Heart failure resulting from cardiac remodeling is the leading cause of morbidity and mortality in the United States and is becoming a major problem worldwide. The remodeling process is defined as progressive increase in ventricular wall volume and myocardial mass. The increase in cardiac mass comes from hypertrophy of cardiomyocytes and proliferation of cardiac fibroblasts, resulting in collagen secretion and resultant fibrosis. Cardiac fibroblasts constitute ≈25% of myocardial tissue volume and account for ≈60% of all cells in the heart. However, the cardiac cell makeup can vary in different species. In any case, fibroblasts are an important component of the heart. The increased cardiac mass during remodeling process is largely because of inappropriate fibroblast proliferation.

Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), a lectin-like 52-kDa receptor for oxidized low-density lipoprotein (LDL), is responsible for binding and uptake of ox-LDL, as well as other ligands exhibiting oxidized phospholipids, including injured and apoptotic cells. In recent studies, LOX-1 deletion was shown to limit cardiac hypertrophy and the remodeling process in mice subjected to sustained hypertension or myocardial ischemia. Part of the decrease in cardiac remodeling with LOX-1 deletion seems to be related to reduction in fibrosis in the heart. In view of markedly reduced collagen deposition and fibrosis in the LOX-1 knockout (KO) mice hearts, we posited that LOX-1 may modulate fibroblast growth, a key factor for cardiac fibrosis.

This study was designed to elucidate the role of LOX-1 on cardiac fibroblast growth and collagen secretion. Our study provides powerful evidence that LOX-1 is a major regulator of cardiac fibroblast growth, cytoskeleton, and function.

Materials and Methods
For a full description, see the online-only Data Supplement.

Results

Confirmation of LOX-1 Deletion
LOX-1 Deletion and Fibroblast Proliferation
In vitro cultures showed that LOX-1 KO cardiac fibroblasts became dramatically larger, some almost 10 times the size of WT mice fibroblasts (results shown in Figure 1D were
obtained on day 7 after cell seeding). The growth of cells in culture was also drastically slower when compared with that of WT counterparts ($P<0.001$) (Figure 1D bottom). This suggests that fibroblasts from LOX-1 KO mice hearts have markedly limited capacity for cell division. Failure of fibroblasts to divide and proliferate results in cells becoming very large.

**LOX-1 Overexpression and LOX-1 KO Fibroblast Proliferation**

To ascertain the role of LOX-1 in cardiac fibroblast growth, exogenous h-LOX-1 cDNA was transfected into fibroblasts from LOX-1 KO mice. After transfection, h-LOX-1 (408 bp) transgene was expressed at high level (Figure 2A and 2B). Importantly, h-LOX-1 cDNA-transfected LOX-1 KO mice fibroblasts exhibited restoration of ox-LDL uptake (Figure 2C). Furthermore, upregulation of h-LOX-1 resulted in a significant reduction in their size (Figure S2 in the online-only Data Supplement). Transfection with LOX-1 cDNA restored the morphology of LOX-1 KO mice cardiac fibroblasts similar to that of WT mice fibroblasts. Most importantly, proliferation pattern of h-LOX-1 cDNA-transfected LOX-1 KO fibroblasts became similar to that of the WT fibroblasts (Figure 2D).

**LOX-1 and CDC42 Expression and Cytoskeleton Organization**

Cell division control protein 42 (CDC42) regulates cell morphology, cell division, and cell cycle. We measured CDC42 in WT and LOX-1 KO fibroblasts by Western blotting and immunocytochemistry. Measurement by both methods showed that CDC42 expression was markedly lower in LOX-1 KO fibroblasts than in WT fibroblasts ($P<0.01$) (Figure 3A and Figure S3). Furthermore, transfection of LOX-1 KO fibroblasts with h-LOX-1 cDNA markedly upregulated CDC42 expression ($P<0.01$) (Figure 3B and Figure S3).

CDC42 is also an important regulator of cytoskeleton organization that determines cell morphology and cell division.11 Immunostaining for F-actin showed dramatic disruption of cytoskeleton with significant changes in strand orientation in LOX-1 KO fibroblasts. Analysis of different cell populations showed that some fibroblasts from LOX-1 KO mice were completely devoid of the protein. More importantly, changes in F-actin organization were reversed after LOX-1 KO cells were transfected with h-LOX-1 gene (Figure 3B).

**LOX-1 and NADPH Oxidase, Reactive Oxygen Species Generation, and Cell Proliferation Signals**

Reactive oxygen species (ROS) are important intracellular signals that stimulate cell proliferation.12 Inhibition of ROS generation has been reported to nearly abolish proliferation of many cell lines.13 LOX-1 is known to be a key mediator of ROS generation.14 Western blotting showed that LOX-1 KO mice cardiac fibroblasts had markedly reduced NADPH oxidase (subtypes p22phox and p47phox) expression and ROS generation (Figure 4A). Importantly, transfection of LOX-1 KO fibroblasts with h-LOX-1 cDNA significantly enhanced p22phox and p47phox expression and ROS generation (Figure 4B).

Nuclear factor (NF)-κB, murine double minute 2 (Mdm2), and serine/threonine-specific protein kinase-1 (Akt1) are
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Important signals for cell proliferation. Western blotting showed that LOX-1 deletion was associated with a marked reduction in phospho-NF-κB-p65 and Mdm2, as well as Akt (Figure 4A). Again, in keeping with other observations, transfection of LOX-1 KO mice cardiac fibroblasts with h-LOX-1 cDNA restored the expression of phospho-NF-κB-p65 and Mdm2, as well as Akt (Figure 4B).

LOX-1 and Cardiac Fibroblast Growth and Cardiac Fibrosis In Vivo

To further study whether LOX-1 regulates cardiac fibroblast growth during state of increased stress, we used a mouse model of sustained hypertension in response to angiotensin (Ang) II. Figure 5A shows absence of LOX-1 in the KO mice. After 4 weeks of continuous infusion, there was significant cardiac fibroblast growth (discoidin domain receptor 2 immunopositivity) and collagen accumulation (Picro-Sirius staining) in between cardiomyocytes and in the perivascular regions throughout the hearts of WT mice (Figure 5B). In keeping with previous observations, there was a significant decrease in collagen in the accumulation LOX-1 KO mice despite similar duration of Ang II administration. The number of fibroblasts in the heart sections was much fewer and collagen deposition much less in the LOX-1 KO mice hearts than in WT mice hearts ($P<0.01$).

Fibronectin is a major mediator of collagen secretion by proliferating fibroblasts. We stained myocardial sections with fibronectin-specific antibody and observed much lower fibronectin expression in LOX-1 mice hearts than in WT mice hearts ($P<0.01$) (Figure 5A).

The results of immunopositivity and Picro-Sirius staining were confirmed by Western blotting (Figure 5C). In addition, the generation of collagen and pro-collagen-1 was markedly reduced in the hearts of LOX-1 KO mice ($P<0.01$ versus WT mice hearts) (Figure 5C).

Discussion

In this study, we provide convincing data that the presence of LOX-1 is essential for cardiac fibroblast growth. Intact LOX-1 function seems to be critical for fibroblast proliferation and cytoskeletal organization because LOX-1 abrogation in fibroblasts resulted in severely altered F-actin distribution, impaired mitosis, polyploidy, and very substantial (some $\leq 10$-fold) increase in size compared with WT mice fibroblasts under identical culture conditions. Moreover, on transfection of the fibroblasts from LOX-1 KO mice with h-LOX-1 cDNA, these cells became essentially indistinguishable from WT fibroblasts in terms of proliferation, morphology, and cytoskeletal organization.

Although several studies have shown that LOX-1 activation influences proliferation of endothelial cells and cardiomyocytes, we now show that cardiac fibroblast growth potential also depends on availability of LOX-1. Cultured LOX-1 KO fibroblasts showed a depletion of CDC42, a key molecule that regulates cytoskeleton assembly, cell-cell adhesion, and cell cycle progression and promotes G1/S progression. The reduction of CDC42 observed in LOX-1 KO fibroblasts could also account for signs of mitotic distress in these cells including polyploidy, increased dimensions, and compromised proliferation.

Studies have shown that a modest oxidant stress is responsible for growth of endothelial cells and other cell lines. Hu et al and Dandapat et al showed that endothelial cell proliferation in response to oxidant stress induced by ox-LDL or Ang II.

Figure 2. Oxidized low-density lipoprotein uptake and growth of Lectin-like oxidized low density lipoprotein receptor-1 (LOX-1) knockout (KO) mice fibroblasts after transfection with empty vector or human LOX-1 cDNA (h-LOX-1). A, Reverse-transcription polymerase chain reaction shows LOX-1 expression in fibroblasts after transfection with empty vector or h-LOX-1; (B) immunostaining shows LOX-1 expression in KO mice fibroblasts after transfection with h-LOX-1; (C) 1,1'-dioctadecyl-3,3',3'-tetramethyl-indocarbocyanine perchlorate labeled oxidized low-density lipoprotein (Dil-ox-LDL) uptake in LOX-1 KO mice fibroblasts after transfection with h-LOX-1; (D) morphology and numbers of LOX-1 KO mice fibroblasts 2 days after transfection with h-LOX-1. Graphs show cell numbers as mean±SD based on 5 independent experiments. DAPI indicates 4',6-diamidino-2-phenylindole.
is mediated via LOX-1. In their studies, LOX-1 antibody, inhibitors of NADPH oxidase, ROS, or NF-κB each inhibited the proliferative effects of ox-LDL or Ang II. In the present study, we show that the loss of LOX-1 in fibroblasts was associated with markedly lower ROS production and reduced expression of Akt1, Mdm2, and NF-κB and dramatically reduced proliferation. Importantly, all these changes were reversed with transfection of cells with h-LOX-1 cDNA, and LOX-1 KO fibroblasts became indistinguishable from WT fibroblasts.

Akt is a necessary survival signal in mammalian cells. Akt 14 Higuchi et al 24 have stressed the importance of the activation of Akt1 and Rac and CDC42 GTPases in rat fibroblast motility. In their studies, Akt1 was observed to be downstream of Rac/CDC42. Furthermore, the Akt dominant-negative state resulted in inhibition of fibroblast motility stimulated by Rac/CDC42. The active Akt1 seemed to localize at the leading edge of fibroblast with Rac and Cdc42. 24

Mdm2 is recognized as a potent regulator of cell growth and serves as an important negative regulator of the p53 tumor suppressor activity. A number of investigators have shown that the activity of Mdm2 is regulated by the NF-κB family of transcription factors, particularly RelA (p65). 25,26 although there are some reports of regulation of Mdm2 expression independent of NF-κB activation. 27 The reduction in the expression of Mdm2 and NF-κB in LOX-1 KO fibroblasts and the reversal of this phenomenon with h-LOX-1 transfection suggest an important role of LOX-1 in the regulation of Mdm2 and thereby in fibroblast development and growth.

A key role of ROS as second messengers in cell signaling is well established. 28 Whereas generation of large amounts of ROS in pathologic states clearly alters cell membrane function and denatures mitochondria, 12,29 low levels of ROS play an important role in functioning as signaling molecules for cell proliferation and migration. 21,22 Cominacini et al 30 showed that ROS upregulate LOX-1 transcription and activate it. Furthermore, LOX-1 activation itself results in activation of NADPH oxidases and generation of ROS, resulting in downstream activation of redox-sensitive transcription factors such as NF-κB. 12,31 Recently, Yu et

**Figure 3.** Cell division control protein 42 (CDC42) expression and cytoskeleton organization in wild-type (WT) and lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) knockout (KO) mice fibroblasts. (A) Western blotting and immunocytochemistry show altered CDC42 expression and F-actin organization in LOX-1 KO mice fibroblasts (vs WT mice fibroblasts); (B) Western blotting and immunocytochemistry show that CDC42 expression and F-actin organization in LOX-1 KO mice fibroblasts transfected with empty vectors or vectors carrying h-LOX-1 cDNA.

**Figure 4.** Reduced nicotinamide-adenine dinucleotide (NADPH) oxidase, reactive oxygen species (ROS) generation, and cell proliferation signals in wild-type (WT) and knockout (KO) mice fibroblasts. (A) NADPH oxidase (p22phox and p47phox subunits), phosphor-nuclear factor (NF)-κB-p65, murine double minute 2 (Mdm2) and serine/threonine-specific protein kinase 1 (Akt1) expression, and ROS generation in WT and lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) KO mice fibroblasts; (B) NADPH oxidase (p22phox and p47phox subunits) phosphor-NF-κB-p65, Mdm2 and Akt1 expression, and ROS generation in KO mice fibroblasts transfected with empty vector or h-LOX-1.
Wang et al showed that enalaprilat (an angiotensin-converting enzyme inhibitor), N-acetyl-cysteine (an antioxidant), and SB203580 (a mitogen-activated protein kinase p38 inhibitor) each inhibited Ang II–stimulated rat cardiac fibroblast proliferation by inhibiting burst of intracellular ROS. In keeping with these observations, Wang et al demonstrated that the angiotensin II type I receptor blocker losartan, as well as aspirin, attenuated fibroblast proliferation and expression of collagens in response to small concentrations (10\(^{-9}\) mol/L) of Ang II. Suppression of proliferation was accompanied with inhibition of NADPH oxidase, ROS generation, and NF-κB phosphorylation and a suppression of LOX-1.

The reversal of changes in intracellular signals, structure, and proliferation potential of LOX-1 KO fibroblasts on transfection with h-LOX-1 cDNA strongly implicates LOX-1 as a primary regulator of fibroblast biology via NADPH oxidase activation, ROS generation, and NF-κB phosphorylation.

We translated the in vitro observations in the in vivo state using WT and LOX-1 KO mice, both subjected to continuous Ang II infusion, which resulted in sustained hypertension and cardiac remodeling characterized by fibroblast growth and collagen accumulation. Particularly noteworthy is the observation of only limited number of fibroblasts and collagen accumulation in the hearts of LOX-1 KO mice subjected to sustained hypertension. The most important signal for collagen generation, that is, fibronectin, was also markedly diminished in LOX-1 KO mice hearts when compared with that in WT mouse hearts. The model of LOX-1 deletion has been extensively used in models of atherogenesis, myocardial ischemia, and hypertension. In all these models, LOX-1 deletion was shown to reduce ROS generation, NF-κB activity, and collagen deposition. The results of the in vivo studies are in concert with the in vitro data of limited fibroblast potential in the absence of LOX-1. However, the LOX-1-Ang II interaction described here seems to be unique, and this relationship is not seen in norepinephrine-induced hypertension.

In summary, we provide strong evidence that LOX-1 is involved in the maintenance of fibroblast cytoskeleton. LOX-1 also seems important in fibroblast division and growth, resulting in collagen formation. These observations may have a bearing on the relative absence of fibrosis and collagen deposition in the hearts of LOX-1 KO mice subjected to the chronic stress of ischemia or sustained hypertension.
Perspective
Fibroblasts play a critical role in cardiac remodeling after the stress of sustained hypertension. In this study, we show that LOX-1, a lectin-like 52-kDa molecule, responsible for binding and uptake of ox-LDL, as well as other ligands exhibiting oxidized phospholipids, plays a key role in the development of fibroblast cytoskeleton. Cytoskeleton development is critical in the cell proliferation. We show that cardiac fibroblasts taken from LOX-1 KO mice hearts do not proliferate and are deficient in related signals, Akt and Mdm2. These in vitro observations were confirmed in the hearts of LOX-1 KO mice that showed much less fibroblast growth, fibronectin, and collagen formation, confirming previous observations of attenuated cardiac remodeling despite sustained hypertension in these mice. These observations elucidate a novel mechanism of fibroblast proliferation and suggest a potential role of LOX-1–based therapies in modulating cardiac remodeling that occurs in stress states.

Sources of Funding
This study was supported, in part, by funds from the Department of Veterans Affairs, Veterans Health Administration, Office of Research and Development, Biomedical Laboratory Research and Development, Washington, DC.

Disclosures
None.

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Lectin-like Oxidized Low-density Lipoprotein Receptor-1 (LOX-1) and Cardiac Fibroblast Growth

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Hypertension. published online October 8, 2012;
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/early/2012/10/08/HYPERTENSIONAHA.112.200659

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Supplementary material

LOX-1 and cardiac fibroblast growth

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Running title: LOX-1 and cardiac fibroblast growth

Word count: Abstract: 250     Complete paper: 4002

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Methods

Animal protocol

C57BL/6 mice (wild-type, WT) were obtained from Jackson Laboratories (Bar Harbor, ME, USA). The homozygous LOX-1 KO mice were developed and backcrossed eight times with C57BL/6 strain to replace the genetic background [8,11]. This investigation conforms to 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). All experimental procedures were performed in accordance with protocols approved by the Institutional Animal Care and Usage Committee.

After anesthesia (pentobarbital sodium, 80 mg/kg, i.p.), animals were implanted an osmotic minipump (Alzet Technical Services, Model 2004) containing saline vehicle or Ang II (Sigma, St Louis, MO) in the subcutaneous space. Ang II was delivered at an infusion rate of 50 ng/min for 28 days. The dosage of Ang II was chosen on the basis of published data [11]. Ang II-treated WT and LOX-1 KO mice developed sustained hypertension (Supplement Figure S1).

Genotyping

Genomic DNA was extracted from the tails of the mice using DNeasy Blood-Tissue Kit according to the manufacturer’s instructions. PCR was performed using a 20 μL reaction volume containing 100 ng/mL DNA, 10μL 2X PCR reaction mixture and 0.3 μM primers for LOX-1 gene: 5’-GGCCAACCATGGCTTGGGAGAATGG-3’ and 5’-CAGCGAACACAGCTCCGTCTTGAAGG-3’; and for neomycin-resistant gene: 5’-AGGATCTCGTCTGACCATGGCGA-3’ and 5’-GAGCGGCGATACCGTAAGCAGCAG G-3’. The products were separated by 1.5 % agarose gel electrophoresis and visualized by ethidium bromide on UV transilluminator.

Cardiac fibroblast culture

Cardiac fibroblasts were isolated from the hearts of 4 week-old WT mice and LOX-1 KO mice with C57BL/6 background as reported previously [12]. Experiments were performed with passages 3 to 5 cells. For gene transfection, LOX-1 KO fibroblasts were seeded in 6-well plates (for protein extraction) and 24-well plates (for cell counting). When the cells reached 70% confluence, they were transfected with PCI-neo plasmid with human LOX-1 cDNA (hLOX-1) or PCI-neo empty plasmid (vector).

Cell counting

Fibroblasts (4×10⁴/well) were seeded in 6-well and cell number was determined using a hemocytometer on days 1 through 13. For the transected fibroblasts (seeded in 24 well plates), cell number was counted 48 hours after transfection.

Dil-ox-LDL uptake

Dil-ox-LDL uptake was determined as described previously [13].

Western blotting

Proteins were extracted from cardiac fibroblasts or heart tissues using standard protocol. Protein samples in 12% SDS-PAGE were transferred to the PVDF membranes on a semi-dry transfer apparatus. The membranes were blocked with 5% non-fat milk in
Tris-buffered saline/0.1% tween (TBS-T), and then incubated with primary antibodies (1:1000) at 4°C overnight. The blots were incubated with HRP-conjugated second antibodies (1:10000) for 1 hr at room temperature. Source of primary antibodies for Western blotting: CDC42, DDR2 and β-actin (ABcam), p-NF-κB (Cell signaling), MDM2, p22phox, p47phox, fibronectin, collagen-1 and procallagen-1 (Santa Cruz), and LOX-1 (gift from Prof T. Sawamura, Osaka, Japan).

**RT-PCR**

RT-PCR was performed as described previously [12]. The primer pair for Akt-1 was F- 5’-GTCTCTAGGGTCAGGCGCAAAATCGTC-3’ and R- 5’-CATCTAAAGGACAAGTCTAGGAG-3’.

**Immunofluorescence assay**

Immunostaining of the cultured cardiac fibroblasts was performed as previously described [12] using standard methods. All samples were imaged with LSM510 Zeiss Laser inverted confocal microscope using LSM510 software.

**ROS measurement**

Flow-cytometry assay was used to evaluate the levels of superoxide anions by dihydroethidium staining as described previously [12].

**Statistical analysis**

Statistical analysis was performed with SPSS 11.5 software. Data are presented as means and standard deviation (SD) from at least 4 independent experiments. Univariate comparisons of means were evaluated using the Student $t$ test, and a $P<0.05$ was considered statistically significant.
Figure S1

Blood pressure in WT and LOX-1 KO mice after 4 weeks of Ang II infusion. Bar groups are mean ± SD from 4 separate animals in each group. *P<0.01 vs. WT sham; # P<0.05 vs. WT+Ang II
Figure S2

Cell size of LOX-1 KO fibroblasts after transfection with empty vector or human LOX-1 cDNA (h-LOX-1). Bar groups are mean ± SD from at least 400 cells in each group. *P<0.01 vs. empty vector
Figure S3

CDC42 expression in WT and KO fibroblasts (left panel) and KO fibroblasts transfected with empty vector or human LOX-1 cDNA (h-LOX-1) (right panel). Bar groups are mean ± SD from at least 3 independent experiments in each group. *P<0.01 vs. corresponding WT fibroblasts of KO fibroblast transfected with empty vector.