LOX-1 deletion decreases collagen accumulation in atherosclerotic plaque in low-density lipoprotein receptor knockout mice fed a high-cholesterol diet

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Aims Collagen, as a component of the extracellular matrix, has been linked to atherosclerotic plaque formation and stability. Activation of LOX-1, a lectin-like oxidized low-density lipoprotein (LDL) receptor-1, exerts a significant role in collagen formation. We examine the hypothesis that LOX-1 deletion may inhibit collagen accumulation in atherosclerotic arteries in LDL receptor (LDLR) knockout (KO) mice.

Methods and results We generated LOX-1 KO and LOX-1/LDLR double KO mice on a C57BL/6 (wild-type mice) background and fed a 4% cholesterol/10% cocoa butter diet for 18 weeks. Vessel wall collagen accumulation was increased in association with atherogenesis in the LDLR KO mice (P < 0.01 vs. wild-type mice), but much less so in the double KO mice (P < 0.01 vs. LDLR KO mice). Collagen accumulation data were corroborated with pro-collagen I measurements. Expression/activity of osteopontin, fibronectin, and matrix metalloproteinases (MMP-2 and MMP-9) was also increased in the LDLR KO mice (P < 0.01 vs. wild-type mice), but not in the mice with LOX-1 deletion (P < 0.01 vs. LDLR KO mice). The expression of NADPH oxidase (p47phox, p22phox, gp91phox, and Nox-4 subunits) and nitrotyrosine was increased in the LDLR KO mice (P < 0.01 vs. wild-type mice) and not in mice with LOX-1 deletion (P < 0.01 vs. LDLR KO mice). Phosphorylation of Akt-1 and endothelial nitric oxide synthase and expression of haem-oxygenase-1 were found to be reduced in the LDLR KO mice (P < 0.01 vs. wild-type mice), but not in the mice with LOX-1 deletion (P < 0.01 vs. LDLR KO mice).

Conclusion LOX-1 deletion reduces enhanced collagen deposition and MMP expression in atherosclerotic regions via inhibition of pro-oxidant signals.

1. Introduction
Atherogenesis involves lipid accumulation, especially oxidized low-density lipoproteins (ox-LDLs),1 endothelial injury,2 chronic inflammation3 and oxidative stress.4 Besides the accumulation of lipids, atherosclerotic regions are characterized by the presence of fibrous elements in different layers of the arterial wall. A new insight into the role of collagen accumulation in plaque development has been gained by understanding the role of extracellular matrix (ECM).5 ECM composition and its metabolic behaviour are decisive factors in the evolution and complications of atherosclerosis. It is evident that the metabolic balance of ECM is regulated in large part by matrix metalloproteinases (MMPs).5

LOX-1, a lectin-like receptor for ox-LDL, is a major receptor responsible for binding, internalization, and degradation of ox-LDL in endothelial cells.6 It has been demonstrated that insertion of LOX-1 plasmids in cardiac fibroblasts that are naturally low expressors of LOX-1 alters the biology of fibroblasts to pro-inflammatory phenotype.7 Further, ox-LDL treatment enhances collagen formation by fibroblasts that can be blocked by anti-LOX-1 antibody. In cultured cardiac myocytes, LOX-1 activation has also been shown to upregulate the expression of pro-collagen I and collagen type I via redox-sensitive pathways.8 A recent study shows that LOX-1 deletion alters signals of myocardial

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remodelling immediately after ischaemia. These observations collectively suggest that LOX-1 may be an important player in the regulation of ECM.

There is growing evidence to support the contributory role of LOX-1 in atherogenesis. Importantly, targeted deletion of LOX-1 reduces atherogenesis in the LDL receptor knockout (LDLR KO) mice fed high-cholesterol diet. In order to gain further insight into the role of LOX-1 in ECM formation in atherogenesis, we studied the effect of LOX-1 deletion on ECM accumulation and MMPs expression in atherosclerotic aortas. We also examined the modulation of oxidative stress and pro-inflammatory signals as the mechanistic basis of ECM modulation.

2. Methods

2.1 Animal protocol

The generation of LOX-1 KO and LOX-1/LDLR double KO mice has been described recently. In brief, C57BL/6 mice (also referred to as wild-type mice) and homozygous LDLR KO mice (on C57BL/6 background) were originally obtained from Jackson Laboratories. The homozygous LOX-1 KO and LOX-1/LDLR double KO mice were back-crossed eight times with C57BL/6 strain to replace the genetic background. C57BL/6 and homozygous LOX-1 KO, LDLR KO and LDLR/LOX-1 double KO (all 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, AR, USA. All male animals were given a high-cholesterol diet (4% cholesterol/10% cocoa butter) for 18 weeks from the age of 6 weeks. This investigation conforms to the Guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health. All experimental procedures were performed in accordance with protocols approved by the Institutional Animal Care and Usage Committee.

2.2 Quantitative analysis of collagen-positive area

Entire aortas from the aortic arch above the aortic valves to the iliac bifurcation were harvested and embedded in paraffin. Cross-sections (5 μm thick) were made at five pre-defined points (proximal ascending aorta, aortic arch, descending aorta, mid-thoracic aorta, and abdominal aorta above the renal arteries). The sections were stained with Masson’s trichrome and Picro-sirus red. The images were captured by digital imaging system and analysed with Image pro software (Media Cybernetics). The presence of area positive for collagen (blue) was recorded for each section, averaged for each mouse and expressed as ratio of entire vessel wall area. Data were obtained from five mice in each group.

2.3 Immunohistochemical staining

Sections (5 μm thick) of aortas made at five pre-defined points as described above were incubated with primary antibody to fibronectin, osteopontin, or nitrotyrosine (Santa Cruz, dilution 1:200) for 2 h at room temperature, rinsed with PBS, and incubated with corresponding biotinylated secondary antibody for 30 min. The slides were then incubated in avidin–biotin complex for 30 min followed by rinse with PBS, then incubated in diamobenzidine, and finally washed in distilled water and counterstained with hematoxylin.

2.4 Protein preparation and analysis by western blot

Aortic specimens were derived from animals at 18 weeks of high-cholesterol diet. Aortic protein was extracted for expression analysis of pro-collagen I, osteopontin, fibronectin, MMP-2, MMP-9, NADPH oxidase p22phox, NADPH oxidase p47phox, NADPH oxidase gp91phox, NADPH oxidase Nox-4, nitrotyrosine, Akt-1, phos-Akt-1 (Ser 473), phos-S1177 endothelial nitric oxide synthase (eNOS), haem-oxygenase-1 (HO-1), and β-actin using standard methodologies of western blot. Band density relative to β-actin was analysed.

2.5 MMP-2 and MMP-9 activity assay

Activity of MMP-2 and MMP-9 in aortic tissues was determined by zymography.

2.6 Statistical analysis

Data are presented as mean ± SE. All data were analysed by a two-way ANOVA with a Bonferroni post hoc test. Four-fold comparisons were performed and an adjusted value of *P* < 0.05 was considered to be significant. All calculations were performed with SPSS version 12.0.

3. Results

3.1 LOX-1 deletion reduces collagen deposition in the aortic wall

Collagen is an important component of atherosclerotic plaque, and its role as a component of ECM has been established. We, therefore, determined accumulation of collagen by specific staining in multiple aortic sections from different animal groups. The results of Masson’s trichrome and Picro-sirus red staining were similar. Representative examples of aortic sections are shown in Figure 1A, and the summary data from Masson’s trichrome staining are shown in Figure 2A. There was extensive deposition of collagen in the aortas of LDLR KO mice, encompassing ~40% of the aortic cross-sectional area. In comparison to the LDLR KO mice, collagen deposition was much less in the aorta of double KO mice (~50% reduction vs. LDLR KO mice, *P* < 0.01). The wild-type control mice also showed some collagen-positive areas (~18% of cross-sectional area), perhaps a result of high-cholesterol diet feeding, and that there were much fewer collagen-positive areas in the LOX-1 KO mice.

In support of the collagen accumulation data, the LDLR KO mice showed a marked increase in pro-collagen I expression (*P* < 0.01 vs. wild-type mice). Deletion of LOX-1 in the LDLR KO mice reduced pro-collagen I expression (*P* < 0.01 vs. LDLR KO mice). Importantly, the pro-collagen I expression was lower in the LOX-1 KO mice when compared with that in the wild-type mice (*P* < 0.01, Figure 2B), indicating that LOX-1 deletion also reduces the basal expression of pro-collagen I.

3.2 LOX-1 deletion reduces expression of osteopontin and fibronectin in the aortic wall

Osteopontin has been shown to interact with fibronectin and to play an important role in ECM organization and stability. Accordingly, we determined the expression of osteopontin and fibronectin in the aortic sections from different groups of mice. Representative examples (immunohistochemistry) are shown in Figures 1B and western analysis in Figure 3. The expression of osteopontin as well as fibronectin was increased in the LDLR KO mice (*P* < 0.01 vs. wild-type mice). In contrast, LDLR/LOX-1 double KO mice showed much less increase in the expression of osteopontin as well as fibronectin (*P* < 0.01 vs. LDLR KO mice).
Figure 1 Representative examples of collagen accumulation (Picro-sirius and Masson’s trichrome staining, A), osteopontin and fibronectin expression, B, and nitrotyrosine staining, C) in selected aortic sections.

Figure 2 Quantitation of collagen accumulation (A) and expression of pro-collagen I by western blot analysis (B). KO, knockout.

Figure 3 Representative examples and quantitation of expression of osteopontin (A) and fibronectin (B), determined by western blot analysis.
3.3 LOX-1 deletion reduces matrix metalloproteinases expression and activity in the aortic wall

Collagen accumulation in various tissues depends not only on its production, but also on its degradation by proteinases, such as MMPs. Therefore, we determined the expression and activity of mouse-specific MMP-2 and MMP-9 (Figure 4A and B). The expression as well as activity of MMP-2 and MMP-9 was found to be increased approximately 100% in LDLR KO (P < 0.01 vs. wild-type mice). On the other hand, deletion of LOX-1 in the LDLR KO mice 'normalized' the expression and activity of MMPs (P < 0.01 vs. LDLR KO mice; P < 0.05 vs. wild-type mice).

3.4 LOX-1 deletion reduces redox-sensitive signalling associated with collagen accumulation

Atherosclerosis involves oxidative stress and inflammation, in keeping with this concept, p47phox, p22phox, gp91phox, and Nox-4 subunits of NADPH oxidase were markedly increased in the LDLR KO mice (P < 0.01 vs. wild-type mice) (Figure 5A). The upregulation of four subunits of NADPH oxidase 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 (P < 0.01 vs. wild-type mice), indicating that LOX-1 deletion reduces the basal expression of four subunits of NADPH oxidase. To confirm the increased production of reactive oxidative species (ROS) in aorta, we examined the presence of nitrotyrosine as an indirect marker of oxidative stress by immunohistochemistry (Figure 1C) and western blot analysis (Figure 5B). Expression of nitrotyrosine was higher in LDLR KO mice (P < 0.01 vs. the wild-type mice), and LOX-1 deletion limited this increase in nitrotyrosine expression.

Next, we studied the expression of Akt-1 protein and its phosphorylation in the aortic tissues. While Akt-1 protein expression was similar in all mice, Akt-1 phosphorylation was reduced by 50% in the LDLR KO mice (P < 0.01 vs. wild-type mice), and LOX-1 deletion in the wild-type and LDLR KO mice 'normalized' Akt-1 phosphorylation (Figure 6A). Akt-1 activation regulates the activity of eNOS. We, therefore, examined phos-S1177 eNOS expression in the aortic tissues. As shown in Figure 6B, the expression of phos-S1177 eNOS was reduced by ~50% in the LDLR KO mice compared with the wild-type mice (P < 0.01), and LOX-1 deletion markedly increased eNOS phosphorylation in the LDLR mice (P < 0.01). It is of note that the basal level of eNOS activity was higher in the LOX-1 KO mice when compared with wild-type mice (P < 0.01).

We also examined the expression of HO-1, another vasodilator species that is relevant in atherogenesis. As shown in Figure 6C, HO-1 expression was reduced in the aortas from LDLR KO mice (vs. wild-type mice). On the other hand, LOX-1 deletion enhanced HO-1 expression in LDLR KO mice. As with eNOS activity, basal levels of HO-1 were higher in the LOX-1 KO mice (vs. wild-type mice, P < 0.01).

4. Discussion

Atherosclerosis has been viewed as uncontrolled plaque growth eventually leading to total occlusion of the artery. New clinical findings suggest that acute coronary events are triggered by thrombosis associated with rupture of the...
atherosclerotic plaque. Clinical data from analysis of human atherosclerotic regions by intravascular ultrasound histology and polarization-sensitive optical coherence tomography suggest that collagen deposition (fibrous tissue) is a significant component of the atherosclerotic region. Detailed analysis of the atherosclerotic plaques in animal tissues also shows that collagen constitutes over 50% of the plaques. It may be postulated that fibrous tissue provides an anchor for smooth muscle cells and monocytes/macrophages. In light of our previous in vitro studies showing that collagen formation by fibroblasts treated with ox-LDL is an oxidant-response that can be blocked by LOX-1 abrogation, we embarked on this in vivo study to examine if LOX-1 deletion in the LDLR KO mice will attenuate collagen deposition. Indeed, our study shows that collagen deposition is markedly reduced in the LDLR KO mice with LOX-1 deletion. Further, this study revealed that the signals involved in collagen deposition in atherosclerotic tissues are similar to those seen in in vitro studies.

4.1 Collagen formation and deposition

While almost all cell types in atherosclerotic regions form collagen, fibroblasts are the primary source of collagen. Our previous studies showed that treatment of fibroblasts with ox-LDL enhances collagen formation, especially when LOX-1 is over-expressed in fibroblasts. Treatment of fibroblasts with angiotensin II (Ang II), which is abundantly present in the atherosclerotic regions, also stimulates fibroblast growth and collagen formation. In addition to collagen formation, there is abundance of collagen degrading enzymes, the MMPs, in the atherosclerotic plaque. Collagen degradation products and MMPs can also be identified in plasma and urine of patients with atherosclerosis. However, these markers lack a specific 'tissue signature'.

Simultaneous increase in MMPs and collagen expression in atherosclerotic tissues suggests that the two processes are inter-related and represent a cellular attempt to regulate the remodelling process. The expression of both MMPs and collagen may be a response to ROS release, a common accompaniment of atherogenesis. We have earlier shown that atherosclerosis is associated with over-expression of LOX-1, a finding reproduced in the LDLR KO mice on high-fat diet. It is of note that LOX-1 activation has been linked to ox-LDL, Ang II, and release of ROS.

We showed that LOX-1 deletion reduced the extent of atherosclerosis in the LDLR KO mice, and also attenuated the expression/activity of MMPs and pro-collagen I. Reduction in pro-collagen I may represent a decrease in oxidant stress in the LOX-1 KO mice. NADPH oxidase is the major source of ROS in the vascular tissues. We measured the expression of NADPH oxidase and nitrotyrosine, and found that the expression of NADPH oxidase (p22phox, p47phox, pg91phox, and Nox-4 subunits) and nitrotyrosine was increased dramatically in the LDLR KO mice. This increase in NADPH oxidase and nitrotyrosine (oxidant stress marker) was much less in mice with LOX-1 deletion.

We do not know the exact source of NADPH oxidase and ROS in the atherosclerotic arteries, but it could be fibroblasts, smooth muscle cells, endothelial cell, and/or inflammatory cells. All these cell types have been shown to generate ROS; it also appeared to be confirmed by nitrotyrosine staining in our study (Figure 1C).

4.2 Osteopontin and fibronectin expression in atherosclerosis

We observed that the expression of osteopontin as well as fibronectin was increased in the aortas of LDLR KO mice compared with wild-type mice. Osteopontin has been

![Figure 6](image-url)
implicated in chemoattraction of monocytes and in cell-mediated immunity.25 It is also important in smooth muscle cell migration.26 Collins et al.27 showed that osteopontin was formed in response to Ang II, and the osteopontin null mice had much less fibroblasts proliferation and much less ECM accumulation after 3 weeks of Ang II infusion. Interestingly, osteopontin null mice also had reduced atherosclerosis and MMPs activity.28

The signal for osteopontin expression seems to involve oxidant stress and related pathways. Xie et al.29 showed that Ang II regulates osteopontin gene expression via ROS-sensitive signalling pathway. Lai et al.30 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 hyperglycaemic conditions in vivo. Gorin et al.31 have similarly shown a relationship between NADPH oxidase activation and fibronectin expression in both in vitro and in vivo conditions. Our previous study10 showed that p38MAPK activity is increased in the LDLR KO mice and much less so in the LDLR KO mice with LOX-1 deletion. The results of the present study coupled with previous work suggest a strong link between NADPH oxidase-induced oxidant stress, osteopontin/fibronectin expression, and MMPs expression. In keeping with these studies, it was not surprising that the expression of osteopontin, fibronectin, and MMPs was lower in the aortas of mice with LOX-1 abrogation that had low levels of NADPH oxidase (p22phox, p47phox, gp91phox, and Nox-4 subunits).

4.3 Endothelial nitric oxide synthase and HO-1 in atherosclerosis and the effect of LOX-1 deletion

Atherosclerotic regions have reduced activity of eNOS.32 This phenomenon was confirmed in the present study (Figure 6) as well as in our previous work.10 Reduction of locally released NO may enhance oxidative stress and subsequently monocyte accumulation, collagen synthesis, and cell proliferation. Protein kinase B/Akt-1 is important in downstream targeting of extracellular PI-3 kinase signalling, and alterations in its activity may be important in the phosphorylation of NOS in response to oxidant stimuli.33 In keeping with this concept, we observed a reduction in Akt-1 phosphorylation and diminished expression of eNOS in the aortas of LDLR KO mice. Importantly, LOX-1 deletion enhanced Akt-1 phosphorylation to a level higher than that in the wild-type mice. Previous in vivo studies have also documented that LOX-1 is key to altered endothelium-dependent vasorelaxation in atherosclerosis.10

There is emerging evidence that HO-1 and its products function as adaptive molecules against oxidative insults.15,16 HO-1 upregulation in turn reduces NADPH oxidase activity34 and NF-κB phosphorylation.35 The signaling of HO-1 in atherogenesis is not clear, but it appears that persistent oxidant stress may reduce the expression of HO-1.36 There is also evidence that Akt-1 activation upregulates HO-1.37 The absence of HO-1 exacerbates atherosclerotic lesion formation,38 suggesting a potential tissue protective role for HO-1 in atherogenesis. In keeping with these observations, we found that atherosclerotic aortas from LDLR KO mice had reduced expression of HO-1. With LOX-1 deletion, there was a marked increase in HO-1 expression. Whether the two events are related or not is not clear, but our observations strongly suggest that oxidant stress and HO-1 are intertwined, and Akt-1/ NF-κB phosphorylation may relate to these alterations.

4.4 Collagen deposition in atherosclerosis and its relevance to LOX-1 deletion

Enhanced expression of collagen appears to be an inherent part of the atherosclerotic process. A host of mediators of oxidative stress, including ox-LDL and Ang II, are present in the atherosclerotic regions and activate NADPH oxidase system. The intense oxidant stress in the atherosclerotic regions stimulates MAPKs and the redox-sensitive transcription factors, such as NF-κB10 followed by upregulation of genes, such as fibronectin, osteopontin, collagen, and MMPs, which result in the formation of collagen. Interestingly, excessive collagen deposition is associated with enhanced release of MMPs. While ox-LDL and Ang II-stimulated LOX-1 activation enhances oxidative stress and inflammation,6 and oxidative stress per se upregulates LOX-1 expression.6 This process may self-amplify leading to intense collagen deposition in atherosclerotic regions over time. These events are summarized in Figure 7.

Attenuation of the expression of the redox-sensitive signals and collagen formation with LOX-1 deletion suggests that LOX-1 could be a relevant therapeutic target in the management of atherosclerosis and vascular remodelling process.
Conflict of interest: none declared.

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