Regulation of TGFβ₁-mediated Collagen Formation by LOX-1

**STUDIES BASED ON FORCED OVEREXPRESSION OF TGFβ₁ IN WILD-TYPE AND LOX-1 KNOCK-OUT MOUSE CARDIAC FIBROBLASTS**

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Transforming growth factor β₁ (TGFβ₁) activation leads to tissue fibrosis. Here, we report on the role of LOX-1, a lectin-like 52-kDa receptor for oxidized low density lipoprotein, in TGFβ₁-mediated collagen expression and underlying signaling in mouse cardiac fibroblasts. TGFβ₁ was overexpressed in wild-type (WT) and LOX-1 knock-out mouse cardiac fibroblasts by transfection with adeno-associated virus type 2 vector carrying the active TGFβ₁ moiety (AAV/TGFβ₁.ACT). Transfection of WT mouse cardiac fibroblasts with AAV/TGFβ₁.ACT markedly enhanced the expression of NADPH oxidases (p22phox, p47phox, and gp91phox subunits) and LOX-1, formation of reactive oxygen species, and collagen synthesis, concomitant with an increase in the activation of p38 and p44/42 mitogen-activated protein kinases (MAPK). The TGFβ₁-mediated increase in collagen synthesis was markedly attenuated in the LOX-1 knock-out mouse cardiac fibroblasts as well as in WT mouse cardiac fibroblasts treated with a specific anti-LOX-1 antibody. Treatment with anti-LOX-1 antibody also reduced NADPH oxidoase expression and MAPK activation. The NADPH oxidase inhibitors and gp91phox small interfering RNA reduced LOX-1 expression, MAPK activation, and collagen formation. The p38 MAPK inhibitors as well as the p44/42 MAPK inhibitors reduced collagen formation without affecting LOX-1 expression in cardiac fibroblasts. These observations suggest that collagen synthesis in cardiac fibroblasts involves a facilitative interaction between TGFβ₁-NADPH oxidase and LOX-1. Further, the activation of MAPK pathway appears to be downstream of TGFβ₁-reactive oxygen species-LOX-1 cascade.

Cardiac fibrosis, characterized by accumulation of matrix proteins in the extracellular space in the perivascular region, is closely associated with the development of heart failure. Among the extracellular matrix proteins, up to 85% are collagens, of which type I (collagen I) and type III (collagen III) constitute two-thirds of total collagens in most tissues (1). Transforming growth factor (TGF)β₁ is one of the most pleiotropic and multifunctional peptides known (2). It exerts potent effects on many different cell types and is involved in a wide variety of biological processes (2). The cellular actions of TGFβ₁ are dependent not only on the cell type but also on its state of differentiation and the cytokine milieu (3). Although there is evidence that TGFβ₁ stimulates fibroblast growth, enhances collagen synthesis, and suppresses collagen degradation (2), the specific effect of TGFβ₁ on the cardiac remodeling process remains unclear. TGFβ₁ is synthesized in cells as a precursor molecule, TGFβ₁Latent. Conversion from cysteine (Cys²²³/²²²) into serine (Ser²²³/²²²) in the TGFβ₁Latent molecule is associated with the formation of TGFβ₁.ACT (active TGFβ₁) (4). It is the TGFβ₁.ACT that appears to be functionally relevant in the process of ischemia-reperfusion (5).

LOX-1 is a lectin-like receptor for oxidized low density lipoprotein (6). It is also up-regulated in response to oxidative stress (7). Activation of LOX-1 enhances the growth of cardiac fibroblasts and promotes collagen synthesis (8, 9). It has been reported that TGFβ₁ can regulate LOX-1 expression in vascular endothelial cells, smooth muscle cells, and monocytes/macrophages (10, 11). Another study showed that LOX-1 blockade reduces TGFβ₁ protein expression in endothelial cells (12). The present study was conducted to examine whether and how LOX-1 mediates TGFβ₁-mediated collagen production.

**EXPERIMENTAL PROCEDURES**

**Materials and Reagents**—Monoclonal antibody against mouse LOX-1 raised in rat with mouse Fc portion of IgG has been reported earlier to block the effect of LOX-1 (13). The following reagents and antibodies were purchased: p38 MAPK inhibitors SB203580 (Sigma) and SB202190 (Calbiochem); p44/42 MAPK inhibitors U0126 (Sigma) and PD98059 (Calbiochem); NADPH oxidase inhibitors apocynin (Aldrich) and diphenyliodonium (Sigma); GeneSilencer® siRNA transfection reagent (Gene Therapy Systems); siCONTROL® non-targeting siRNA and siGENOME SMARTpool® NADPH oxidase gp91phox subunit siRNA (Dharmacon); human recombinant TGFβ₁ (rTGFβ₁) and mouse nonspecific IgG (Sigma); all pri-
mary antibodies for Western blot analysis except anti-LOX-1 antibody (Santa Cruz Biotechnology). 2'7'-dichlorodihydrofluorescein diacetate was purchased from Cayman. U0126, PD98059, SB203580, SB202190, diphenyliodonium, and apocynin were dissolved in Me2SO. The final concentration of Me2SO was 0.1% in these experiments.

Animals—C57BL/6 mice (also referred to as WT mice) were obtained from Jackson Laboratories. The homozygous LOX-1 knock-out (KO) mice were developed as described recently (13) and backcrossed eight times with the C57BL/6 strain to replace the genetic background. Male C57BL/6 and LOX-1 KO mice weighing 22–26 g were utilized at 8–10 weeks of age. All animals received humane care in compliance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals published by the National Institutes of Health.

Construction of AAV/TGFβ1/ACT and Generation of Recombinant AAV Stocks—The TGFβ1 mutant TGFβ1/ACT cDNA was generated, sequenced, and ligated into an adeno-associated virus (AAV) vector, dl6–95, as described recently (14). Recombinant AAV vector hereafter will be referred to as AAV/TGFβ1/ACT. The generation of AAV/Neo virus has been described earlier (15). Virus stocks were generated as described previously (15). The titer of purified virus, in encapsidated genomes per milliliter (eg/ml), was calculated by dot-blot hybridization and determined to be \(10^{11}\) eg/ml.

Cell Culture and AAV Vector Infection—Mouse (WT and LOX-1 KO) cardiac fibroblasts were isolated and cultured as described earlier (16). Cells cultured to fifth passage were used in all experiments. To transfect the cultured cells, AAV vectors were added to cell culture dishes at multiplicity of infection (m.o.i.) of \(10^3\) and incubated with the cells for 72 h at 37 °C in 5% CO2/95% air. The infection efficiency was evaluated by AAV/GFP expression using fluorescent microscopy. AAV/GFP was obtained from the University of North Carolina Gene Therapy Center, and the titer was \(10^{12}\) virus particles/ml (3.1 \(10^{10}\) infectious units).

Experimental Protocols—Cardiac fibroblasts were transfected with AAV vector (or only culture medium) for 72 h in the absence or presence of anti-LOX-1 antibody (10 μg/ml), nonspecific IgG (10 μg/ml), apocynin (600 μM), diphenyliodonium (10 μM), U0126 (10 μM), PD98059 (10 μM), SB203580 (10 μM), SB202190 (10 μM), gp91phox siRNA (50 nM), or non-targeting siRNA (50 nM), or Me2SO (as vehicle control). These concentrations were chosen on the basis of published data (13, 17–20).
and modified in accordance with the results of pilot experiments. In other experiments, cardiac fibroblasts were treated with rTGFβ1 (2 ng/ml) for 24 h. At the end of the experiments, culture medium was collected for the determination of soluble collagen production and fibroblasts were examined for measurement of reactive oxygen species (ROS) release and expression of specific proteins.

**Evaluation of Cell Growth**—Cell growth was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma). Meanwhile, cell number was determined in triplicate using a hemocytometer. Results were normalized to the conversion of MTT and cell number in control cultures as 100%, respectively.

**Soluble Collagen Assay**—Soluble collagen production was determined by the Sircol assay (Biocolor) as described earlier (21) and expressed as μg/10⁶ cells.

**Measurement of ROS in Cardiac Fibroblasts**—Intracellular ROS generation was measured with the use of 2',7'-dichlorodihydrofluorescein diacetate (10 μM) fluorescent signal, a cell-permanent indicator for ROS, as described previously (14). Results were displayed in a ratiometric fashion normalized for permeant indicator for ROS, as described previously (14).

**Western Blot Analysis**—The expression of TGFβ1, collagen type I, collagen type III, LOX-1, p38, and p44/42 MAPK isoforms, NADPH oxidase subunits (p22phox, p47phox, and gp91phox), or β-actin was assessed by Western analysis using standard methodologies (14). Densities of protein bands relative to β-actin were analyzed.

**Statistical Analysis**—Data, based on at least four separate experiments, are expressed as means ± S.E. All values were analyzed by using one-way analysis of variance and the Newman-Keuls-Student t test. The significance level was chosen as p < 0.05.

**RESULTS**

**AAV Facilitates Transfection of Fibroblasts**—In pilot experiments, AAV/GFP vectors were added to cell culture dishes at an m.o.i. of 0–10³ and incubated with the cells for 72 h at 37 °C in 5% CO₂/95% air. More than 90% of the cells showed GFP expression at m.o.i. of 10³. There was no difference in GFP expression in cardiac fibroblasts from WT and LOX-1 KO mice (Fig. 1A). Because the most optimal transfection was observed at m.o.i. of 10³, transfection in subsequent experiments with AAV vectors was performed at m.o.i. of 10³.

**Increase in TGFβ1 Expression**—After 72 h of AAV infection, fibroblasts were harvested for Western blotting. As shown in Fig. 1B, total TGFβ1 expression was increased in AAV/TGFβ1ACT-transfected cells, indicating successful delivery of the transgene. AAV/Neo alone had no effect on TGFβ1 expression. Note that there was no difference in TGFβ1 expression in cardiac fibroblasts from WT and LOX-1 KO mice.

**AAV/TGFβ1ACT Transfection Facilitates Cell Growth**—AAV/TGFβ1ACT transfection had a stimulatory effect on fibroblast growth, and the number of fibroblasts increased by ~50% (versus fibroblasts kept under control conditions). This effect was much less evident in fibroblasts from LOX-1 KO mice (p < 0.01 versus AAV/TGFβ1ACT-transfected fibroblasts from wild-type mice) (Fig. 2, A and B). AAV/Neo alone had no effect on cell growth.

**Interaction between TGFβ1ACT, LOX-1 Expression, and Collagen Synthesis in Fibroblasts**—AAV/TGFβ1ACT transfection increased the expression of collagen type I and III (p < 0.01 versus fibroblasts kept under control conditions) in WT mouse cardiac fibroblasts, but this effect was much less pronounced in fibroblasts from LOX-1 KO mice (p < 0.01 versus AAV/TGFβ1ACT-transfected fibroblasts from WT mice) (Fig. 3A). Note that AAV/Neo alone had no effect on collagen expression. We also measured soluble collagen in the medium bathing the fibroblasts, and in keeping with collagen I and III expression data, soluble collagen increased in the supernates of AAV/TGFβ1ACT-transfected cardiac fibroblasts (Fig. 3B). To further confirm the effect of AAV/TGFβ1ACT on collagen expression, we treated cells with rTGFβ1 directly and found that the effects of rTGFβ1 were consistent with that of transfection with AAV/TGFβ1ACT (Fig. 3C). These observations suggest that LOX-1 plays an important role in TGFβ1-induced collagen production.

Because the observations in LOX-1 KO mouse cardiac fibroblasts may reflect effects of genes other than LOX-1 in modulation of TGFβ1-mediated collagen synthesis, the role of LOX-1 in the effects of TGFβ1ACT was confirmed by use of a specific
FIGURE 4. TGFβ1ACT transfection, NADPH oxidase, LOX-1, MAPKs, and collagen expression. The expression of collagen type I and III was increased in AAV/TGFβ1ACT-transfected fibroblasts, and this increase was inhibited by anti-LOX-1 antibody as well as the NADPH oxidase inhibitors apocynin and diphenyliodonium (DPI), the p38 MAPK inhibitors SB203580 and SB202190, the p44/42 MAPK inhibitors U0126 and PD98059 (A and B), and gp91phox siRNA (C). TGFβ1ACT-mediated LOX-1 expression was inhibited by treatment of fibroblasts with anti-LOX-1 antibody, the NADPH oxidase inhibitors, and gp91phox siRNA, but not by MAPK inhibitors. Nonspecific IgG and non-targeting siRNA had no effect on collagen and LOX-1 expression. Error bars, ± S.E.

FIGURE 5. Effect of AAV/TGFβ1ACT transfection on NADPH oxidase expression and ROS generation. AAV/TGFβ1ACT transfection enhanced the expression of NADPH oxidase subunits p22phox, p47phox, and gp91phox (A and C) and ROS generation measured by 2′,7′-DCF fluorescence (B). The treatment of cells with anti-LOX-1 antibody and the NADPH oxidase inhibitors apocynin and diphenyliodonium (DPI) reduced the expression of NADPH oxidase (p22phox, p47phox, and gp91phox) and DCF fluorescence. AAV/Neo or nonspecific IgG alone had no effect on the expression of NADPH oxidase and DCF fluorescence. gp91phox siRNA, but not non-targeting siRNA, effectively inhibited the basal and AAV/TGFβ1ACT-mediated expression of gp91phox. Error bars, ± S.E.

Interaction between TGFβ1, NADPH Oxidase, and LOX-1—Oxidant stress up-regulates the expression and activation of LOX-1 (7), and activation of LOX-1 itself can stimulate the formation of ROS (22, 23). NADPH oxidase activation is a major source of ROS in cardiac fibroblasts (19). We, therefore, measured intracellular ROS generation and NADPH oxidase expression in fibroblasts transfected with AAV/TGFβ1ACT. Indeed, AAV/TGFβ1ACT transfection enhanced the expression of NADPH oxidase (p22phox, p47phox, and gp91phox subunits) as well as dihydrofluorescein (DCF) fluorescence reflecting intracellular ROS generation. The treatment of cells with anti-LOX-1 antibody reduced the expression of NADPH oxidase (all three subunits) (Fig. 5A). In addition, anti-LOX-1 antibody treatment reduced TGFβ1ACT-induced increase in DCF fluorescence (Fig. 5C), reflecting a reduction in intracellular ROS generation, concomitant with suppression of collagen production. Nonspecific IgG had no effect on TGFβ1ACT-induced expression of NADPH oxidase and ROS generation (Fig. 5, A and C). Further, treatment of fibroblasts with apocynin and diphenyliodonium, the NADPH oxidase inhibitors, attenuated the expression of LOX-1 as well as collagen expression despite forced up-regulation of TGFβ1ACT (Fig. 4, A and B). To confirm the role of NADPH oxidase, we conducted experiments using gp91phox subunit knockdown methodology. As shown in Figs. 5B and 4C, gp91phox siRNA markedly inhibited the expression of gp91phox, LOX-1, and collagen induced by TGFβ1ACT. The non-targeting siRNA had no effect.

Signaling of TGFβ1-LOX-1-mediated Collagen Formation—To study the intracellular signaling mechanism of LOX-1-TGFβ1 interaction, the AAV/TGFβ1ACT-transfected cardiac fibroblasts from WT mice anti-LOX-1 antibody. As shown in Fig. 4A, the expression of collagens was less in cells treated with anti-LOX-1 antibody than in fibroblasts kept under control conditions (p < 0.01). Note that nonspecific IgG had no effect on AAV/TGFβ1ACT-induced collagen expression (Fig. 4B).

Interaction between TGFβ1, NADPH Oxidase, and LOX-1—Oxidant stress up-regulates the expression and activation of collagens was less in cells treated with anti-LOX-1 antibody than in fibroblasts kept under control conditions (p < 0.01). Note that nonspecific IgG had no effect on AAV/TGFβ1ACT-induced collagen expression (Fig. 4B).
inhibitors, indicating that NADPH oxidase activation plays a significant role in TGFβ1- mediated LOX-1 expression and that NADPH oxidase activation is upstream of MAPKs.

Next, we measured the expression of redox-sensitive p38 and p44/42 MAPKs. As shown in Fig. 6, A and B, the expression of p38 or p44/42 MAPKs was not altered by TGFβ1 up-regulation. However, TGFβ1 up-regulation markedly increased the phosphorylation of both p38 and p44/42 MAPKs, which was inhibited by anti-LOX-1 antibody, apocynin, diphenyliodonium (DPI), and MAPK inhibitors. AAV/Neo transfection or nonspecific IgG had no effect. Error bars, ± S.E.

DISCUSSION

In this study, we set out to examine the hypothesis that LOX-1 plays an important role in collagen generation in mouse cardiac fibroblasts in response to TGFβ1. Toward that goal, we transfected WT and LOX-1 KO mouse cardiac fibroblasts with TGFβ1 using AAV as the delivery vector. In previous studies, AAV has been shown to transfect smooth muscle cells, cardiomyocytes, and fibroblasts very efficiently (14, 24, 25). Indeed, we observed that there was >90% AAV/TGFβ1 transfection efficiency at an m.o.i. of 105. In keeping with the well established role of TGFβ1 in the genesis of fibrosis, the forced up-regulation of TGFβ1 in WT mouse cardiac fibroblasts dramatically enhanced the expression of collagens (types I and III) and increased the level of soluble collagen in the fibroblast supernates. We used TGFβ1 to up-regulate TGFβ1 because previous studies (5, 14) have shown that the active moiety of TGFβ1 results in significantly greater biological effects than the latent form of TGFβ1.

The suggestion of a role of LOX-1 in the biological effects of TGFβ1 comes from studies that indicate that TGFβ1 influences the pro-inflammatory effect of oxidized low density lipoprotein (12). In these studies, pretreatment of endothelial cells with anti-LOX-1 antibody attenuated the synthesis of TGFβ1 in response to oxidized low density lipoprotein. Minami et al. (11) provided evidence that TGFβ1 (0.1–10 ng/ml) induces LOX-1 expression in a transcriptional manner in both bovine aortic endothelial and smooth muscle cells in a dose- and time-dependent fashion. Draude and Lorenz (10) described TGFβ1-mediated stimulation of oxidized low density lipoprotein uptake in freshly isolated and cultured human monocytes with simultaneous severalfold increase in LOX-1 mRNA. All these observations suggest a link between LOX-1 and TGFβ1 in the biology of a variety of cell types.

This study provides conclusive evidence that TGFβ1-mediated collagen formation in fibroblasts involves a critical role of LOX-1. Forced overexpression of TGFβ1 in WT mouse cardiac fibroblasts induced a 3–4-fold increase in LOX-1 expression. The evidence for the critical role of LOX-1 in TGFβ1-mediated collagen formation was obtained by two different approaches: first, the use of fibroblasts from LOX-1 KO mouse hearts and second, the use of a specific monoclonal anti-LOX-1 antibody. Both these approaches resulted in a marked inhibition of TGFβ1-mediated collagen formation. It is noteworthy that TGFβ1-mediated fibroblast growth was also blocked in fibroblasts from LOX-1 KO mice.

In other experiments, we observed that NADPH oxidase expression was greatly increased in WT mouse cardiac fibroblasts transfected with AAV/TGFβ1. The activity of TGFβ1 has been shown to be associated with generation of ROS (26, 27). This was confirmed in the present studies by examination of NADPH oxidase (p22phox, p47phox, and gp91phox subunits) expression and direct measurement of intracellular ROS as DCF fluorescence. Further, ROS have been shown to activate LOX-1 (7) and LOX-1 activation itself leads to ROS generation.
(22, 23). We observed that the inhibition of NADPH oxidase by apocynin, diphenyliodonium, or gp91phox siRNA reduced LOX-1 expression and collagen formation in WT mouse cardiac fibroblasts despite forced up-regulation of TGFβ1ACT. Interestingly, treatment of fibroblasts with the anti-LOX-1 antibody itself reduced the up-regulation of NADPH oxidase (p22phox, p47phox, and gp91phox subunits) despite forced up-regulation of TGFβ1. These observations provide compelling evidence for a positive feedback between TGFβ1 and NADPH oxidase-mediated ROS generation, and LOX-1 (summarized in Fig. 7).

Next, we examined the role of redox-sensitive MAPKs in TGFβ1-mediated collagen formation. The AAV/TGFβ1ACT-transfected WT mouse cardiac fibroblasts did not exhibit any change in p38 or p44/42 MAPK protein levels, but their phosphorylation was increased 2–3-fold. MAPK phosphorylation was blocked by the treatment of cells with NADPH oxidase inhibitors as well as anti-LOX-1 antibody. On the other hand, treatment of fibroblasts with the MAPK inhibitors significantly reduced the expression of collagens in fibroblasts transfected with AAV/TGFβ1ACT without effect on LOX-1 expression (Fig. 4), suggesting that MAPK activation is downstream of ROS generation in response to TGFβ1-NADPH oxidase-LOX-1 cascade (Fig. 7).

In essence, this study provides the missing link between TGFβ1 activation and collagen formation in the heart during pro-oxidant states such as hypertension and ischemia-reperfusion injury. Identification of LOX-1 as an important molecule in TGFβ1-mediated collagen synthesis provides a new target for therapy of disease states characterized by excessive tissue remodeling.

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