Nicotinamide Adenine Dinucleotide Phosphate Oxidase 4 Mediates the Differential Responsiveness of Atrial Versus Ventricular Fibroblasts to Transforming Growth Factor-β

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Background—Atrial fibrosis, a common feature of atrial fibrillation, is thought to originate from the differential response of atrium versus ventricle to pathological insult. However, detailed mechanisms underlying the regional differences remain unclear. The aim of this study was to investigate the related factor(s) in mediating atrial vulnerability to fibrotic processes.

Methods and Results—We first compared the response of cultured atrial versus ventricular fibroblasts with transforming growth factor-β (TGF-β), a key mediator of myocardial fibrosis. Atrial fibroblasts showed a stronger response to TGF-β1 in producing extracellular matrix protein (collagen and fibronectin) than ventricular fibroblasts. Furthermore, TGF-β1 activated its downstream signaling (Smads) and induced pronounced oxidative stress, including up-regulation of nicotinamide adenine dinucleotide phosphate oxidase in atrial fibroblasts, and to a lesser extent in ventricular fibroblasts. Nicotinamide adenine dinucleotide phosphate oxidase inhibitors and small-interfering RNA for Nox4 eliminated TGF-β1-induced difference between atrial and ventricular fibroblasts, suggesting the crucial role of Nox4 in mediating the atrial-ventricular discrepancy. Small-interfering RNA for Smad3 also suppressed the differential responsiveness of atrial versus ventricular fibroblasts to TGF-β1, including Nox4 activation, implicating a crosstalk between nicotinamide adenine dinucleotide phosphate oxidases and Smad. In vivo, the increased TGF-β1 responsiveness and Nox4 expression were documented in the atria of transgenic mice with cardiac overexpression of TGF-β1.

Conclusions—Atrial fibroblasts show greater fibrotic and oxidative responses to TGF-β1 than ventricular fibroblasts. Nox4-derived reactive oxygen species production mediates the susceptibility of atrial fibroblasts to TGF-β1 via activating TGF-β1/Smad signaling cascade, which provides a novel insight into the pathogenesis of atrial fibrosis. (Circ Arrhythm Electrophysiol. 2013;6:790-798.)

Key Words: atrial fibrillation • atrial fibrosis • NADPH oxidase • oxidative stress • TGF

Atrial fibrillation (AF) is the most common sustained arrhythmia in clinical settings. Atrial fibrillation, involving nonmyocyte growth and extracellular matrix protein deposition, plays a critical role in the pathogenesis of AF. Atrial fibroblasts, which constitute the major component of nonmyocytes, are the main origin of extracellular matrix proteins in the atrium. Emerging evidence suggests that atrial fibroblasts behave differently from ventricular fibroblasts in response to fibrotic insult. In comparison with ventricular fibrosis, atrial fibrosis is more prone to develop in a variety of animal models. Therefore, investigation of the mechanisms underlying the differential behavior between ventricular and atrial fibroblasts may be important for understanding the pathogenesis of AF.

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Transforming growth factor β (TGF-β) is a major contributor to atrial fibrillation. TGF-β1 activates atrial fibroblasts and promotes their differentiation into myofibroblasts, which in return stimulates the production of extracellular matrix proteins, such as collagen I, III, and fibronectin. Transgenic mice with cardiac overexpression of TGF-β1 show selective atrial fibrosis and AF property despite equivalent TGF-β1 expression in the atria and ventricles. It is conceivable that the differential responsiveness of atrial versus ventricular fibroblasts to TGF-β1 may determine atrial vulnerability to fibrosis.

Numerous clinical and experimental studies have associated oxidative stress with the pathogenesis of AF, especially in relation to atrial fibrillation. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (Noxs) by producing reactive oxygen species (ROS) have emerged as the major source of oxidative stress in the atrium. Because oxidative stress induced by TGF-β1 may contribute to the development of atrial fibrillation, we hypothesize that NADPH oxidase-derived ROS may participate in the differential susceptibility to atrial versus ventricular fibrosis.
We first evaluated in parallel the fibrotic response of atrial and ventricular fibroblasts to TGF-β1, especially focusing on extracellular matrix protein expression. We then compared TGF-β1–induced NADPH oxidase–derived ROS production between atrial and ventricular fibroblasts and explored the potential role of NADPH oxidases in mediating TGF-β1.

Figure 1. A, After 24 hours of serum deprivation, cardiac fibroblasts were treated with or without 5 ng/mL TGF-β1 for 24 hours. The expression of fibronectin, collagen I, III, Nox2/4, p-Smad3, and tubulin protein was evaluated by western blot as described in Materials and Methods. The expression of tubulin was used as an internal control. B, The relative expression levels of indicated proteins were quantified by densitometry and normalized to the control level, which was set at 1.0. Each value represents the mean±SE of 4 independent experiments. P<0.05; *represents the significant difference among groups. Nox2/4 indicates nicotinamide adenine dinucleotide phosphate oxidase 2/4; and TGF-β1, transforming growth factor β1.

Figure 2. A, Intracellular ROS production was measured by fluorescent DCF dye as described in Materials and Methods. After 24 hours of serum deprivation, cardiac fibroblasts were treated with or without 5 ng/mL TGF-β1. Time-dependent changes of intracellular reactive oxygen species are shown. B, An identical paradigm was followed as described in A. After 24 hours of serum deprivation, atrial fibroblasts were preincubated with Nox inhibitors (plumbagin, DPI, and gp91-dstat) for 2 hours and subsequently treated with 5 ng/mL TGF-β1. C, An identical paradigm was followed as described in A. After deprivation of serum and transfection of indicated siRNAs for 48 hours, atrial fibroblasts were treated with 5 ng/mL TGF-β1. Each value represents the mean±SE of 4 independent experiments. P<0.05; *,†,#represent the significant differences among groups. DPI indicates diphenyleneiodonium; Nox, nicotinamide adenine dinucleotide phosphate oxidase; si, small interfering; and TGF-β1, transforming growth factor β1.
signaling. Finally, we sought to determine whether the findings obtained in vitro could be verified in transgenic mice with cardiac overexpression of TGF-β1.

Materials and Methods

Complete details are presented in the online-only Data Supplement, expanded Methods section.

Cell Cultures

Atrial and ventricular fibroblasts were obtained from left atria and ventricles of adult male Wistar rats (euthanized with ketamine [100 mg/kg] and xylazine [11.5 mg/kg], IP) using the collagenase and trypsin digestion methods.

MHC-TGFcys33ser Transgenic Mice

The MHC-TGFcys33ser transgenic mice (generous gift from Loren J. Field, James Whitcomb Riley Hospital for Children, IN) are generated by the mouse α-cardiac MHC promoter and sequences encoding the human TGF-β1 cDNA as described.5,6

Results

TGF-β1 Induces Different Responses Between Atrial and Ventricular Fibroblasts

We first compared the effect of TGF-β1 on cultured atrial versus ventricular fibroblasts. Treatment of atrial fibroblasts with TGF-β1 induced the expression of collagen and fibronectin, 2 major cardiac extracellular matrix proteins.2 Atrial fibroblasts...
showed a stronger response to TGF-β1 in producing collagen I, III, and fibronectin than ventricular fibroblasts (Figure 1A and 1B). However, there was no difference in the distribution/expression of TGF-β1 receptor between atrial and ventricular fibroblasts (Figure 1 in the online-only Data Supplement). Because Smad3 is the main downstream target of TGF-β1, we further assessed the effect of TGF-β1 on the activation of Smad3 using a specific antibody against phospho-Smad3. TGF-β1 increased phospho-Smad3 expression in atrial but not in ventricular fibroblasts (Figure 1A and 1B).

Previous study shows that TGF-β1 stimulates Nox4-derived ROS production and thereby activates cardiac fibroblast. Both Nox2 and Nox4 are the main isoforms of NADPH oxidases in the myocardium. Therefore, we assessed the effect of TGF-β1 on the expression of Nox2/4 and ROS production in atrial and ventricular fibroblasts. The expression of Nox4 and its activity was significantly increased by TGF-β1 treatment in atrial but less in ventricular fibroblasts, and Nox2 expression was unaffected in both fibroblasts (Figure 1A and 1B; Figure 2 in the online-only Data Supplement). Consistent with Nox4 up-regulation, TGF-β1 robustly enhanced ROS formation in atrial fibroblasts, with a much smaller response in ventricular fibroblasts (Figure 2A). The ROS-enhancing effect of TGF-β1 in atrial fibroblasts was attenuated by 10 μmol/L diphenyleneiodonium (DPI; a nonspecific NADPH oxidase inhibitor) and 10 μmol/L plumbagin (a specific Nox4 inhibitor), but to a lesser extent by 50 μmol/L gp91-dstat (a specific Nox2 inhibitor; Figure 2B; Figure 3 in the online-only Data Supplement), supporting the essential role of Nox4 in TGF-β1–induced ROS production. To further confirm the involvement of Nox4 and Smad3 in TGF-β1–induced ROS elaboration, small-interfering RNAs (siRNAs) specific for Smad3 and Nox2/4 were applied. All Smad3 and Nox2/4 siRNAs suppressed the up-regulation of ROS production by TGF-β1 in atrial fibroblasts, but to a lesser extent in Nox2 siRNA-transfected cells (Figure 2C; Figure 3 in the online-only Data Supplement). The specificity of siRNAs was documented by western blot (Figure 4 in the online-only Data Supplement). Together, these experimental findings suggest that TGF-β1, via the activation of Smad3, may induce stronger Nox4-derived ROS production in atrial fibroblasts.

Nox4 Is Involved in TGF-β1–Induced Atrial-Ventricular Difference

The next experiments were designed to assess the potential role of Nox4 in differential extracellular matrix protein responses of atrial fibroblasts. The effect of TGF-β1 on increasing collagen I and fibronectin expression was abolished by Nox4 inhibitors (DPI and plumbagin), but not by Nox2 inhibitor (gp91-dstat; Figure 3A). Furthermore, transfection of both Nox4 and Smad3 siRNAs, but not Nox2 siRNA, attenuated TGF-β1–induced up-regulation of collagen I and fibronectin in atrial fibroblasts (Figure 3B and 3C). The fact that the difference between atrial and ventricular fibroblasts in response to TGF-β1 can be blocked by Nox4 and Smad3 inhibitors implies the potential role of Nox4 and Smad3 in mediating atrial-selective responses.

Previous studies demonstrate a crosstalk between NADPH oxidases and Smad in mediating TGF-β1–induced differentiation of cardiac and renal fibroblasts. The next experiments investigated whether NADPH oxidases are involved in TGF-β1–induced activation of Smad3, including its phosphorylation and nuclear translocation. Pretreatment of both fibroblasts with Nox4 inhibitors and transfection of Nox4 siRNA prevented TGF-β1–stimulated phosphorylation of Smad3 (Figure 3A–3C). Transfection of Smad3 siRNA also suppressed the up-regulation of Nox4 in TGF-β1–treated fibroblasts (Figure 3B and 3C). Furthermore, in a gain-of-function study, we transfected fibroblasts with a wild-type Nox4 plasmid to overexpress Nox4. Overexpression of Nox4 in atrial fibroblasts augmented TGF-β1–induced collagen I, fibronectin, and phospho-Smad3 expressions (Figure 3D), implicating that Nox4 and Smad3 are mutually regulated.

In agreement with its effect on Smad3 phosphorylation, TGF-β1 promoted more prominent nuclear translocation of Smad3 in atrial than in ventricular fibroblasts (4.1±0.3 versus 2.7±0.1 fold; P<0.05; Figure 4A and 4B). The treatment of both fibroblasts...
with Nox4 inhibitor (DPI) and transfection of Nox4 siRNA attenuated TGF-β1–induced nuclear translocation of Smad3, with more inhibitory effect on atrial fibroblasts (75.8±5.8% versus 45.4±8.1% for DPI; 49.5±2.9% versus 37.2±19.3% for Nox4 siRNA transfection, respectively; \( P < 0.05 \); Figure 4A and B). These findings provide further evidence that there is a cross-talk between NADPH oxidases and Smad in TGF-β1 signaling.

**Nox4 Mediates TGF-β1–Induced Atrial-Ventricular Difference in Collagen Transcriptional Activity**

Because there is difference between atrial and ventricular fibroblasts in TGF-β1–induced collagen expression, the following experiments were designed to assess the regulatory level in determining this difference. At the transcriptional level, TGF-β1 concentration dependently increased collagen I mRNA level and its promoter activity in atrial and ventricular fibroblasts but to a lesser extent in ventricular fibroblasts (Figure 5A and 5B). Furthermore, the promoting effect of TGF-β1 on collagen transcriptional activity could be blocked by Nox4 inhibition, including DPI, plumbagin, and Nox4 siRNA transfection (Figure 5B). At the post-transcriptional level, the half-time of collagen I mRNA remained unaffected by TGF-β1 treatment in both fibroblasts, suggesting that changes in mRNA stability were not involved (Figure 5 in the online-only Data Supplement). These experimental results point to Nox4-dependent transcriptional regulation as the basis of the greater response of collagen protein expression to TGF-β1 in atrial fibroblasts.

Previous studies indicate that the TGF-β1–Smad pathway is operated via the activation of genes with Smad4-binding elements in their promoter regions. Bioinformatic analysis identified 2 putative Smad4-binding elements in the promoter region...
of collagen I gene. Mutation analysis of the collagen I promoter showed that TGF-β1–increased transcriptional activity was only evident when the promoter constructs contained the proximal Smad4-binding element (Figure 5C). To directly test in vivo binding of Smad4 to the collagen I promoter, chromatin immunoprecipitation assay was performed using a specific Smad4 antibody. TGF-β1 caused significantly larger amount of Smad4 bound to the collagen I promoter in atrial than in ventricular fibroblasts (9.2±0.5 versus 5.5±0.2 fold; P<0.05; Figure 5D). Transfection of Nox4 siRNA reduced TGF-β1–induced binding of Smad4 to the collagen I promoter in atrial fibroblasts but to a less extent in ventricular fibroblasts (39.2±3.7% versus 15.9±5.5%; P<0.05; Figure 5D). These experimental results document that TGF-β1/Smads signaling is more sensitive to Nox4 inhibition in atrial than in ventricular fibroblasts.

**Proliferative Response to TGF-β1 Is Similar Between Atrial and Ventricular Fibroblasts**

In addition to the accumulation of extracellular matrix, increased cell numbers of fibroblasts may contribute to the development of myocardial fibrosis. We, therefore, compared proliferation and viability between atrial and ventricular fibroblasts in response to TGF-β1. BrdU assay showed that TGF-β1 speeded cell proliferation in a similar rate between atrial and ventricular fibroblasts (Figure 6A–6B). Although TGF-β1 was both increased in the atria and ventricles of MHC-TGFcys33ser transgenic mice, the relative collagen to TGF-β1 ratio was significantly higher in atria than in ventricles (Figure 6A–6C). These results suggested that an increased responsiveness of TGF-β1 contributes to greater collagen deposition in the atria of MHC-TGFcys33ser transgenic mice. Furthermore, ROS production and Nox4 expression in fibroblasts (identified by colocalization with vimentin), NADPH activity, and Smad3 phosphorylation were more prominent in atria than in ventricles (Figures 7 and 8; Figure 9 in the online-only Data Supplement). In contrast with Nox4, Nox2 expression exhibited no atrial-ventricular difference in MHC-TGFcys33ser transgenic mice (Figure 8; Figure 9 in the online-only Data Supplement). In addition, these changes have no left–right difference (Figures 6 and 7). These in vivo data are compatible with in vitro findings of differential atrial-ventricular fibroblast responses of Nox4 and ROS to TGF-β1.

**Discussion**

The main findings of this study are as follows: (1) atrial fibroblasts are more responsive to TGF-β1 with respect to the expression of extracellular matrix proteins (collagen and fibronectin), especially at the transcriptional level, (2) TGF-β1 provokes more intense oxidative stress and promotes more active Smad signaling in atrial than in ventricular fibroblasts, which is mediated by the differential atrial-ventricular expression of Nox4, (3) Nox4-derived oxidative stress is involved in TGF-β1–induced up-regulation of collagen transcription, which accounts for the differential atrial-ventricular collagen expression in response to TGF-β1, and (4) in transgenic mice with cardiac overexpression of TGF-β1, the atrial-ventricular difference in collagen expression parallels that of TGF-β1 responsiveness and Nox4 expression.

We demonstrated that TGF-β1 increased the transcriptional activity of collagen and activated the Smad signaling in atrial
fibroblasts. TGF-β1 also stimulated more Nox4 expression in atrial than in ventricular fibroblasts, which was reflected by a stronger ROS production. Furthermore, Nox4 inhibitors attenuate TGF-β1–dependent Smad signaling cascade, including activation of Smad3 and binding of Smad4 to the collagen promoter. Therefore, we conclude that the higher production of Nox4–derived ROS induced by TGF-β1 is essential for the discrepant atrial-ventricular collagen expression in cardiac fibroblasts.

The different characteristics in atrial-ventricular remodeling have been the subject of intensive investigation.3–8 Atria-selective fibrosis was found in TGF-β1–overexpressing mice.5,6 In dogs with tachypacing-induced heart failure, fibrosis is more substantial in left atrium than in left ventricle.3,4 A recent study shows that atrial fibroblasts behave differently from ventricular fibroblasts in either in vitro or in vivo experiment.4 In comparison with ventricular fibroblasts, atrial fibroblasts respond greatly to growth factors in its proliferative activity. This atria-selective response is attributed to platelet-derived growth factor.4 Our study further demonstrated that Nox4–derived ROS significantly contributes to the atria-specific diversity. These findings implicate that TGF-β1 alone is not sufficient to cause fibrosis and Nox4–dependent ROS generation may potentiate the effect of TGF-β1 to promote fibrosis.

Previous studies demonstrate a crosstalk between NADPH oxidases and Smad in mediating TGF-β–induced differentiation of cardiac fibroblasts.18,20,21 In this study, we provide further evidence that Nox4 participates in TGF-β1/Smad signaling...
to induce differential atrial-ventricular collagen expression. Both Smad and Nox4 are essential for the up-regulation of collagen expression by TGF-β1 because both inhibitors may block this process. TGF-β1 induces only modest increase in phospho-Smad3 in ventricular fibroblasts, which accounts for the lower transcriptional activity and expression of collagen. The discrepancy in Nox4 expression and ensuing ROS production may contribute to the differential atrial-ventricular expression of phospho-Smad3 in response to TGF-β1.

NADPH oxidase-derived ROS production plays an important role in cardiovascular pathophysiology. Both Nox2 and Nox4 are expressed in cardiac fibroblasts and contribute to cardiac fibrosis.19 Nox2 is involved in angiotensin II– and pressure overload-induced cardiac fibrosis, which is significantly attenuated in Nox2−/− mice taking infusion of angiotensin II22 or aortic constriction.23 A recent study also shows that Nox2−/− mice with myocardial infarction exhibit less cardiac hypertrophy and fibrosis than wild-type mice.24 Because Nox4 does not require cytosolic factors, such as p47phox, p67phox, and the small GTPase Rac for its activation, its expression level may fully determine the ROS production.25–27 Recent studies have demonstrated that Nox4 is up-regulated in the heart by hypertrophic stimuli and presents a major source of oxidative stress in the failing heart.25–27 Cardiac-specific Nox4−/− mice exhibit significant attenuation of cardiac hypertrophy and fibrosis during pressure overload compared with wild-type mice.25 In contrast, overexpression of Nox4 in mouse hearts exacerbates cardiac dysfunction and fibrosis in response to pressure overload.25,26 However, another study provides contradictory results.27 Because most previous studies focus on ventricular fibrosis, our study has extended our knowledge about the role of NADPH oxidases, especially Nox4, in atrial fibrosis. Actually, numerous clinical and experimental studies have associated oxidative stress and TGF-β with AF.18,20 Nox2 has been shown to contribute to ROS production in the atria of AF patients, whereas the role of Nox4 remains unclear in AF.5,6 Despite comparable overexpression of TGF-β1 in the atria and ventricles of transgenic mice with cardiac overexpression of TGF-β1, we found that the ratio of collagen to TGF-β1 increases in the atria compared with ventricles. Because TGF-β1 is the key contributor to collagen production in the heart, it is conceivable that the responsiveness of collagen to TGF-β1 is greater in the atria. The involvement of Nox4, but not Nox2, in the responsive differences between atria and ventricles is documented by a higher expression of Nox4 in the atria. Nevertheless, because pathological changes other than atrial fibrosis are also involved in the pathogenesis of AF, whether the inference obtained from these transgenic mice can be applied to AF management needs to be further clarified.

TGF-β1 is well-known to promote extracellular matrix protein production from cardiac fibroblasts in vivo. We found that TGF-β1 did not induce collagen production in ventricular fibroblasts, which conflicts with previous studies. The explanation for this discrepancy is that we apply TGF-β1 in 10% fetal bovine serum, rather than the fetal bovine serum–free cultural conditions used by other groups.28,29 The effect of TGF-β1 on fibroblast proliferation also contradicts some of previous studies. Burststein’s study in dogs reports that there are species differences or discrepancies in experimental methods may account for this inconsistency.

Previous studies indicate that atrial fibrosis is prominent in transgenic mice with cardiac overexpression of TGF-β1, which may make it an ideal model for studying the pathogenesis of AF.5,6 Although comparable overexpression of TGF-β1 in the atria and ventricles of transgenic mice with cardiac overexpression of TGF-β1, we found that the ratio of collagen to TGF-β1 increases in the atria compared with ventricles. Because TGF-β1 is the key contributor to collagen production in the heart, it is conceivable that the responsiveness of collagen to TGF-β1 is greater in the atria. The involvement of Nox4, but not Nox2, in the responsive differences between atria and ventricles is documented by a higher expression of Nox4 in the atria. Nevertheless, because pathological changes other than atrial fibrosis are also involved in the pathogenesis of AF, whether the inference obtained from these transgenic mice can be applied to AF management needs to be further clarified.

Figure 8. A, The expression of TGF-β1, Nox2/4, and p-Smad3 in the atria and ventricles of TGF transgene mice and wild-type controls (WT) was detected by western blot. B, The relative expression levels of each protein are quantified by densitometry and normalized to the levels of control, which was set at 1.0. Each value represents the mean±SE of 6 rats. *P<0.05; †represents the significant differences among groups. GAPDH indicates glyceraldehyde 3-phosphate dehydrogenase; Nox, nicotinamide adenine dinucleotide phosphate oxidase; and TGF, transforming growth factor.

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**Disclosures**

None.
Atrial fibrosis constitutes a common feature of atrial fibrillation, which may originate from the differential response of atrium versus ventricle to pathological insult. The aim of this study was to investigate the related factor(s) in mediating atrial vulnerability to fibrotic processes. We first compared the response of cultured atrial versus ventricular fibroblasts to transforming growth factor-β (TGF-β), a key mediator of myocardial fibrosis. Atrial fibroblasts showed a stronger response to TGF-β1 in producing extracellular matrix protein (collagen and fibronectin) than ventricular fibroblasts. Furthermore, TGF-β1 activated its downstream signaling (Smads) and induced pronounced oxidative stress including up-regulation of nicotinamide adenine dinucleotide phosphate oxidase 4 (Nox4) in atrial fibroblasts and to a lesser extent in ventricular fibroblasts. Nox inhibitors and small-interfering RNA for Nox4 eliminated TGF-β1–induced difference between atrial and ventricular fibroblasts suggesting the crucial role of Nox4 in mediating the atrial-ventricular discrepancy. Small-interfering RNA for Nox4 also suppressed the differential responsiveness of atrial versus ventricular fibroblasts to TGF-β1 including Nox4 activation, implicating a crosstalk between Nox and Smad. In vivo, the increased TGF-β1 responsiveness and Nox4 expression were documented in the atria of transgenic mice with cardiac overexpression of TGF-β1. In conclusion, atrial fibroblasts show greater fibrotic and oxidative responses to TGF-β1 than ventricular fibroblasts. Nox4-derived reactive oxygen species production mediates the susceptibility of atrial fibroblasts to TGF-β1 via activating TGF-β1/Smad-signaling cascade, which provides a novel insight into the pathogenesis of atrial fibrosis and atrial fibrillation.

**CLINICAL PERSPECTIVE**

**References**


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Data Supplement (unedited) at:

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SUPPLEMENTAL MATERIAL

Materials

Most chemicals were purchased from Sigma (St. Louis, MO) unless otherwise indicated.

Cell cultures

The animal study was approved by the Institutional Animal Care and Use Committee of Chang Gung Memorial Hospital, and performed in accordance 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). Atrial and ventricular fibroblasts were obtained from left atria and ventricles of adult male Wistar rats (euthanized with ketamine [100 mg/kg] and xylazine [11.5 mg/kg], IP) using the collagenase and trypsin digestion methods as described,\textsuperscript{1,2} and grown in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS). Death of rats was confirmed by cessation of respiration. Only cells from early passages (1-2) at 80 to 90% confluence were used. The fibroblastic character of cells was determined by positive staining for vimentin (>95%) and negative staining for desmin and von Willebrand factor. When cultures reached confluence, the medium was replaced with serum-free DMEM for 24 hours. The cells were then pre-incubated with or without the tested inhibitors or small interfering (si) RNAs and treated with or without TGF-β1 for an
additional 24 hours in DMEM with 10% FBS.

**Western blot analysis**

Western blot was performed as described previously. Equal amounts of protein in SDS-PAGE sample buffer were sonicated and subjected to electrophoresis on 8% SDS-polyacrylamide gels. After transfer to PVDF membranes (Stratagene, The Netherlands), proteins were incubated with primary antibodies against collagen I, III, Nox2/gp91phox, Nox4, TGF-β1 receptor (Abcam, Cambridge, MA), phospho-Smad3 (p-Smad3) (R&D Systems, Minneapolis, MN), fibronectin, and tubulin (Santa Cruz, Delaware Avenue, CA). Signals were detected by ECL-detection (Amersham, Netherlands) and quantified by densitometry. Signal-bands were in the linear immunoreactive range and expressed relative to tubulin.

**Real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR)**

Total cellular RNA was extracted using TRIzol reagent (Life Technologies, Rockville, MD) and real-time quantitative RT-PCR was performed as described previously. GAPDH mRNA was used as the internal control.

**Detection of intracellular oxidative stress**

Intracellular oxidative stress from reactive oxygen species (ROS) was measured with fluorescent dye (2, 7-dichlorofluorescein diacetate [DCF-DA]), a cell-permeable
indicator for ROS. The ROS-dependent fluorescence was detected by confocal microscopy (Leica TCS SP2, Carl Zeiss, Jena, Germany) with excitation at 488 nm and emission at 530 nm. Cardiac fibroblasts were pre-incubated with 10 μmol/L DCF-DA for 30 minutes (37°C). Excitation of DCF-DA was achieved using the 488-nm band of an argon laser. Emission was recorded with a longpass LP 515 filter set. Two-dimensional 514×514 pixel images were acquired.

**Measurement of intracellular ROS by flow cytometry**

Cardiac fibroblasts were plated in 6-cm culture dish with 1×10^6 per well. After cardiac fibroblasts treating with TGF-β1 for 30 minutes, cells were harvested by trypsinization, collected by centrifugation, and suspended in medium containing 20 μmol/L DCF. After incubating with DCF 30 minutes at 37 °C in dark, cells were collected by centrifugation, suspended, and stored in fresh PBS. The stained cells were analyzed by flow cytometry (BD LSRII System, BD Biosciences, San Jose, CA). Measurement was taken at 510–540 nm after excitation of cells at 488 nm with an argon ion laser. Cell content was quantified using FCS Express V3 software.

**Measurement of NADPH-dependent superoxide production by chemiluminescence**

Superoxide production was measured from homogenized cardiac tissues (around 2 mg) or fibroblasts by lucigenin-enhanced chemiluminescence using a luminometer (Plate CHAMELEON, Hidex, Finland). Lucigenin (5 μmol/L) and NADPH (100 μmol/L) were each added, and the photon outputs were measured. Chemiluminescence was counted
through a 7 mm diameter window, and normalized to cellular protein concentrations or
dry weight.

**Immunohisto- and cytochemical analyses**

Immunohisto- and cytochemical studies were performed by confocal microscopy
using primary antibodies against collagen I, Nox2/4, p-Smad3, TGF-β1 receptor, and
vimentin (Sigma) followed by FITC (green) or Cy3 (red, Chemicon, Temecula,
CA)-conjugated secondary antibodies. Immunocytochemical cells were grown on 25
mm glass coverslips. The cells treated with or without 5 ng/mL TGFβ1 were removed
from the media, and washed in PBS, and fixed in 4% paraformaldehyde for 20
minutes at room temperature (RT). The coverslips were rinsed in PBS and placed in
0.5% Triton X-100 for 15 minutes at RT. The cells were then washed twice with PBS
and blocked with 2% BSA for 1 hour at RT. For immunohistochemical analysis,
frozen tissue sections were cut at 5 μm and stored at -80°C for future usage. Frozen
sections were washed with PBS and blocked with 2% BSA for 30 minutes at RT.
Immunohisto- and cytochemical samples were exposed to primary antibodies with
1:100 dilution in BSA at 4°C over night or at RT for 1 hour. The coverslips were
washed 3 times with PBS for 2 minutes each and then exposed to FITC (green) or
Cy3 (red, Chemicon, Temecula, CA)-conjugated secondary antibodies with 1:200
dilution in PBS for 60 minutes at RT. The coverslips were washed 3 times with PBS
for 2 minutes, and mounted in chambers for observation. Nuclei were visualized by DAPI-staining. The expression levels of target proteins were calculated as protein-occupied area in the tissue divided by the nuclear area. Nuclear expression of p-Smad3 was calculated as the p-Smad3-occupied area in the nucleus divided by total nuclear area. For each analysis, at least 3 random fields were chosen to observe >30 cardiac fibroblasts.

**Small interfering (si) RNA and expression vector**

Cardiac fibroblasts were cultured at a density of 80-90% confluence on the day of transfection. Chemically synthesized siRNAs for Nox2/4, Smad3, and their control siRNAs were purchased from Dharmacon (Lafayette, CO) and transferred into cells with DharmaFECT-1 according to manufacturer’s instructions. Wild-type Nox4 plasmid was purchased from ORIGENE (Rockville, MD).

**RNA stability assay**

RNA stability assay was performed as previously described.² Cardiac fibroblasts were cultured in serum-free medium for 24 hours and pre-incubated with actinomycin D (5 μg/mL) for 2 hours and subsequently treated with or without TGF-β1 (5 ng/mL). The cells were harvested at 0, 6, 24, and 48 hours and collected for RNA extraction and quantified by real-time RT-PCR. All values were expressed relative to the basal value at 0 hours.
**Promoter activity assay**

Fragments of rat collagen I promoter (nucleotides –3494 to +144) were amplified by PCR with primers designed from the published nucleotide sequence and were sub-cloned into the pGL3Basic vector (Promega, Madison, WI) at *Bgl*II and *Hind*III sites. Two putative Smad4 binding elements (SBEs, –2194 to –2189 and –2084 to –2079, respectively) were found at the collagen I promoter. Site-directed mutations of these 2 SBE sites were constructed by PCR using the same strategy. For transient transfection assays, cardiac fibroblasts at 50-60% confluence were transfected with indicated plasmids using TransIT-LT1 reagent (Life Technologies). The transfection efficiency by this method was approximately 60%. After an additional 24 hours, samples were sent for measurement of luciferase activity with an assay system (Dual-Luciferase® Reporter, Promega). Luciferase activities were measured with a luminometer (Luminoskan TL PMS, Thermo Labsystems), and normalized to cellular protein concentrations.

**Chromatin immunoprecipitation (ChIP) assay**

ChIP assay was performed as described elsewhere. Briefly, serum-deprived cardiac fibroblasts were treated with or without TGF-β1 (5 ng/mL) for 24 hours, cross-linked with 1% formaldehyde, and sonicated. Soluble chromatin was precleared with 30μL of 50% protein G (Sigma) containing 5μg ssDNA at 4°C for 30 minutes. Then, the
remaining lysate was used for immunoprecipitation with anti-Smad4, or rabbit nonspecific IgG (Sigma) as a negative control. After incubation at 4°C overnight, 30μL of protein G were added and immunocomplexes were recovered. Wash the immunocomplexes twice with RIPA A buffer (150 mmol/L NaCl, 1% NP40, 1% deoxycholic acid, 50 mmol/L Tris-Cl pH9.0, 5 mmol/L EDTA, and 0.1% SDS), three times with RIPA B buffer (300 mmol/L NaCl, 1% NP40, 1% deoxycholic acid, 50 mmol/L Tris-Cl pH9.0, 5 mmol/L EDTA, and 0.1% SDS), three times with LiCl wash buffer (150 mmol/L NaCl, 300 mmol/L LiCl, 1% NP40, 1% deoxycholic acid, 50 mmol/L Tris-Cl pH9.0, 5 mmol/L EDTA, and 0.1% SDS), and twice with TE buffer (10 mmol/L Tris-Cl pH8.0 and 1 mmol/L EDTA). The immunocomplexes were eluted twice with 150μL of elution buffer (50 mmol/L NaHCO3 and 1% SDS) at 37°C for more than 30 minutes. For the reversal of cross-links, 18μL of 5 mol/L NaCl and 1μL RNase A were added at 67°C overnight. The DNA was extracted with 300μL phenol/chloroform/isoamyl alcohol for more than twice. Then, the supernatant was mixed with 30μL of 5 mol/L NaCl and 20μg of glycogen to precipitate the DNA fragment and eluted in TE buffer. The precipitated DNA was quantified by real-time PCR using SYBR Green. A (–2169 to –1830) fragment of the rat collagen I promoter was amplified using a primer pair (5'-CAATTCTAGTAGTCTCCTAGCC-3' and 5'-AACTCAGTAAAAGTCTGCCTT-3').
**Bromodeoxyuridine incorporation (BrdU) assay**

The proliferative activities of fibroblasts were determined by BrdU incorporation using an ELISA detecting kit (Roche, Mannheim, Germany) following manufacturer’s instructions. Briefly, cardiac fibroblasts were seeded in 96-well plates with equal cell number per well. Cardiac fibroblasts were exposed to TGF-β1 (5 ng/mL) and incubated with BrdU (10 µmol/L) for an additional 24 hours, and fixed in FixDenat solution. The BrdU incorporation was measured by the absorbance of each well at 450 nm with an ELISA plate reader. Data were expressed as a percentage of the control.

**MTT assay**

The viability of fibroblasts was detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma) following manufacturer’s instructions. Cardiac fibroblasts were seeded in 96-well plates. After treatment with or without TGF-β1 (5 ng/mL), the media were replaced with 0.5 mg/mL MTT in PBS and incubated for 2 hours at 37°C. Cell viability was assessed by an ELISA reader at 540 nm.

**MHC-TGFcys33ser transgenic mice**

The MHC-TGFcys33ser transgenic mice (generous gift from Loren J. Field, James Whitcomb Riley Hospital for Children, IN) are generated by the mouse α-cardiac
MHC promoter and sequences encoding the human TGF-β1 cDNA as described.\textsuperscript{3, 4}

The resulting pups were screened using diagnostic PCR amplification. The DBA/2 strain mice were designated as controls.

**Statistical analysis**

All values were expressed as the mean ± SEM. Differences between 2 groups (with versus without TGF-β treatment; wild type versus TGF-β transgene) were compared by unpaired \( t \)-test. Two-way repeated measures ANOVA models followed by post hoc Bonferroni test were applied with factors including cardiac regions and treatment groups. For multiple groups without repeated measures, one-way ANOVA with post hoc Scheffe test was used. A 2-tailed value of \( P<0.05 \) was considered statistically significant. Analyses were performed using GraphPad Prism 4.0 (GraphPad, San Diego, CA) software.

Two-way repeated measures ANOVA models followed by post hoc Bonferroni test were used to compare protein expression (expressed as fold-changes over baseline) at 24 hours (Figure 1 and Supplemental Figure 1), NADPH activity at 24 hours (Supplemental Figure 2), and ROS production at 30 minutes (Figure 2A and Supplemental Figure 3A) across cardiac regions (atrium versus ventricle) and between treatment groups (with versus without TGF-β treatment). Both factors were treated as repeated factors. In the case of a significant interaction, treatment group were
compared for each region.

One-way ANOVA with post hoc Scheffe test was used to compare ROS generation at 30 minutes (Figures 2B, C, and Supplemental Figures 3B and C), protein expression at 24 hours (Figures 3A-C and Supplemental Figure 4), and Smad3 nuclear translocation at 6 hours (Figure 4) between treatment groups. Two-way repeated measures ANOVA models were also used to compare protein expression (Figure 3D), mRNA expression (Figure 5A), collagen I promoter activity (Figures 5B and C), and ChIP assays (Figure 5D) across cardiac regions (atrium versus ventricle) and between treatment groups (TGF-β with versus without Nox inhibitors/expressions). Finally, two-way repeated ANOVA models were used to compare protein expression in vivo across cardiac regions (LA versus LV; RA versus RV) and between groups (wild type versus TGF-β transgene) (Figures 6-8, and Supplemental Figures 8 and 9).

References


Supplemental Figure 1:

A. After 24 hours of serum deprivation, cardiac fibroblasts were treated with or without 5 ng/mL TGF-β1 for 24 hours. The distribution/expression of TGF-β1 receptor was evaluated by confocal immunohistochemistry as described in Materials and Methods. Relative intensities of TGF-β1 receptor are quantified and normalized to the levels of control, which was set at 1.0.

B. The expression of TGF-β1 receptor was evaluated by western blot. The relative expression level of TGF-β1 receptor were quantified by densitometry and normalized to the control level, which was set at 1.0. Each value represents the mean ± SE of 4 independent experiments. P<0.05; *: the different symbol represents the significant difference among groups.
Supplemental Figure 2:

After 24 hours of serum deprivation, cardiac fibroblasts were treated with or without 5 ng/mL TGF-β1 for 24 hours. NADPH-dependent superoxide production was measured with lucigenin-enhanced chemiluminescence. Each value (mean ± SE, n=6) is expressed as a fold of NADPH activity relative to the control condition. P< 0.05; *. the different symbol represents the significant differences among groups.
Supplemental Figure 3:

A. Intracellular ROS production was measured by fluorescent DCF dye and detected by flow cytometry as described in Materials and Methods. An identical paradigm was followed as described in Figures 2A-C. After 24 hours of serum deprivation, cardiac fibroblasts were treated with or without 5 ng/mL TGF-β1 for 30 minutes.

B. After 24 hours of serum deprivation, atrial fibroblasts were pre-incubated with indicated Nox inhibitors and subsequently treated with or without 5 ng/mL TGF-β1 for 30 minutes.

C. After deprivation of serum and transfection of indicated siRNAs for 48 hours, atrial fibroblasts were treated with or without 5 ng/mL TGF-β1 for 30 minutes.

Each value represents the mean ± SE of four independent experiments. P< 0.05; *, †: the different symbols represent the significant differences among groups.

A=atrium; V=ventricle
Supplemental Figure 4:

After deprivation of serum and transfection of indicated siRNAs for 48 hours, atrial fibroblasts were treated with or without 5 ng/mL TGF-β1 for 24 hours. The expression of Nox2/4 and tubulin protein was evaluated by western blot. Each value represents the mean ± SE from 4 independent experiments. P< 0.05; *: the different symbol represents the significant differences among groups.
Supplemental Figure 5:

Collagen I mRNA stability was evaluated by stopping transcription with actinomycin D (5 µg/mL), as described in Materials and Methods. The relative expression levels of mRNA were quantified and normalized to the control level, which was set at 1.0. Each value represents the mean ± SE of 4 independent experiments.
Supplemental Figure 6:

Growth-arrested cardiac fibroblasts were treated with or without 5 ng/mL TGF-β1 for the indicated times. The BrdU incorporation into fibroblasts was assayed by BrdU cell proliferation ELISA, as described in the Methods. Each value (mean±SE [n=6]) is expressed as the fold of BrdU incorporation of controls.
Supplemental Figure 7:

Growth-arrested cardiac fibroblasts were treated with or without 5 ng/mL TGF-β1 for the indicated times. The viability of fibroblasts was determined by MTT assay as described in the Methods. Each value (mean±SE [n=6]) is expressed as the fold of controls.
Supplemental Figure 8:

NADPH-dependent superoxide production was measured with lucigenin-enhanced chemiluminescence from atria and ventricles of MHC-TGFcys^{33}ser transgenic mice compared to controls. Each value (mean ± SE, n=6) is expressed as a fold of NADPH activity relative to the control condition. P< 0.05; *: the different symbol represents the significant differences among groups.
Supplemental Figure 9:

A. An identical paradigm was followed as described in Figure 6. Representative confocal images show Nox2 expression in the atria and ventricles of MHC-TGFcys^{33}ser transgenic mice compared to controls.

B. Relative intensities of Nox2 staining in vimentin-expressing area are quantified. Data are means ± SE (a total of >3 fields and 30 cells with scanning and averaging). P< 0.05; *: the different symbol represents the significant differences among groups.