NAD(P)H Oxidase 4 Mediates Transforming Growth Factor-β1–Induced Differentiation of Cardiac Fibroblasts Into Myofibroblasts

Ioan Cucoranu, Roza Clempus, Anna Dikalova, Patrick J. Phelan, Srividya Ariyan, Sergey Dikalov, Dan Sorescu

Abstract—Human cardiac fibroblasts are the main source of cardiac fibrosis associated with cardiac hypertrophy and heart failure. Transforming growth factor-β1 (TGF-β1) irreversibly converts fibroblasts into pathological myofibroblasts, which express smooth muscle α-actin (SM α-actin) de novo and produce extracellular matrix. We hypothesized that TGF-β1–stimulated conversion of fibroblasts to myofibroblasts requires reactive oxygen species derived from NAD(P)H oxidases (Nox). We found that TGF-β1 potently upregulates the contractile marker SM α-actin mRNA (7.5±0.8-fold versus control). To determine whether Nox enzymes are involved, we first performed quantitative real time polymerase chain reaction and found that Nox5 and Nox4 are abundantly expressed in cardiac fibroblasts, whereas Nox1 and Nox2 are barely detectable. On stimulation with TGF-β1, Nox4 mRNA is dramatically upregulated by 16.2±0.8-fold (n=3, P<0.005), whereas Nox5 is downregulated. Small interference RNA against Nox4 downregulates Nox4 mRNA by 80±5%, inhibits NADPH-driven superoxide production in response to TGF-β1 by 65±7%, and reduces TGF-β1–induced expression of SM α-actin by 95±2% (n=6, P<0.05). Because activation of small mothers against decapentaplegic (Smads) 2/3 is critical for myofibroblast conversion in response to TGF-β1, we also determined whether Nox4 affects Smad 2/3 phosphorylation. Depletion of Nox4 but not Nox5 inhibits baseline and TGF-β1 stimulation of Smad 2/3 phosphorylation by 75±5% and 68±3%, respectively (n=7, P<0.0001). We conclude that Nox 4 mediates TGF-β1–induced conversion of fibroblasts to myofibroblasts by regulating Smad 2/3 activation. Thus, Nox4 may play a critical role in the pathological activation of cardiac fibroblasts in cardiac fibrosis associated with human heart failure. (Circ Res. 2005;97:0-0.)

Key Words: Nox4 ■ human cardiac fibroblasts ■ transforming growth factor ■ reactive oxygen species ■ Smad 2/3

Heart failure remains the leading cause of hospital admissions in the United States, with more than 550 000 new patients diagnosed each year.1 Regardless of etiology, cardiac fibrosis is a major contributor to cardiac remodeling associated with heart failure.2 It is characterized by expansion of the interstitial compartment due to increased deposition of extracellular matrix by activated myofibroblasts.2 Cardiac myofibroblasts are specialized contractile fibroblasts formed by irreversible acquisition of contractile proteins such as smooth muscle α-actin (SM α-actin) in response to potent fibrogenic cytokines.3 The expression of SM α-actin is regulated by transforming growth factor-β1 (TGF-β1), a primary fibrogenic growth factor in heart failure that is downstream of many of the pro-fibrotic actions of other profibrotic growth factors, such as angiotensin II, aldosterone, and norepinephrine.4 TGF-β1 is upregulated in failing human hearts and various experimental models of cardiac hypertrophy,4 and functional blockade of TGF-β1 prevents cardiac interstitial fibrosis induced by pressure overload in rats.5 There is increasing evidence that oxidative stress plays a critical role in the development and progression of cardiac remodeling associated with heart failure.6 Oxidative stress is increased in human heart failure7,8 and animal models of cardiac hypertrophy and fibrosis (induced by angiotensin II,9,10 aldosterone,11 myocardial infarction,12 tachycardia-induced cardiomyopathy,13,14 and aortic coarctation10,15). The first evidence that elevated oxidative stress can cause cardiomyopathy was provided by Li et al,16 who showed that deletion of superoxide dismutase (a main scavenger of intracellular superoxide) caused dilated cardiomyopathy and cardiac fibrosis in mice.16 Reactive oxygen species (ROS) modulate extracellular matrix remodeling by mediating cardiac fibroblast function and also by stimulating collagen turnover via activation of matrix metalloproteinases, enzymes critical for extracellular matrix remodeling.17 ROS also stimulate release and activation of cytokines. For example, in cell culture, exposure of cardiac fibroblasts to superoxide stimulates their proliferation by increasing the production of TGF-β1.18

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NADPH oxidases (Nox) are multisubunit enzymes that generate superoxide by transferring electrons from NADPH to molecular oxygen.\textsuperscript{19} Although the prototype catalytic subunit gp91phox (Nox2) was originally discovered in neutrophils, there are also multiple homologues in nonphagocytic cells with wide range tissue distribution: Nox1, Nox3, Nox4, Nox5, and the dual oxidases Duox1 and Duox2.\textsuperscript{20} In phagocytes, ROS originating from Nox participate in bacterial killing, whereas in nonphagocytic cells they are required for cellular responses to cytokines and growth factors.\textsuperscript{21} Fibroblasts also produce ROS via Nox in response to growth factors. For example, lung fibroblasts endogenously release ROS from Nox in response to TGF-\textbeta1.\textsuperscript{22} An important consequence of fibroblast stimulation with TGF-\textbeta1 is a phenotypical conversion into myofibroblasts. Although NADPH oxidases are activated by TGF-\textbeta1, it is unknown whether their activation is required for upregulation of SM-\alpha-actin, the key element that defines the acquisition of contractile phenotype by myofibroblasts.

In the current study, we sought to (1) determine whether ROS derived from NADPH oxidases are important for TGF-\textbeta1–induced myofibroblast differentiation, (2) identify the catalytic Nox subunit that is required for SM-\alpha-actin expression in response to TGF-\textbeta1, and (3) discover the mechanism by which Nox-derived ROS mediate the conversion to the myofibroblast phenotype. These data provide new insights into the mechanisms underlying the pathological activation of fibroblasts in cardiac fibrosis induced by TGF-\textbeta1.

Materials and Methods

Cell Culture

Primary human cardiac fibroblast cells (catalog number ACBRI 5118) were purchased at passage 2 from Cell Systems (Kirkland, WA) and grown in fibroblast growth medium-2 (Cambrex, Baltimore, Md). All cells were harvested at passage 3 to 6 and used at 50% to 70% confluence for transfection experiments and 80% to 90% confluence for time course experiments (see online data supplement at http://circres.ahajournals.org for characterization of human cardiac fibroblasts).

Western Blotting

Human cardiac fibroblasts were sonicated in lysis buffer. After separation by SDS-PAGE and transfer to nitrocellulose membranes, signals were detected with specific corresponding antibodies, visualized with enhanced chemiluminescence, and quantified by laser densitometry (see detailed Methods in data supplement).

Immunofluorescent Cytochemistry

Single and double-label fluorescent immunocytochemistry was performed on cells plated onto glass coverslips as described previously.\textsuperscript{23} The antibodies used were rabbit polyclonal anti-\textalpha-actin (1:50 dilution) and mouse monoclonal anti-smooth muscle actin (clone 1A4, 1: 100 dilution, Sigma). Similar coverslips treated with secondary antibodies alone did not show specific staining.

Quantitative Real-Time Polymerase Chain Reaction

Quantification of human Nox 1, Nox 2, Nox 4, Nox 5, human smooth muscle specific-\alpha actin and 18S rRNA was performed by amplification of cDNA using the LightCycler real-time thermocycler as described previously\textsuperscript{24} (see detailed Methods in online data supplement).

Detection of Intracellular Superoxide Using High Performance Liquid Chromatography

To measure intracellular release superoxide, we uses the superoxide-specific fluorescent dihydrodethidium and quantified the superoxide specific signal by using high performance liquid chromatography as previously described\textsuperscript{24,25} (see online data supplement).

Measurement of NADPH-Dependent Superoxide Production

Membrane samples from fibroblasts were prepared as described previously\textsuperscript{26} (see online data supplement).

Transfection With Small Interference RNA

Human cardiac fibroblasts were trypsinized and plated on 100-mm dishes at 30% to 50% confluence 24 hours before transfection. To downregulate Nox4, Nox5, and small mothers against decapentaplegic (Smad) 2, scrambled small interference RNA (siScr) and small interference RNA (siRNA) against human Nox4 (siNox4, sense sequence: 5'-ACUGAGGUAACGUGAUUGU-3', anti-sense sequence: 3'-CAUCCACGUGACCUCAGUUU-3'), human Nox5 (siNox5: sense sequence 5'-GUGGUGAGUAAUACUCGGAUCCT-3', anti-sense sequence 5'-GUCGAAUAAGUCCACCTT-3'), Smad 2 (sense sequence 5'-GUCGCAUGAAAAAGACUAAATT-3', anti-sense sequence 5'-UUAGAUGUUUCUAGGGACCT-3'), were purchased from Ambion. Individual siRNAs (at 25 to 50 nmol/L), oligofectamine and Opti-MEM were mixed and incubated at room temperature for 20 minutes. siRNA-oligofectamine complexes were added to cells for 24 hours, after which siRNA-oligofectamine complexes were removed and cells were washed and placed in serum-free fibroblast growth medium (Cambrex) for 72 hours. Subsequently, cells were treated with or without TGF-\textbeta1 (10 ng/mL) for the indicated time and harvested for RNA or protein extraction, immunocytochemistry, or superoxide assay. To control for possible nonspecific effects of siRNAs, multiple scrambled siRNAs were used, including sequence specific Nox4 scrambled (which has the same nucleotide content but in random order), siLuc (siRNA against luciferase), and the universal negative control siRNA commercially available from Ambion.

Statistical Analysis

Results are expressed as mean±standard error of mean (SEM). Statistical significance was assessed by using paired Student’s t test or 1-way or 2-way ANOVA with appropriate post-hoc analysis (Scheffe’s) to exclude possible interactions between various variables within subgroups using Origin software version 7.5. A value of P<0.05 was considered to be statistically significant.

Results

TGF-\textbeta1 Upregulation of Smooth Muscle \alpha-Actin in Human Cardiac Fibroblasts Is ROS Sensitive

TGF-\textbeta1 is the most potent stimulus for differentiation of fibroblasts into myofibroblasts. This phenotype is characterized by upregulation of SM-\alpha-actin, which confers contractile behavior to myofibroblasts. To determine whether ROS are necessary for expression of SM-\alpha-actin in response to TGF-\textbeta1, we exposed cardiac fibroblasts to the ROS inhibitors diphenylene iodonium (an inhibitor of flavin-containing oxidoreductases such as NADPH oxidases), ebselen (a non-specific H2O2 scavenger), or N-acetyl cysteine (a glutathione precursor and scavenger of H2O2) and then examined the expression of SM-\alpha-actin at the RNA and protein levels after stimulation with TGF-\textbeta1 (10 ng/mL) for 24 hours. As shown in Figure 1A, TGF-\textbeta1 upregulated SM-\alpha-actin 7.5±0.8 fold (n=4, P=0.001), and all ROS inhibitors potently abrogated
TGF-β1 Differentiation of Myofibroblasts

Nox4 and TGF-β1 Differentiation of Myofibroblasts

TGF-β1 Stimulates NADPH Oxidase Activity in Human Cardiac Fibroblasts

NADPH oxidases are important sources of ROS in human fibroblasts. It is well established that TGF-β1 activates NADPH oxidases and thereby stimulates ROS release from fetal lung fibroblasts. Therefore, we examined whether TGF-β1 stimulates NADPH-driven superoxide production in human cardiac fibroblasts by performing a 24-hour time course. Indeed, TGF-β1 induces NADPH-driven superoxide production from cardiac fibroblast membranes (Figure 2). After an initial stimulation at 1 hour (64±13%), the membrane-derived NADPH-driven superoxide production returns to baseline and increases again at 18 and 24 hours (2.4±0.4-fold, n=3, P=0.002). These results confirm that TGF-β1 chronically increases NADPH-driven superoxide production from membranes of human cardiac fibroblasts.

TGF-β1 Upregulates Nox4, but not Nox1, Nox2, or Nox5 in Human Cardiac Fibroblasts

Because TGF-β1 induced an increase in NADPH-driven ROS production in cardiac fibroblasts that was sustained over 24 hours, we hypothesized that it is mediated by increased expression of a NADPH oxidase. To identify which Nox is upregulated, we stimulated cardiac fibroblasts with TGF-β1 and quantified the expression of various Nox enzymes at the level of RNA or protein. At baseline, Nox4 and Nox5 mRNA were abundantly expressed, whereas Nox 1 or Nox 2 (gp91phox) were expressed at very low levels (limit of detection of the assay, data not shown). After stimulation with TGF-β1, Nox 4 mRNA was potently upregulated by 16.2±0.8-fold at 24 hours (n=3, P<0.005) starting as early as 2 hours, with a peak at 24 hours. In contrast, Nox 5 mRNA was progressively reduced by 75±13% at 24 hours (n=4, P<0.005; Figure 3A) whereas Nox 1 and Nox 2 were unaffected (data not shown). These data suggest that upregu-
lation of Nox4 may be important for TGF-β1 effects on cardiac fibroblasts.

If Nox4-mediated release of ROS is required for SM α-actin expression, induction of Nox4 protein should precede the expression of SM α-actin on stimulation with TGF-β1. Therefore, we stimulated cells with TGF-β1 and harvested them at various time points for Western blotting of Nox4 or SM α-actin proteins. To identify Nox4 protein, we performed Western blotting using a rabbit polyclonal antibody. This antibody identifies an ∼112 to 120 KDa band that is specifically blocked by preincubation with the peptide against which this antibody was raised (supplemental Figure I). Furthermore, this band was specifically upregulated by TGF-β1 and was inhibited by depleting fibroblasts of Nox4 using siRNA (see below). These experiments showed that after stimulation with TGF-β1, Nox4 expression increases as early as 4 hours (2.1 ± 0.1-fold), whereas SM α-actin increase starts at 16 hours (1.88 ± 0.3-fold). Maximum upregulation of both Nox4 and SM α-actin occurred at 24 hours (3.9 ± 0.2-fold for Nox4 and 3.4 ± 0.8-fold, n = 4, for SM α-actin; Figure 3B). The fact that expression of Nox4 preceded the increase in SM α-actin by 12 hours suggests that Nox4-derived ROS are important for upregulation of SM α-actin after treatment with TGF-β1.

Figure 3. Nox 4 is upregulated, whereas Nox 5 is downregulated, by TGF-β1. A, Human cardiac fibroblasts were treated for 1 to 24 hours with TGF-β1 (10 ng/mL) and harvested and RNA was extracted. Nox4 and Nox5 mRNA were quantified using quantitative RT-PCR. Data are mean ± SEM from 4 experiments normalized to 18S. *P < 0.005 vs control. B, Cells were stimulated with TGF-β1 and harvested as above and protein was extracted. Nox4 and SM α-actin proteins were quantified by Western blotting. Data are mean ± SEM from 3 experiments. *P = 0.001 vs control; **P = 0.01 vs control.

Figure 4. Transfection of human cardiac fibroblasts with Nox 4 siRNA reduces Nox 4 expression and NADPH-driven superoxide production in response to TGF-β1. A, Cells were transfected with siScr or siNox4 as above and proteins were extracted. Shown is a representative Western blot for Nox4 and SM-α-Actin expression of SM-α-Actin, Fibronectin, Collagen A1, and Connective Tissue Growth Factor

On the basis of these observations, we performed additional studies to determine whether Nox4 is necessary for TGF-β1 expression of SM-α-actin. We downregulated the expression of Nox4 protein using transfection with small interference oligonucleotide RNA directed against Nox4 (siNox4). First, we confirmed that siNox4 is able to decrease Nox4 mRNA levels. On transfection with siNox4, both basal and TGF-β1-induced expression of Nox4 mRNA were dramatically reduced (74 ± 2% and 78 ± 3% respectively; n = 4; P < 0.05; Figure 4B). Similarly, Nox4 protein was po tently inhibited by siNox4 (Figure 4A). Transfection with fluorescein-labeled siScr showed that siRNA entered more than 95% of cells (supplemental Figure II). We also found that Nox5 mRNA was not inhibited by transfection with siNox4, demonstrating...
the specificity of siNox4 sequence (data not shown). To confirm the functional effect of siNox4, we measured NADPH-driven superoxide production from membranes of cells in which Nox4 was depleted. Transfection with siNox4 reduced basal and TGF-β1-stimulated superoxide production at 24 hours by 32±4% and 44±5%, respectively (n=3, P<0.01 versus siScr; Figure 4C), demonstrating that Nox4 participates in NADPH-driven superoxide production from fibroblast membranes both basally and on chronic stimulation with TGF-β1. Furthermore, deletion of Nox4 and not Nox5 also inhibited intracellular superoxide in response to acute TGF-β1 stimulation (30 minutes; supplemental Figure III). Because at 30 minutes the absolute TGF-β1–induced superoxide production was blocked acutely by ~50%, it is possible that other sources of superoxide may account for residual superoxide production.

To assess whether Nox4 is required for SM α-actin expression, we quantified SM α-actin at the RNA and protein levels after transfection of cells with siNox4. SM α-actin mRNA was reduced by more than 90% by the siRNA against Nox4, whereas SM α-actin protein was inhibited by 66±8% under basal conditions and by 75±7% after stimulation with TGF-β1 (n=3, P<0.001 versus siScr; Figure 5A). Next, to determine whether Nox4 mediates the conversion to the myofibroblast phenotype, we performed immunofluorescent cytochemistry for Nox4 and SM α-actin after transfection with siNox4. Transfection with siNox4 abolished the myofibroblast phenotype (data not shown). In contrast to Nox4, downregulation of Nox5 did not prevent TGF-β1 stimulation of SM α-actin, demonstrating that Nox4 but not Nox5 is required for this response (supplemental Figure IV).

Another important effect of TGF-β1 is stimulation of production of extracellular matrix proteins such as fibronectin and collagen 1. Because it has been demonstrated both in vitro and in vivo that cytokine connective tissue growth factor (CTGF) production is potently increased in response to TGF-β1 and in turn stimulates collagen secretion by the fibroblast, we tested the effect of Nox4 on these responses. We found that depletion of Nox4 inhibited basal and TGF-β1–induced CTGF protein by 51±6% (n=3, P<0.01; Figure 5B), fibronectin production by 61±5% (n=3, P<0.003; Figure 5B), and collagen I A1 mRNA upregulation by 47±7% (n=3, P<0.01; data not shown). Taken together, these results demonstrate that Nox4-derived ROS are critical for differentiation of cardiac fibroblasts in myofibroblasts and extracellular matrix protein production in response to TGF-β1.

**Nox4-Derived ROS Are Required for Chronic Smad 2/3 Activation in Response to TGF-β1**

Recent studies have demonstrated that Smads mediate TGF-β1 induction of SM α-actin in human lung fibroblasts. On phosphorylation by TGF-β1 type I receptor, Smad2 and Smad3 become active, form a heterotrimeric complex with Smad4, and translocate to the nucleus, where the complex activates target gene transcription. To verify that Smad 2/3 phosphorylation mediates TGF-β1–induction of SM α-actin in our cells, we reduced Smad 2 levels by transfecting fibroblasts with siSmad2 and performed Western blotting for phospho-Smad 2/3, total Smad 2/3, and SM α-actin. The siSmad2 effectively blocked not only phosphorylation of Smad 2/3 and total Smad 2 by >90%, but also SM α-actin induction in response to TGF-β1 at 24 hours (n=3; Figure 6A) and Nox4 mRNA at 4 hours (data not shown).

To identify a mechanism by which Nox4-derived ROS are important for Smad 2/3 activation in response to TGF-β1, we first tested the ROS sensitivity of Smad activation. As previously reported, TGF-β1 potently stimulated Smad 2/3 activation with a time course of activation that started as early as 5 minutes and was sustained up to 72 hours (data not shown). Pretreatment with DPI, ebselen, or NAC partially inhibited Smad 2/3 phosphorylation by 51±8%, 52±6%, and 71±5% (n=3, P=0.001 versus control; Figure 6B), respectively. To specifically test whether Nox4 is the source of ROS responsible for TGF-β1–induced Smad 2/3 activation, we depleted cells of Nox4 using transfection with siNox4 and measured Smad 2/3 phosphorylation after 1 hour and 24 hours of stimulation with TGF-β1. At 24 hours, Smad 2/3 phosphorylation was potently inhibited by 75±5% and 68±5% in basal and TGF-β1 stimulated cardiac fibroblasts, respectively (n=7, P<0.0001). At 1 hour, siNox4 inhibited TGF-β1–induced phosphorylation of Smad 2/3 by 52±2% (n=3, P<0.01), whereas depletion of Nox5 did not have any effect (supplemental Figure V). Together with the requirement of Smads for SM α-actin expression, these data show...
Figure 6. Nox4 mediates TGF-β1-induced SM-α actin by altering phosphorylation of Smad2/3. A, Smooth muscle α-actin induction by TGF-β1 is mediated by Smad 2/3. Cells were transfected with siScr or siSmad 2 and stimulated with TGF-β1 (10 ng/mL) for 24 hours. Proteins were extracted and Western blotting for SM-α-actin, phospho-Smad 2/3, total Smad 2, and CDK4 (for loading control) was performed. This is a representative blot and the experiment that was repeated 3 times. B, Chronic Smad 2/3 activation by TGF-β1 is ROS-dependent. Human cardiac fibroblasts were pretreated for 2 hours with DPI (10 μmol/L), ebselen (Ebs; 20 μmol/L), or NAC (10 mmol/L), stimulated with TGF-β1 (10 ng/mL) for 24 hours, and harvested, and proteins were extracted. Smad 2/3 activation was quantified by performing Western blot analysis using anti-phospho-Smad 2/3 (normalized to total Smad 2 levels). Data are mean±SEM from 3 experiments normalized to control. *P<0.001 vs control; #P=0.001 vs control; **P<0.0001 vs control. C, Chronic Smad 2/3 activation by TGF-β1 requires Nox 4. Cells were transfected with siScr or siNox4 as above and proteins were extracted. The top panel shows a representative Western blot for phospho-Smad 2/3. The blot was stripped and re-blotted for CDK4 to show equal loading. Mean±SEM of densitometric data from 7 experiments. *P<0.0001 vs siScr; **P<0.01 vs siScr; #P<0.0001 vs siScr +TGF-β1.

that TGF-β1 induces ROS production from Nox4 as early as 30 minutes that subsequently participate in the phosphorylation and activation of Smad 2/3, leading to the long term induction of Nox4 and SM α-actin expression and conversion to myofibroblasts

Discussion

In the present study, we provide evidence that Nox4 is essential for differentiation of human cardiac fibroblasts into myofibroblasts in response to TGF-β1. We demonstrate that (1) TGF-β1 upregulates Nox4, ROS production, and SM α-actin in human cardiac fibroblasts; (2) the change in SM α-actin expression and the development of the myofibroblast phenotype in response to TGF-β1 require Nox4-derived ROS; and (3) Nox4 modulates SM α-actin expression by controlling long-term activation of Smad 2/3. These new findings support the notion that Nox4 is critical for modulation of contractile phenotype in response to TGF-β1.

The excessive interstitial fibrosis from failing hearts is produced by the activated myofibroblast in response to regulatory signals such as angiotensin II, aldosterone, or stretch via paracrine release of TGF-β1. Therefore, it has been hypothesized that the phenotypic conversion of fibroblasts into specialized myofibroblasts is a key process mediating cardiac fibrosis. In the present study, we used TGF-β1 as a stimulus for myofibroblast differentiation because this cytokine is a key element that mediates the excessive fibrogenic reaction both in cardiac fibroblast culture and in animals and humans with heart failure. It is well known that TGF-β1 stimulates NADPH oxidase activity and ROS release in human lung fibroblasts. The link between ROS and the acquisition of the myofibroblast phenotype was first suggested by Vozenin-Brotons et al, who showed that superoxide mediates the conversion of skin fibroblasts into myofibroblasts via paracrine release of TGF-β1 in a skin model of wound healing. The source and mechanisms by which ROS mediate myofibroblast differentiation, however, had not been determined before our current study.

The NADPH oxidases are multisubunit enzymes originally discovered in neutrophils. The neutrophil oxidase consists of 5 subunits: 2 membrane-spanning components (the small subunit p22phox and the large catalytic subunit Nox2) and 3 cytosolic components (rac1, p67phox and p47phox). Similar oxidase systems have now been identified in nonphagocytic cells and have been shown to be the primary source of ROS that mediates angiotensin II-induced vascular myocyte hypertrophy (reviewed in Griendling et al). The activation and structure of each cardiovascular NADPH oxidase is determined by the type of catalytic subunit (Nox homologue). For example, Nox1 and Nox2 require recruitment of the cytosolic factors p47phox and p67phox (or their homologues NoxO1 and NoxA1, respectively); Nox5 requires intracellular calcium release, and Nox4 appears to be intrinsically active and requires only p22phox and possibly Rac for activity. Using RT-PCR and Western blot analysis, we demonstrated that 2 catalytic subunits expressed in cardiac fibroblasts are regulated by TGF-β1 (Nox4 is upregulated whereas Nox5 is downregulated). The fact that each cell contains multiple catalytic subunits suggests that each Nox is coupled with
different cellular functions. For example, Nox1 is involved in mitogenic stimulation of vascular myocytes\textsuperscript{23} and Nox5 has been implicated in growth and apoptosis,\textsuperscript{3,4} whereas Nox4 has been associated with growth inhibition, because overexpression of Nox4 in NIH 3T3 cells causes senescence and growth arrest.\textsuperscript{35} Importantly, TGF-\textbeta 1 is also known to inhibit mitogenic growth by upregulating cell cycle inhibitors,\textsuperscript{36} which would suggest that TGF-\textbeta 1 and Nox4 participate in similar cellular processes.

NADPH oxidase subunit expression and activity are increased in various models of cardiac hypertrophy and heart failure.\textsuperscript{37,38} Recently, several studies have established a role for NADPH oxidases in angiotensin II-induced cardiac hypertrophy in vitro and in vivo.\textsuperscript{9,10,15,39} Very little is known, however, about the role of NADPH oxidases in fibroblast function. In the current study, we found that downregulation of Nox4 prevented myofibroblast formation, CTGF expression, and production of extracellular matrix proteins such as fibronectin and collagen 1A1. Because fibronectin, collagen, and CTGF are essential for extracellular matrix remodeling in response to TGF-\textbeta 1,\textsuperscript{22} the current data support the notion that Nox4 activation in fibroblast in response to TGF-\textbeta 1 is vital to development of cardiac fibrosis.

Other Nox proteins have also been implicated in cardiac fibrosis. For example, one study demonstrated that in vivo deletion of Nox2 reduced angiotensin II-induced cardiac fibrosis (suppressor concentration).\textsuperscript{9} Nox2 does not appear to mediate the cardiac fibroblast response, however, because transfection with siNox2 had no effect on SM \alpha-actin protein induction in response to TGF-\textbeta 1 (unpublished observations). The hypothesis that different Nox enzymes couple with different agonists is also supported by 2 other in vivo studies. Maytin et al\textsuperscript{16} and Byrne et al\textsuperscript{10} showed that Nox2 knockout did not prevent cardiac fibrosis or hypertrophy in a pressure-overload model of cardiac hypertrophy, although this model was shown to require ROS and resulted in increased cardiac levels of Nox4. Our observations, taken together with these studies, suggest that Nox4 may mediate TGF-\textbeta 1-induced fibrosis and pressure-overload (ie, stretch), whereas Nox2 is required for angiotensin II-induced cardiac fibrosis. Because we found that Nox2 was not involved in TGF-\textbeta 1 differentiation of human cardiac myofibroblasts, this may suggest that Nox4 substitutes for Nox2 in TGF-\textbeta 1-induced cardiac fibrosis, these data provide insight into novel mechanisms with potential therapeutic implications for heart failure. Further studies are necessary to determine whether Nox4-derived ROS are also involved in vivo in models of cardiac fibrosis.

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ONLINE DATA SUPPLEMENT

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**Cell Culture**

We tested our observations on three different primary cardiac fibroblasts bought from Cell Systems at passage two and two other isolated by us from failing human hearts (explanted from patients undergoing heart transplant for end-stage cardiomyopathy) as primary cardiac fibroblasts at passage 0. All these cells were characterized by immunohistochemistry to show that they are fibroblasts: they were positive for vimentin (marker for fibroblasts) and negative for desmin (marker for smooth muscle cells) and von Willebrandt factor (marker for endothelial cells). In agreement with others, these fibroblasts differentiate with passage into myofibroblasts. In our cells at passage 0-2 there are very few myofibroblasts (<5%, defined as the presence of SM α-actin in stress fibers), but with passage more cells convert to myofibroblasts (50% by passage 9). For the current experiments, we used cells no later than passage 6 (myofibroblast content up to 10-20%), which allows us to measure a good response to TGF-β1 (conversion fibroblasts into myofibroblasts of up to 90%).

**Materials**

The rabbit polyclonal Nox4 antibodies (one for immunocytochemistry and one for western blotting) were generously provided by Dr David Lambeth (Emory University). The specificity of these antibodies was documented by ablation of the signal after pre-treatment with a blocking peptide against which antibodies were raised (Figure 1, supplemental data). Mouse monoclonal anti-gp91phox (clone 54.1, 1:50 dilution) was generously provided by Dr Mark T. Quinn. Commercially available antibodies used were as follows: rabbit polyclonal to phospho-Smad 2 and total Smad 2 (Cell Signaling, Beverly, MA), rabbit polyclonal to CDK4 (sc-260, Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal anti-smooth muscle actin (clone 1A4, Sigma, St. Louis,
MO). Oligofectamine and Opti-MEM media were bought from Invitrogen. Real time PCR was performed using a LightCycler real-time thermocycler (Roche, Indianapolis, IN). Universal 18S rRNA primers (Alternate 18S Internal Standards) were from Ambion (Austin, TX). All other oligonucleotide primers were from Genosys Biotechnologies (The Woodlands, TX). SYBR green I 10,000X stock was purchased from Molecular Probes (Eugene, OR). All other chemicals and reagents, including Dulbecco's modified Eagle's medium with 25 mmol/L HEPES and 4.5 g/L glucose, were from Fisher Scientific (Pittsburgh, PA) or Sigma (St. Louis, MO).

**Western blotting**

Cells were grown in FGM-2 media in 100-mm dishes and were placed in serum-free media for 16 hours before experiments. After treatment, cells were washed two times with ice-cold PBS and placed on ice. Cells were lysed by incubating for 30 minutes on ice with 500 µl of ice-cold lysis buffer, pH 7.4 ((in mM) 50 HEPES, 5 EDTA, 50 NaCl), 1% Triton X-100, protease inhibitors (10 µg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin), and phosphatase inhibitors ((in mM) 50 sodium fluoride, 1 sodium orthovanadate, 10 sodium pyrophosphate). Solubilized proteins were centrifuged at 14,000 × g in a microcentrifuge (4 °C) for 10 min, and supernatants were stored at −80 °C. Extracted protein was quantified by the Bradford assay. Proteins (40 µg per lane) were separated using 9% (for Nox4) or 10% (for Smad2/3, CDK4 and SM α-actin) polyacrylamide gels using SDS-PAGE, and transferred to Hybond-ECL nitrocellulose membranes. Membranes were blocked for 1 hour at room temperature with PBS containing 5% non-fat dry milk and 0.2% Tween 20 (PBST). The blots were incubated overnight at 4°C with the respective primary antibodies in PBS containing 1% non-fat dry milk and 0.2% Tween 20. Next, membranes were washed four times for 5 minutes in PBST, and incubated with secondary antibodies (HRP-conjugated goat anti-rabbit or sheep anti-mouse antibody, 1:1,000) for 1 h at room temperature in PBST.
Both primary (mouse monoclonal and rabbit polyclonal) and secondary (anti mouse or anti-rabbit) HRP conjugate antibodies were used at a 1:1,000 dilution. Signals were detected using enhanced chemiluminescence and quantified by laser densitometry.

**Measurement of NADPH-dependent superoxide production**

Membrane samples from fibroblasts were prepared as described previously. Human cardiac fibroblasts were harvested, washed twice with ice-cold 50 mM phosphate buffer (PBS), scraped, centrifuged at 400g (10 min), and resuspended in 1 ml of lysis buffer (50 mM phosphate (treated for 2h with 5g/100ml Chelex-100 and filtered) containing the protease inhibitors aprotinin (10 µg/ml), leupeptin (0.5 µg/ml), pepstatin (0.7 µg/ml), and PMSF (0.5 mM) (pH 7.4)). Cells were sonicated (power: 4 watts, using Microson 2425 from Misonix Inc; Farmingdale, NY, USA) for 10 s on ice and centrifuged at 28,000g for 15 min at 4°C. The membrane pellet was resuspended in 150 µl of lysis buffer and protein concentration was measured using the Bradford microplate method.

Twenty µg of protein were added to 1 mM 1-hydroxy-3-carboxy-pyrrolidine (CPH), 200 µM NADPH, and 0.1 mM diethylenetriaminepentaacetic acid (DTPA) in a total volume of 100µL of Chelex-treated PBS. CPH is a nitrene spin trap that provides quantitative measurements of $O_2^{•−}$ radicals with high sensitivity. ESR spectroscopy was used for quantitative measurements of $O_2^{•−}$ production. Superoxide formation was assayed as NADPH (200 µM)-dependent, SOD-inhibitable formation of 3-carboxyproxyl (CP•). The ESR spectra were recorded using an EMX ESR spectrometer (Bruker) as published by monitoring the ESR amplitude of the low-field component of the ESR spectrum of CP•. Superoxide dismutase (50 U/ml) added directly to the sample inhibited 95-98% of CP• production.

**Detection of intracellular superoxide using high performance liquid chromatography**
To evaluate intracellular production of superoxide, we measured the formation of hydroxyethidium from dihydroethidium (DHE) oxidation of superoxide using high performance liquid chromatography (HPLC) analysis as recently reported\(^3\),\(^4\). Cells were washed on ice three times with chilled Krebs/HEPES buffer, pH 7.4. Cells growing on 100 mm dish were incubated for 20 min at 37°C in 2.5 mL of Krebs/HEPES-buffer containing 10 µmol/L DHE. Cells were scraped in 100 µl of Krebs/HEPES buffer, transferred to glass homogenizer with 300 µL methanol, homogenized for 1 min. Fifty µL of homogenate were taken for protein measurements, and the remainder was filtered through a 0.22 µm syringe filter. Separation of ethidium, hydroxyethidium and dihydroethidium in the filtered samples was performed on a C-18 reverse phase column (Nucleosil 250-4.5 mm, Sigma-Aldrich, St. Louis, MO, USA) using a Beckman HPLC System equipped with both UV and fluorescence detectors. The mobile phase was composed of a gradient containing 60% acetonitrile and 0.1% trifluoroacetic acid. Dihydroethidium, ethidium and hydroxyethidium were separated by a linear increase in acetonitrile concentration from 37% to 47% in 23 min at a flow rate of 0.5 ml/min. Fluorescence detection at 580 nm (emission) and 480 nm (excitation) was used to monitor hydroxyethidium production. UV absorption at 355 nM was used for detection of dihydroethidium.

Hydroxyethidium was expressed as nanomoles per mg protein. Protein concentration was measured by Bradford assay. For each sample extra dishes were incubated with PEG-SOD (50 U/ml) overnight and 50 U/ml of MnSOD (Sigma) was added 5 min prior to addition of dihydroethidium. PEG-SOD inhibited the DHE signal
by 50%-60%. For quantification of intracellular superoxide we compared the superoxide dismutase inhibitable signal for each sample.

**Quantitative real-time PCR**

Quantification of human Nox 1, Nox 2, Nox 4, Nox 5, human smooth muscle specific-alpha actin and 18S rRNA was performed by amplification of cDNA using the LightCycler real-time thermocycler as described previously.\(^5\) Optimized amplification conditions were 100 nmol/L primers for Nox1, Nox2, Nox4, Nox5 and SM α-actin (Table 1, on-line supplement), 4 mmol/L MgCl\(_2\), annealing at 68°C; for 18S, 50 nmol/L universal 18S rRNA primers, 4 mmol/L MgCl\(_2\) and annealing at 62°C; extension at 72°C. Copy numbers were calculated by the instrument software from standard curves generated from human Nox 1, Nox 2, Nox 4, Nox 5, SM α–actin and 18S templates.
TABLE 1

PCR primers used for amplification of Nox enzymes and SM α-actin

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence 5' to 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nox1 (+1)</td>
<td>TTCACCAATTCACCGATTGAATGGATGGTC</td>
</tr>
<tr>
<td>Nox1 (-2)</td>
<td>GACCTGTACAGATGCGAGGCTGTC</td>
</tr>
<tr>
<td>Nox2 (+1)</td>
<td>GTCACACCTTCCATCCATTCTCAAGTCAGT</td>
</tr>
<tr>
<td>Nox2 (-2)</td>
<td>CTGAGACTCATCCCAGCCAGTGAGGTAG</td>
</tr>
<tr>
<td>Nox4 (+1)</td>
<td>CTGAGAGCTGCCAGCGGAAAGGAAG</td>
</tr>
<tr>
<td>Nox4 (-2)</td>
<td>GTCACACCTTCATCCATTCTCAAGTCAGT</td>
</tr>
<tr>
<td>Nox5 (+1)</td>
<td>GTCGCTCTGCTGCTGCTGCTGCT</td>
</tr>
<tr>
<td>Nox5 (-2)</td>
<td>TGATGGTGAGGGGTGATCGGACTCATAG</td>
</tr>
<tr>
<td>SM α-actin (+1)</td>
<td>GCAGCCAGCAAGCAGTCAGGAGAT</td>
</tr>
<tr>
<td>SM α-actin (-2)</td>
<td>AGCCAGAGCATTGTGACACACACACAG</td>
</tr>
</tbody>
</table>

Figure 1S. A representative Western blot of human cardiac fibroblasts using the rabbit anti-Nox4 antibody. Note that in human cardiac fibroblasts, pre-treatment with blocking peptide blocks a 112-120 KDa band and not the 80 KDa band as reported in rat vascular smooth muscle cells, perhaps representing a heterodimer with p22phox. The same band is downregulated by pre-treatment with siNox4 (not shown).
Figure 2S. Representative phase contrast image of fluorescein-labeled siScr oligo RNAs. Cells were transfected overnight with fluorescein-labeled siScr oligo RNA as in methods (green). Note that >95% cells are transfected with green-fluorescein labeled oligoribonucleotides.

![Figure 2S](image)

Figure 3S. Transfection of human cardiac fibroblasts with Nox4 siRNA but not Nox5 siRNA reduces intracellular-released superoxide production in response to TGF-β1.

Cells were transfected with control scrambled siRNA (siScr), siNox4 or siNox5 RNA/oligofectamine complexes (as in methods) and stimulated with TGF-β1 (10 ng/ml) for 30 min. *O$_2^-$ was measured using HPLC resolution of hydroxyethidium signal in cell
membranes (see methods). Mean ± SEM of data from 3 experiments. * p<0.05 versus no TGF-β1; # p<0.001 versus siScr+TGF-β1, **, p=0.5 versus siScr+TGF-β1.

Figure 4S. Nox5 is not required for TGF-β1-induced SM-α actin in human cardiac fibroblasts

Cells were transfected as in Figure 3S, stimulated for 24 hours with TGF-β1 (10 ng/ml) harvested for RNA extraction and SM-α actin quantified by quantitative real-time PCR (bottom panel) or Nox5 (top panel). Note that Nox5 mRNA message was reduced by 80% by si Nox5. Mean ± SEM of data from 3 experiments. * p<0.001 versus no TGF-β1, # p<0.001 versus siScr, **, p=0.2 versus siScr+TGF-β1.
Cells were transfected with siScr, siNox4 or siNox5 as above, stimulated for 1 hour with TGF-β1 (10 ng/ml) and proteins were extracted. Shown is a representative Western blot for phospho-Smad 2/3 and total CDK4 (for loading). Mean ± SEM of data from 3 experiments *, p<0.001 versus siScr+TGF-β1, **, p=0.3 versus siScr+TGF-β1.

**Figure 5S.**
References


