±0.4	4.2±0.6**	5.5±0.9**	6.9±1.2**	10.4±1.3**
LOX-1 KO Sham	2.1±0.3	2.2±0.5	2.3±0.3	2.2±0.4	2.3±0.4
LOX-1 KO I–R	2.3±0.4	2.8±0.3	3.2±0.7#+	4.0±0.4#+	6.5±1.2#+
$+dp/dt_{max}$ (mmHg/s)					
WT Sham	6889±639	6590±787	6479±323	6689±643	6743±622
WT I–R	6785±645	2765±556**	2832±514**	2249±485**	1824±558**
LOX-1 KO Sham	6708±722	6830±698	6738±470	6484±814	6609±412
LOX-1 KO I–R	5909±433	5012±460#+	4182±554#+	4350±348##++	4209±520##++
$-dp/dt_{max}$ (mmHg/s)					
WT Sham	6629±615	6539±539	6750±625	6420±361	6509±662
WT I–R	6867±317	2598±151**	2566±136**	1464±213**	1148±164**
LOX-1 KO Sham	6657±559	6761±401	6688±568	6561±598	6239±443
LOX-1 KO I–R	6121±240	5068±321#+	4676±261#+	4330±474##++	4038±254##++
Heart rate (beats/min)					
WT Sham	484±40	469±48	478±34	473±33	461±39
WT I–R	478±18	259±34**	242±42**	315±15**	327±10**
LOX-1 KO Sham	494±33	477±21	468±17	478±13	496±27
LOX-1 KO I–R	490±13	427±34++	423±19++	430±12++	424±17++

n=4. **P<0.01 vs. WT Sham; #P<0.05, ##P<0.01 vs. LOX-1 KO Sham;+P<0.05,++P<0.01 vs. WT I–R.
WT-wild-type; I–R-ischemia–reperfusion.

LVEDP indicate diastolic dysfunction. Importantly, despite similar period of I–R in the LOX-1 mice, there was much less deterioration of cardiac function parameters indicating a significant preservation of cardiac function (P<0.01 vs. wild-type mice).

As shown in Fig. 2 B, there were no differences in AAR in the two groups of mice. In the wild-type mice, I–R resulted in extensive infarct (64.1%± 10.6% of AAR). In contrast, LOX-1 KO mice had much smaller infarct (21.4%±3.5% of AAR, P<0.01 vs. wild type mice).

3.4. Myocardial ischemia–reperfusion induces oxidative stress

There is release of large amounts of ROS in the early stages of reperfusion, and NADPH oxidase activation is a major source of ROS in this process [7,8]. Release of ROS causes peroxidation of lipids in the heart. We measured MDA, an index of lipid peroxidation, 8-isoprostane, a nonenzymatic metabolite of ROS, and the expression of NADPH oxidase in the mice hearts. As shown in Fig. 2C and D, I–R caused a significant increase in MDA and 8-isoprostane levels in the wild-type mice hearts. In contrast, LOX-1 KO mice hearts had much lower MDA and 8-isoprostane (P<0.01 vs. wild-type mice), indicating much less ROS release. As shown in Fig. 3, both $p22^{phox}$ and $p47^{phox}$ subunits (mRNA and protein) of NADPH oxidase were markedly increased during I–R in both wild-type and LOX-1 KO mice (vs. sham I–R mice), but the LOX-1 KO mice had a much smaller increase (P<0.01 vs. the wild-type mice).

3.5. LOX-1 ablation reduces collagen deposition and MMPs expression

In the wild-type mice, I–R resulted in a marked increase in collagen accumulation (vs. sham I–R mice). In contrast, LOX-1 KO mice had much less collagen accumulation (P<0.01 vs. wild-type mice). The results of Masson's trichrome and Picro-sirius red staining were similar. Representative examples are shown in Fig. 4 (upper left panel), and the summary data are shown in Fig. 4 (lower left panel).

In support of the collagen data, procollagen-I expression (both mRNA and protein) was found to be markedly increased during I–R in the wild-type mice (P<0.01 vs. sham I–R mice), and the increase was much less in the LOX-1 KO despite I–R (Fig. 4, upper and lower right panel). Next,

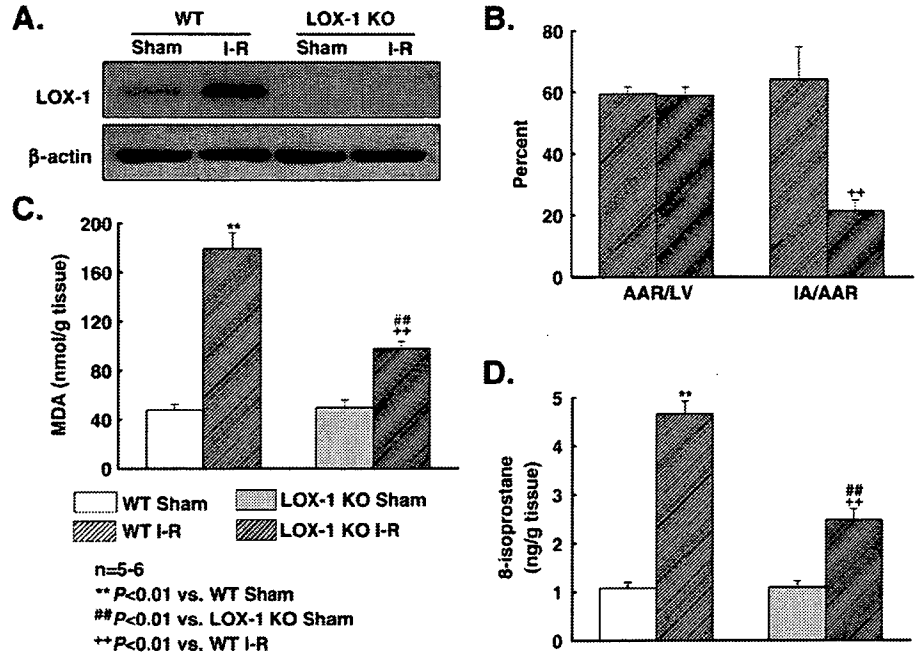

Fig. 2. (A) Western blot analysis of LOX-1 expression in the myocardium; (B) Infarct area (IA) as percent of area at risk (AAR) in different groups of mice; (C) Levels of MDA in the heart tissue; (D) Levels of 8-isoprostane in the heart tissue. WT: wild-type mice; I–R: ischemia–reperfusion.

we determined the expression of MMPs in the heart tissues (Fig. 5). Expression (both mRNA and protein) of MMP-2, MMP-3 and MMP-9 in the wild-type mice was also increased following I–R ($P<0.01$ vs. wild-type mice). It is of note that the basal expression of MMP-2 and MMP-3 was significantly lower in the LOX-1 KO mice than in the wild-type mice. Nonetheless, expression of all three MMPs following I–R remained lower in the LOX-1 KO mice as compared to the wild-type mice.

3.6. LOX-1 ablation attenuates expression of osteopontin and fibronectin

Osteopotin has been shown to interact with fibronectin and plays a role in matrix organization, stability and wound healing [13]. As shown as in Fig. 6, expression (both mRNA and protein) of osteopontin as well as fibronectin increased significantly during I–R in both wild-type and LOX-1 KO mice, but the absolute levels of both remained much lower in the LOX-1 KO mice than in the wild-type mice ($P<0.01$).

4. Discussion

4.1. Summary of main findings

Using state-of-the-art gene knockout technology, we provide convincing evidence that LOX-1 plays a potent role in the induction of myocardial infarct and dysfunction during I–R since the LOX-1 KO mice exhibited a much smaller infarct size and much less cardiac functional deterioration than the wild-type mice despite similar degree of I–R. Importantly, we provide conclusive evidence of expression of remodeling signals following a brief period of I–R in the wild-type mice and attenuation of these signals in the LOX-1 KO mice. The salutary changes in cardiac function in the LOX-1 KO mice were associated with attenuated NADPH oxidase expression, much less ROS release, and decreased collagen deposition.

4.2. Over-expression of collagen-I and metalloproteinases during ischemia–reperfusion

One of the early changes after the onset of ischemia is LV stiffness which persists during chronic ischemia and results in diastolic dysfunction. Although diastolic dysfunction during chronic ischemia has been ascribed to excessive collagen synthesis, enhanced collagen expression 1–2 h after onset of ischemia described in this study is a relatively novel finding. In earlier *in vitro* studies, we found that a brief exposure of cardiac fibroblasts to Ang II results in enhanced collagen-I synthesis [9,24]. We also showed that exposure of cardiac fibroblasts to anoxia-reoxygenation stimulates fibroblast growth as well as collagen type I synthesis and protein expression [25]. Collagen synthesis in response to Ang II and anoxia-reoxygenation was thought to be a response to the release of ROS. In this process, activation of MAP kinases and the redox-sensitive transcription factor NF-κB were noted to play an important role. It is of note that the cytokine TNFα that is released during I–R can also induce collagen synthesis in myofibroblasts [10].

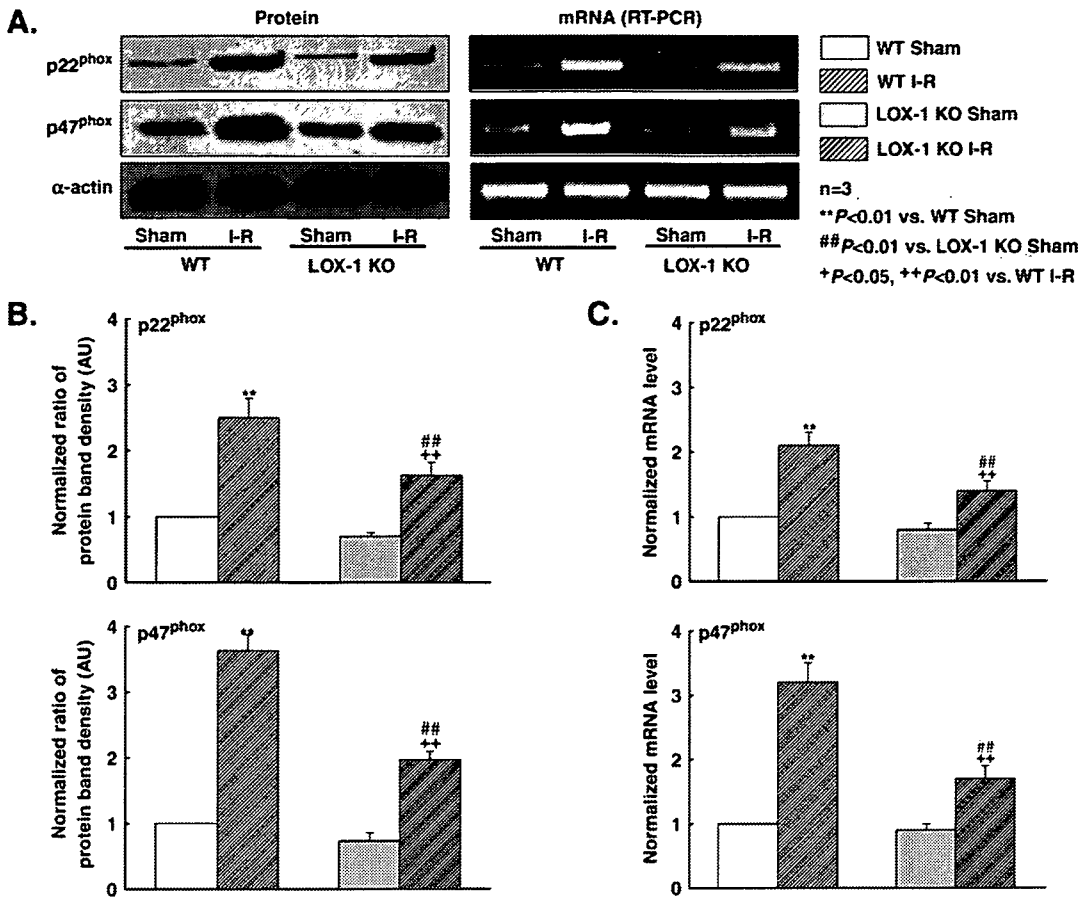

Fig. 3. Expression of NADPH oxidase $p22^{phox}$ and $p47^{phox}$ subunits. (A) Representative protein and mRNA bands determined by Western blot and RT-PCR, respectively; (B) Quantitative protein data; (C) Quantitative mRNA data determined by real time RT-PCR. WT: wild-type mice; I–R: ischemia–reperfusion.

Generally, collagen expression in the heart has been thought to occur several days or weeks after myocardial infarction [26–28]. It is of note that Takino et al [29] showed release of carboxyterminal propeptide of type I procollagen (PICP) in patients with myocardial infarction soon after deployment of reperfusion strategy. Release of PICP peaked at 2–3 weeks after myocardial infarction and correlated with end-diastolic volume. Our observations of increased collagen signals early after I–R are in concordance with the *in vitro* studies from our laboratory [9,24,25] and the *in vivo* study by Takino et al [29].

The marked increase in collagen noted in the present *in vivo* study was found to be associated with release of MMPs (−2, −3 and −9). The release of MMPs early after I–R has been frequently observed in the *in vivo* setting [30], and is thought to contribute to cardiac dilation and rupture during the acute stage of myocardial ischemia. Li et al [16] showed MMP release, expression of adhesion molecules and neutrophil accumulation in the I–R myocardium following a brief period of I–R in the Sprague Dawley rats. *In vitro* studies have also demonstrated that MMP release (by fibroblasts) during exposure of cardiac fibroblasts to anoxia-reoxygenation is triggered by redox-sensitive signals [25].

4.3. Inhibition of collagen-I and MMPs by LOX-1 deletion

Simultaneous release of MMPs and upregulation of collagen expression following I–R suggests that the two processes are inter-related and represent a cellular attempt to regulate the remodeling process. As discussed previously, expression of both MMPs and collagens may be a response to ROS release. Li et al [16] observed that I–R in the rat hearts was associated with over-expression of LOX-1, a finding reproduced in the present study in the wild-type mice. It is of note that LOX-1 activation has been linked to the release of ROS [31]. LOX-1 also acts as an adhesion molecule [18], and this may explain as to why the LOX-1 antibody administration before I–R reduced adhesion molecule expression and neutrophil accumulation in hearts exposed to I–R [16].

We showed that LOX-1 deletion reduces the expression of MMPs and procollagen-I at transcriptional level. We also documented a marked reduction in diastolic function as $-dP/dt_{max}$ fell and LVEDP rose in the wild-type mice subjected to I–R. These alterations may well relate to the increase in collagen signal and deposition in the I–R myocardium. Importantly LOX-1 KO mice had significantly less decline in $-dP/dt_{max}$ and much less increase in LVEDP in concert with a reduction in collagen deposition in the myocardium.

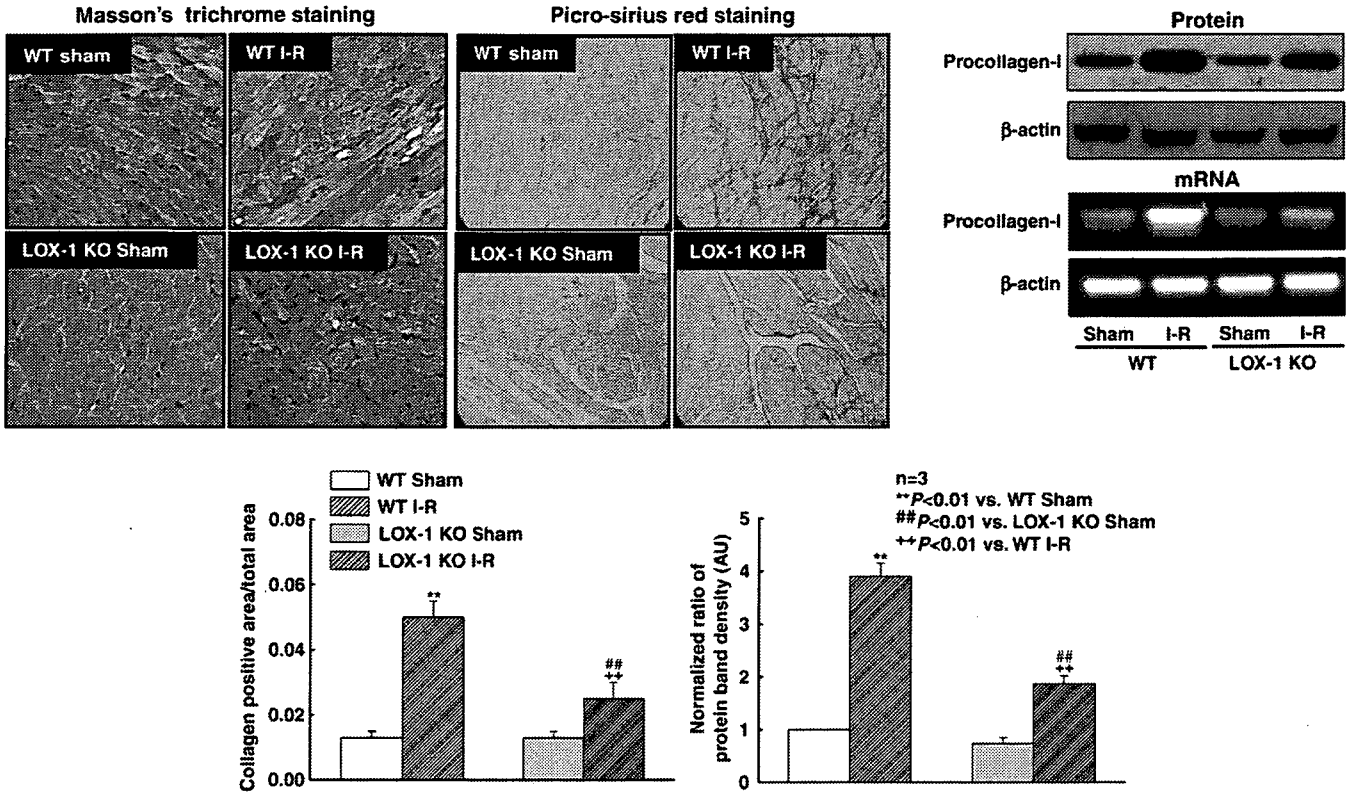

Fig. 4. Collagen and procollagen-I changes following ischemia–reperfusion (I–R). Upper left panel: collagen in representative heart sections (original magnification ×20); Lower left panel: quantitative data on collagen accumulation in heart sections; Upper right panel: representative procollagen-I bands (mRNA and protein); Lower right panel: quantitative data on procollagen-I protein. WT: wild-type mice; I–R: ischemia–reperfusion.

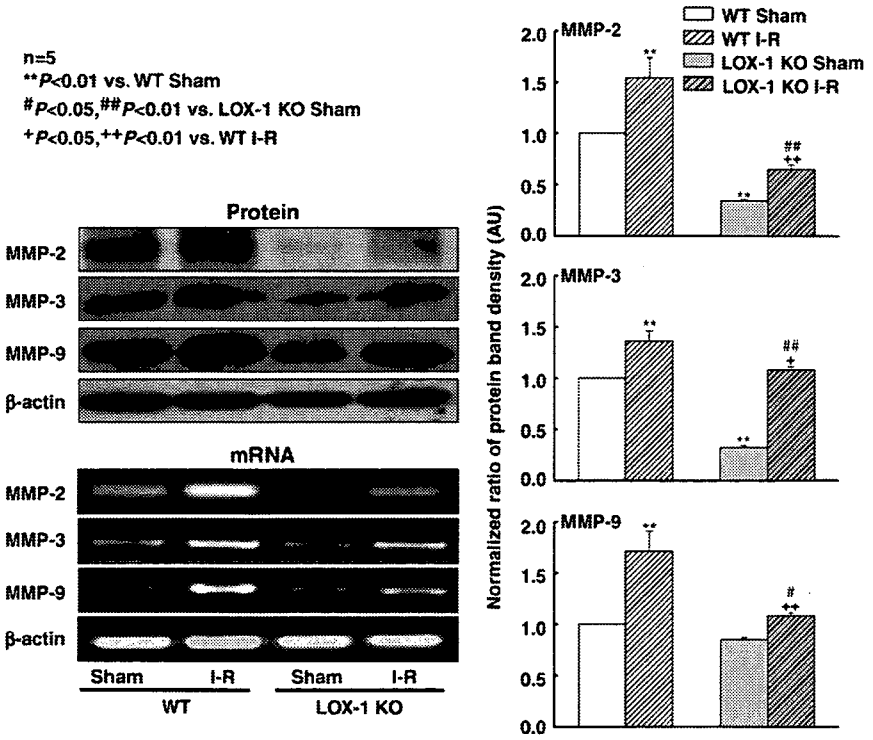

Fig. 5. Expression of matrix metalloproteinases (MMPs) determined by RT-PCR and Western analysis. WT: wild-type mice; I–R: ischemia–reperfusion.

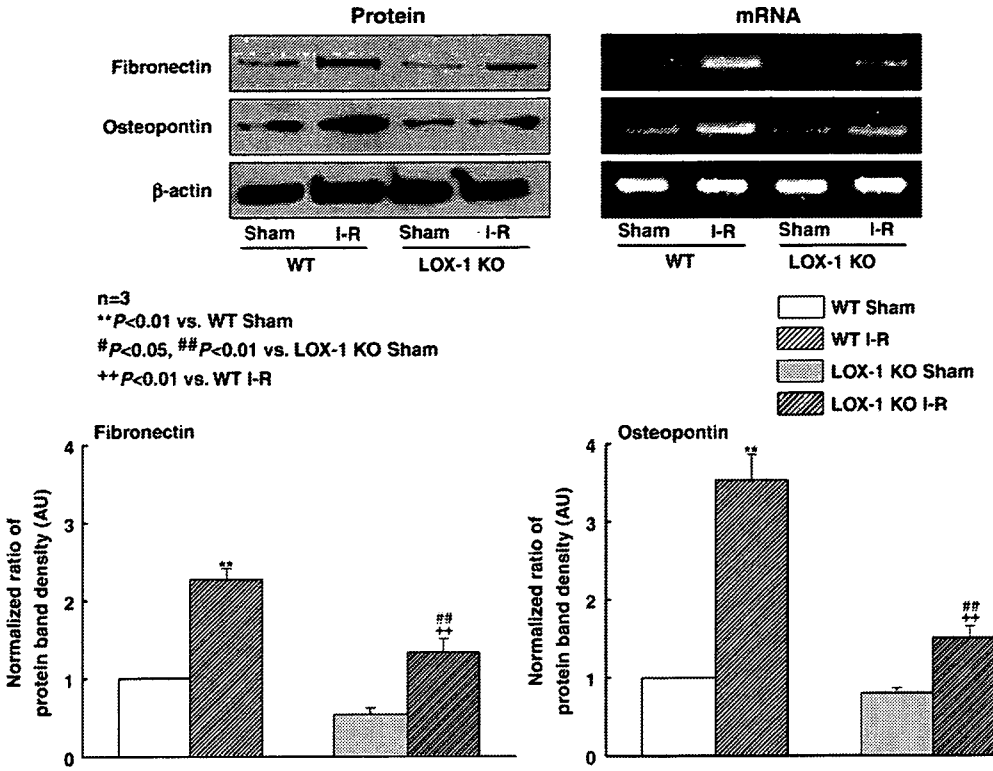

Fig. 6. Expression of fibronectin and osteopontin determined by RT-PCR and Western analysis. WT: wild-type mice; I–R: ischemia–reperfusion.

Reduction in procollagen-I may represent a decrease in oxidant stress in the LOX-1 KO mice. NADPH oxidase is the major source of ROS in the heart [7]. We measured NADPH oxidase expression and the myocardial MDA and 8-isoprostane levels, and found that NADPH oxidase (both $p22^{phox}$ and $p47^{phox}$ subunits) expression and ROS release and resultant lipid peroxidation increased dramatically in the wild-type mice subjected to I–R. This increase in NADPH oxidase and ROS release and resultant lipid peroxidation were much less in the LOX-1 KO mice. The absence of increase in NADPH oxidase and ROS release and resultant lipid peroxidation during I–R in the LOX-1 KO mice is in keeping with a previous report indicating that LOX-1 activation enhances superoxide anion generation [31].

We do not know the exact source of ROS in the present experiments, but it could be cardiomyocytes, fibroblasts, endothelial cell and/or neutrophils. All these cell types have been shown to generate ROS [32].

4.4. Osteopontin and fibronectin expression soon after myocardial ischemia

We observed that the expression of osteopontin as well as fibronectin increased in wild-type mice exposed to I–R. Osteopontin is an adhesion protein implicated as an important mediator of the profibrotic effects of Ang II in the heart. Osteopontin also acts as an adhesion molecule and has been implicated in chemoattraction of monocytes and in cell-mediated immunity [33]. It is also important in smooth muscle migration [34]. The plasma levels of the secreted glycophosphoprotein osteopontin have been associated with the presence and extent of coronary artery disease, especially with coronary calcification and restenosis after coronary intervention [11,12]. In the myocardium exposed to ischemia, osteopontin has been shown to interact with fibronectin suggesting its possible role in matrix organization, stability and wound healing [13]. Kossmehl et al [13] showed that the expression of MMPs, fibronectin and osteopontin was significantly elevated in the infarct area in porcine hearts subjected to 2 h of ischemia and 4 h of reperfusion. Simultaneously, large amounts of PICP were released in the perfusate. Fibroblast-like cells from the infarct area exhibited an enhanced ostepontin and fibronectin expression compared to fibroblasts derived from the control non-infarcted myocardium. Trueblood et al [27] examined the importance of osteopontin in the osteopontin null mice and found that the LV chamber dilation after myocardial infarction was approximately twice as great in osteopontin null mice as in the wild-type mice. Procollagen-I accumulation was also much less in the osteopontin null mice. Collins et al [35] showed that osteopontin is formed in response to Ang II, and the osteopontin null mice had much less cardiac fibroblasts proliferation and much less ECM accumulation after three weeks of Ang II infusion. Interestingly, osteopontin null mice had reduced MMP-2 and MMP-9 activity [36].

The signal for osteopontin expression seems to involve oxidant stress and related pathways. Xie et al [37] showed that p42/44 MAPK is a critical component of the ROS-sensitive signaling pathway activated by Ang II that regulates osteopontin gene expression. In another study in apoE KO mice [38], vitamin E decreased aortic 8-isoprostane and reduced both aortic macrophage infiltration and osteopontin expression. Lai and coworkers [14] demonstrated that osteopontin, via activation of NADPH oxidase-derived superoxide anion formation, promotes upregulation of MMP-9 in primary aortic myofibroblasts and smooth muscle cells under hyperglycemic conditions *in vitro*. Thus, there appears to be a strong link between NADPH oxidase-induced oxidant stress, osteopontin expression and MMP expression and activity. Gorin et al [39] have similarly shown a relationship between NADPH oxidase activation and fibronectin generation in both *in vitro* and *in vivo* conditions. In keeping with these studies, it was not surprising that the expression of osteopontin, fibronectin and MMPs was lower in the hearts of LOX-1 KO mice that had low levels of NADPH oxidase (both $p22^{phox}$ and $p47^{phox}$ subunits) and reduced myocardial 8-isoprostane and MDA content.

4.5. Signal for cardiac remodeling after ischemia–reperfusion

It is now amply evident that a host of mediators are expressed during I–R, 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, osteopontin and collagen leads to myocardial diastolic dysfunction. Attenuation of the expression of these signals in LOX-1 KO mice suggests that LOX-1 could be a relevant therapeutic target in the management of ischemia-associated myocardial dysfunction.

Acknowledgments

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Original article

LOX-1 abrogation reduces myocardial ischemia–reperfusion injury in mice

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Abstract

LOX-1 is a newly described lectin-like receptor for oxidized-LDL (ox-LDL), which is over-expressed in the ischemic myocardium. To examine the pathogenic role of LOX-1 in the determination of ischemia–reperfusion (I–R) injury to the heart, we developed LOX-1 knockout (KO) mice, and subjected these mice to 60 min of left coronary artery occlusion followed by 60 min of reperfusion. I–R in the LOX-1 KO mice resulted in a significant reduction in myocardial injury as well as in accumulation of inflammatory cells in the I–R myocardium and lipid peroxidation ($P<0.01$ vs. wild-type mice). Concomitantly, there was significant preservation of cardiac function in the LOX-1 KO mice despite I–R ($P<0.01$ vs. the wild-type mice). The phosphorylation of oxidative stress-sensitive mitogen-activated protein kinase (p38MAPK) and protein kinase B/Akt-1, expression of nitrotyrosine and inducible nitric oxide synthase (iNOS), and superoxide dismutase activity were enhanced during I–R in the wild-type mice. These alterations in p38MAPK, Akt-1 and iNOS were much less pronounced in the LOX-1 KO mice. The superoxide dismutase activity increased further in the LOX-1 KO mice. These observations provide compelling evidence that LOX-1 may be a key modulator of myocardial I–R injury, and its effect is mediated by pro-oxidant signals. LOX-1 may be a potential target for therapy of myocardial ischemic injury.

Keywords: LOX-1; Ischemia–reperfusion; Mitogen-activated protein kinase; Protein kinase B/Akt-1; Inducible nitric oxide synthase

1. Introduction

Ischemic myocardium is characterized by oxidative stress (release of reactive oxygen species [ROS]), inflammation and cell injury [1]. ROS are released in the early stages of ischemia–reperfusion (I–R) and act as chemoattractants for inflammatory cells; together they induce cell injury. ROS can oxidize low-density lipoproteins (LDL) [2], and the oxidized-LDL (ox-LDL) can injure cell membranes [3], further stimulate ROS generation [4] and increase inducible nitric oxide synthase (iNOS) expression [5], all part and parcel of I–R injury. Actually, perfusion of isolated hearts with ox-LDL *per se* results in myocardial injury [6]. Further, plasma levels of soluble ox-LDL are markedly elevated in patients with acute coronary syndromes [7], and the ischemic tissues contain large amounts of ox-LDL [8].

LOX-1, a lectin-like receptor for ox-LDL, was identified primarily on endothelial cells [9–11]. LOX-1 binds to and internalizes ox-LDL [9]. LOX-1 is upregulated by ROS, and itself stimulates the formation of ROS [10,11]. Its activation leads to

Abbreviations: iNOS, inducible nitric oxide synthase; I–R, ischemia–reperfusion; KO, knockout; LOX-1, a lectin-like receptor for oxidized low-density lipoprotein; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-kappa B; NO, nitric oxide; ox-LDL, oxidized low-density lipoprotein; ROS, reactive oxygen species.

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