NADPH Oxidase 4 Induces Cardiac Fibrosis and Hypertrophy Through Activating Akt/mTOR and NFκB Signaling Pathways

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Background—NADPH oxidase 4 (Nox4) has been implicated in cardiac remodeling, but its precise role in cardiac injury remains controversial. Furthermore, little is known about the downstream effector signaling pathways activated by Nox4-derived reactive oxygen species in the myocardium. We investigated the role of Nox4 and Nox4-associated signaling pathways in the development of cardiac remodeling.

Methods and Results—Cardiac-specific human Nox4 transgenic mice (c-hNox4Tg) were generated. Four groups of mice were studied: (1) control mice, littermates that are negative for hNox4 transgene but Cre positive; (2) c-hNox4 Tg mice; (3) angiotensin II (AngII)—infused control mice; and (4) c-hNox4Tg mice infused with AngII. The c-hNox4Tg mice exhibited an ≈10-fold increase in Nox4 protein expression and an 8-fold increase in the production of reactive oxygen species, and manifested cardiac interstitial fibrosis. AngII infusion to control mice increased cardiac Nox4 expression and induced fibrosis and hypertrophy. The Tg mice receiving AngII exhibited more advanced cardiac remodeling and robust elevation in Nox4 expression, indicating that AngII worsens cardiac injury, at least in part by enhancing Nox4 expression. Moreover, hNox4 transgene and AngII infusion induced the expression of cardiac fetal genes and activated the Akt-mTOR and NFκB signaling pathways. Treatment of AngII-infused c-hNox4Tg mice with GKT137831, a Nox4/NFκB inhibitor, abolished the increase in oxidative stress, suppressed the Akt-mTOR and NFκB signaling pathways, and attenuated cardiac remodeling.

Conclusions—Upregulation of Nox4 in the myocardium causes cardiac remodeling through activating Akt-mTOR and NFκB signaling pathways. Inhibition of Nox4 has therapeutic potential to treat cardiac remodeling. (Circulation. 2015;131:643-655. DOI: 10.1161/CIRCULATIONAHA.114.011079.)

Key Words: fibrosis • hypertrophy • mTORC1
■ NADPH oxidase ■ NFκB ■ reactive oxygen species

Left ventricle remodeling, characterized by interstitial fibrosis and cardiomyocyte hypertrophy, is a common response to acute and chronic cardiac injury observed in various heart diseases in humans and animal models, commencing with hypertension, valvular disease, coronary artery disease, and cardiomyopathy that frequently eventuates in heart failure.1,2 Over the past decade, strong evidence has implicated a common denominator, reactive oxygen species (ROS), in the development of cardiovascular pathology including cardiac remodeling.3 However, the failure of clinical trials with antioxidant compounds has underscored the need for better antioxidant therapies and a more thorough understanding of the source(s) and contribution of ROS in these diseases.4 Thus, it is important to identify the enzymatic source(s) of ROS so that specific targeting of the enzyme system can be developed to efficiently abolish the deleterious effects of ROS in pathological conditions.

Among the various potential sources of ROS, NADPH oxidases have emerged as major enzymes responsible for production of superoxide (O2·−) and hydrogen peroxide. Thus far, 7 homologs/isoforms of these enzymes have been found in humans and animals, and are referred to as the Nox family of NADPH oxidases: Nox1 to 5, Duox1, and Duox2. These enzymes share the capacity to transport electrons across the plasma membrane and to generate O2·− and other downstream ROS, such as hydrogen peroxide (H2O2). Nox enzymes are professional ROS-producing enzymes distinguishing themselves from other sources where

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ROS are produced as a byproduct. In addition to Nox2, Nox4 is a major source of ROS in the heart. Both enzymes share sequence homology, but have distinct characteristics. For example, although Nox2 requires cytosolic factors for its activation, Nox4 is constitutively active and is regulated primarily at the level of its expression. Nox2 localizes primarily in the plasma membrane, whereas Nox4 is found in intracellular membranes such as mitochondria, endoplasmic reticulum, and nuclear membranes. Emerging data suggest a contradictory role of Nox4 in cardiac remodeling with considerable debate on whether Nox4 is protective or deleterious during cardiac response to injury. For example, upregulation of Nox4 by hypertrophic stimuli promotes apoptosis and mitochondrial dysfunction in the myocardium. Also, increased oxidative stress in the nucleus caused by Nox4 mediates the oxidation of histone deacetylase 4 resulting in cardiac hypertrophy. In contrast to these results, Zhang et al reported that Nox4-null mice develop exaggerated contractile dysfunction, hypertrophy, and cardiac dilatation in response to chronic pressure overload, whereas Nox4 transgenic mice are protected. Importantly, signaling molecules targeted by Nox4 in the myocardium are incompletely defined.

The aim of this study was to elucidate the role of Nox4 in the development of cardiac remodeling and explore potential signal transduction pathways that may mediate the effect of Nox4. We generated a cardiac-specific human Nox4 transgenic mouse model (c-hNox4Tg) and investigated (1) the effect of overexpression of Nox4 on cardiac fibrosis and hypertrophy, (2) the genes that are activated by Nox4 in the heart that modulate cardiac remodeling, (3) the effector signaling pathways regulated by Nox4, and (4) the therapeutic potential of inhibiting Nox4 on cardiac remodeling. Because the renin angiotensin system is activated in cardiac disease, we also examined the role of Nox4 in mediating the effects of angiotensin II (AngII) in the myocardium.

Methods

Generation of Inducible Cardiac-Specific Human Nox4 Tg Mice

Lox-Stop-lox-human Nox4 Tg mice (LSL-hNox4Tg) on a mixed genetic background of C57BL/6 were created in collaboration with Taconic Farm, Inc. The transgene is driven by a β-actin promoter. Cardiomyocyte-specific human Nox4 Tg mice (c-hNox4Tg) were generated by crossing homozygote LSL-hNox4Tg mice to tamoxifen-inducible myocardial-specific Cre (αMHC-Cre) mice with a genetic background of C57BL/6 (see Figure IA in the online-only Data Supplement for details about the transgene construct). Experiments were conducted in 4 groups of 16-week-old male mice: (1) Control mice (CTL), the littersmates that are hNox4 negative but Cre positive; (2) c-hNox4Tg mice, littersmates that are positive for both hNox4 and Cre; (3) AngII-infused CTL mice (wild type+AngII); and (4) AngII-infused c-hNox4 Tg (Tg+AngII). Six mice were used in each group (n=6). All protocols concerning the use of animals were approved by the Institutional Animal Care and Use Committee at the University of Texas Health Science Center at San Antonio.

An expanded Materials and Methods section is in the online-only Data Supplement.

Tamoxifen Induction, AngII Infusion, and GKT137831 Treatment

All 4 groups of 16-week-old mice described above were treated with tamoxifen at a dose of 1 mg/mL in the drinking water for 7 days. At the end of tamoxifen treatment, either AngII infusion or vehicle control (water) was infused intraperitoneally via Alzet micro-osmotic pumps (model 1004) as previously described. AngII was infused at a concentration of 1 μg·kg⁻¹·min⁻¹ for 14 days. GKT137831 was mixed in standard chow diet, and treatment with a dose of 40 mg·kg⁻¹·d⁻¹ was started at the same time as tamoxifen treatment and lasted for a total of 3 weeks. Mice were anesthetized and tissues were harvested at the end of the treatment (Figure 1B in the online-only Data Supplement).

To evaluate the potential influence of tamoxifen or Cre activity, multiple CTL mice including C57B/6J, αMHC-Cre, and LSL-hNox4 were treated with vehicle or tamoxifen in the drinking water as for the experimental groups above. Cardiac characteristics were compared. No difference was observed for all the parameters tested without or with tamoxifen treatment (Table I in the online-only Data Supplement).

Statistical Analysis

Data are expressed as mean±standard error of the mean or mean±standard deviation. Normality analysis was performed before any test by using the Shapiro-Wilk and Anderson-Darling statistics. When normally distributed, 2-tailed, unpaired or paired Student’s t tests were performed for 2-group comparisons and 1-way analysis of variance was followed by the Tukey post hoc test for multigroup comparisons. When samples were not normally distributed, the Mann-Whitney U test for 2-group comparisons and the Kruskal-Wallis test for multigroup comparisons followed by the Dunn test were performed. Differences with P<0.05 were considered as statistically significant. The n numbers for each group are indicated in the figure legends.

Results

Characterization of Cardiac-Specific Human Nox4 Tg Mice

To characterize cardiac-specific hNox4 Tg mice, real-time polymerase chain reaction analysis was performed with the use of total RNA prepared from the left ventricles. The human Nox4 (hNox4) transgene mRNA is exclusively expressed in cardiac-specific human Nox4 Tg mice (c-hNox4 Tg), and levels of hNox4 mRNA were 8-fold higher than in endogenous mouse Nox4 mRNA (Figure 1A). The specificity of c-hNox4 expression was confirmed by using liver tissue; as shown in Figure 1B, no expression of hNox4 mRNA was detected. The transgene was not detected in other tissues of the Tg mice (data not shown). Western blot analysis shows that total expression of Nox4 protein was 10-fold higher in c-hNox4 Tg mice than in CTL mice (Figure 1C through 1E). These results indicate significant tissue-specific expression of the transgene. In addition, cardiac Nox activity is consistently increased in the Tg mice in comparison with CTL mice (Figure II in the online-only Data Supplement).

Next, we examined the phenotype of the Tg mice infused with AngII or vehicle. Both Tg and CTL littermates exhibited similar body weight and size. No obvious behavioral abnormalities were observed after 1 week of tamoxifen treatment and following 2 weeks of AngII or vehicle (saline) infusion. However, AngII-infused mice displayed a significant increase in heart weight. Heart weight to body weight ratio and mean blood pressure were markedly augmented in AngII-infused mice in comparison with the CTL mice. Heart weight and heart weight to body weight ratio were significantly higher in Tg+AngII mice than in CTL+AngII mice. The mean blood pressure of Tg+AngII mice was slightly elevated in comparison with CTL+AngII mice, but the difference did not reach statistical significance. There was no significant difference in
heart rate between all groups (Table II in the online-only Data Supplement).

**AngII Infusion Increases Cardiac Nox4 Expression in CTL and Tg Mice, But Does Not Alter the Expression of Other Nox Enzymes**

We first examined the effect of AngII on cardiac Nox4 expression in CTL and in Tg mice. Infusion of AngII into CTL mice significantly increased Nox4 protein levels in the left ventricle (LV; Figure 2A, 2C, and 2D). This increase was similar to that observed in Nox4 Tg mice. AngII infusion into Tg mice resulted in further increase in Nox4 expression in the LV, likely representing the additive effect of the mouse endogenous Nox4 (induced by AngII) and the human Nox4 introduced by the transgene. Note that 2 Nox4 antibodies recognizing different epitopes of the protein showed similar results (Figure 2A). The

![Figure 1.](image1.png)

**Figure 1.** Myocardial-specific expression of human Nox4 transgene (hNox4-Tg). Expression of mouse (mNox4) and human Nox4 mRNAs in LV (A) and livers (B) was analyzed by RT-PCR. Protein expression of Nox4 in LV tissues was analyzed by Western blotting (C), and quantitative analyses are shown in D and E (the fold changes). GAPDH was used as internal control. The data shown are from 3 of 6 mice in each group. *P<0.001 hNox4-Tg vs CTL. CTL indicates control; LV, left ventricle; Nox4, NADPH oxidase 4; and RT-PCR, real-time polymerase chain reaction.

![Figure 2.](image2.png)

**Figure 2.** Expression of Nox4 and other Nox isoforms in LV tissues. LV tissue of the 4 groups was harvested at the end of angiotensin II (AngII) or vehicle infusion and subjected to Western blotting. A, Representative immunoblot showing Nox4 expression analyzed by using Nox4 antibody from Santa Cruz (sc) or Epitomics (ep). B, Representative immunoblot showing protein expression of Nox1, Nox2, and Nox3. C through G, Quantitative densitometric analyses of the immunoblot data. *P<0.01 vs the rest groups. #P<0.01 vs the Tg or CTL-AngII. GAPDH was used as internal control. n=6. CTL, control; LV, left ventricle; ND, no significant difference among all groups; Nox4, NADPH oxidase 4; Tg, transgene; and WT, wild type.
first antibody is a rabbit polyclonal immunoglobulin G against an epitope corresponding to amino acids 201 to 300 mapping within an internal region of Nox4 of human Nox4 (sc-30141, Santa Cruz). The second antibody is a rabbit monoclonal immunoglobulin G against a peptide sequence within the NADPH-binding domain of Nox4 that is conserved between human and mouse Nox4 protein sequences (Epitomics, Burlingame, CA). Both antibodies recognize human and mouse Nox4. The mechanism by which AngII upregulates Nox4 levels remains to be determined. Nox4 is regulated via both transcriptional and translational mechanism, as well. It is well established that the constitutive activity of the Nox4 catalytic unit is potentiated by p22phox. We have found that AngII not only increases the levels of Nox4, but also the regulatory subunit p22phox. Analysis using quantitative polymerase chain reaction with the LV tissues from AngII-infused mice and CTL mice revealed that the level of p22phox mRNA is increased by AngII. Furthermore, AngII increased the level of p22phox protein associated with Nox4 as demonstrated by immunoprecipitation/immunoblot, which may represent a means whereby AngII contributes to Nox4 activation (Figure III in the online-only Data Supplement).

Next, we determined the effect of AngII and the transgene on the levels of other Nox isoforms. As shown in Figure 2B, LV tissues express abundant levels of Nox1. Overexpression of Nox4 and administration of AngII to CTL and Tg mice did not have any effect on Nox1 levels (Figure 2B and 2E). Similarly, LV tissues from Tg mice or from AngII-infused CTL or Tg mice showed no change in the levels of Nox2 and Nox3 (Figure 2B, 2F, and 2G). These results indicate that AngII treatment enhances the levels of Nox4 and amplifies the pathological effect of Nox4 on cardiac remodeling. Furthermore, overexpression of hNox4 does not alter the expression of other Nox isoforms in the LVs.

**Transgenic Nox4 and AngII Additively Promote ROS Production in LV**

The role of AngII-induced ROS in the pathogenesis of cardiac injury is established. Furthermore, our results above demonstrate a potentiating effect of AngII in the Nox4 Tg mice on the expression of Nox4 in the LV. The enhanced expression of Nox4 was associated with increased NADPH oxidase activity (Figure II in the online-only Data Supplement) and increase in ROS production. Dihydroethidium staining was used to detect ROS production in frozen sections of LV tissue. In this assay, the fluorescent intensity of dihydroethidium represents the production of superoxide. Dihydroethidium-positive staining was significantly increased in the LV of Tg mice and CTL+AngII mice. Moreover, a robust increase in dihydroethidium-positive staining was observed in the LV of Tg+AngII mice in comparison with CTL mice, indicating that AngII enhances the effect of the transgene to increase ROS generation (Figure IV in the online-only Data Supplement). ROS production was confirmed by using a fluorescent sensor-HyPer (Figure II in the online-only Data Supplement).

**Overexpression of Nox4 Leads to Cardiac Interstitial Fibrosis**

To investigate the role of Nox4 in cardiac remodeling, we examined fibrotic changes induced by Tg hNox4 and AngII treatment by using Masson trichrome staining of the LV. Interestingly, overexpression of hNox4 alone significantly induced interstitial fibrosis with a 3-fold increase in collagen deposition (Figure 3A, blue area) over CTL mice. AngII infusion tended to induce more fibrosis than the hNox4 transgene alone, but the difference between the both groups was not significant. The Tg+AngII mice exhibited more severe interstitial fibrosis, ≈8-fold higher than in CTL mice (Figure 3A and 3B). Cardiac fibrosis is induced because of changes in the expression of specific markers. Thus, the expression of several molecular markers of cardiac fibrosis was examined at the mRNA level. As shown in Figure 3C and 3D, the expression of the fibrotic cytokines, transforming growth factor-beta1 (TGF-β1) and connective tissue growth factor mRNAs was significantly increased in the LV of Tg mice similar to that in AngII-infused CTL mice. Notably, AngII enhanced the expression of TGF-β1 and connective tissue growth factor in the Tg mice in comparison with that in CTL or Tg mice alone. A similar pattern of increased expression of fibrotic markers, fibronectin, collagen I(a1), and collagen III (a1) was observed in AngII-infused CTL and Tg mice and in Tg mice alone (Figure 3E through 3H). No change in plasminogen activator inhibitor 1 expression was observed (Figure 3I). These results demonstrate that AngII, and its downstream target Nox4, as well, induces fibrotic responses in the LV.

The fact that fibrosis develops in the LV of c-hNox4Tg mice suggests that overexpression of Nox4 in the myocardium induces interstitial collagen deposition by producing and secreting fibronectin, TGF-β1, or cytokines/growth factors that may act in a paracrine manner to prime the differentiation/proliferation of interstitial mesenchymocytes/myofibroblasts. Analysis of terminal deoxynucleotidyl transferase dUTP nick end labeling fluorescent staining shows that the number of apoptotic cardiac myocytes was markedly increased in Tg mice (P<0.001 versus CTL), and that AngII alone induces myocardial apoptosis in CTL mice and potentiates the effect of the hNox4 transgene to enhance cell death (Figure V in the online-only Data Supplement). We further performed histological analysis to determine the relationship of cell death and the development of fibrosis. Results suggest that increased expression of Nox4 in the myocardium causes ROS injury, including myocyte death, thereby inducing paracrine and autocrine cytokines and growth factors leading to the differentiation of interstitial fibroblasts into myofibroblasts and deposition of extracellular matrix/collagens in the LV (Figures VI and VII in the online-only Data Supplement). Because cardiomyocyte apoptosis mainly induces reparative fibrosis, we quantified reparative fibrosis and perivascular fibrosis. The results showed that overexpression or AngII induced reparative fibrosis 2- to 3-fold more than perivascular fibrosis, suggesting an important effect of myocyte death on cardiac fibrosis (Figure VIII in the online-only Data Supplement).

To confirm the role of the Nox4 transgene in inducing cardiac fibrosis, we examined another line of the myocardial-specific hNox4 Tg mice. This line displayed an ≈3.5-fold increase in Nox4 protein expression and increased fibrosis, but not hypertrophy of the LV, in comparison with CTL mice.
Collectively, the data indicate that even a modest increase in Nox4 induces cardiac remodeling.

Nox4 Mediates AngII-Induced Cardiac Hypertrophy

Cardiac-specific hNox4 overexpression alone did not change LV weight or the ratio of LV to body weight (Table II in the online-only Data Supplement and Figure 4B). To determine the effect of Nox4 overexpression on cardiomyocyte hypertrophy, we performed morphometric analysis by using hematoxylin and eosin and wheat germ agglutinin staining of the LV cross-sections. There was no significant difference in the size of cardiomyocytes between Tg and CTL mice (Figure 4A and 4C; Figure VIIIB and VIIIC in the online-only Data Supplement), whereas AngII infusion increased myocyte size by ≈32% in CTL+AngII (P<0.05) and 58% in Tg+AngII mice (P<0.001) in comparison with CTL mice receiving vehicle. Myocyte size was significantly larger in AngII-infused Tg mice than in AngII-infused CTL mice (P<0.05). Furthermore, immunoblot analysis showed that protein expression of atrial natriuretic peptide (ANP), myocardin, β-myosin heavy chain (MyH7 or βMHC), and tropomyosin were increased by Nox4 transgene or AngII infusion (Figure 4D through 4H). AngII and the transgene had an additive effect on inducing the expression of all these hypertrophic markers (Figure 4D through 4H). These data suggest that Nox4 mediates AngII-induced cardiac hypertrophy by reactivating a set of fetal cardiac genes that are normally expressed in the heart only before birth.18,19
Nox4 Activates Akt/mTOR/NFκB Signaling

Signaling pathways that mediate the reactivation of cardiac fetal genes in postnatal hearts in response to fibrotic/hypertrophic stimuli remain under investigation. Furthermore, little is known about the role of Nox4 in the amplification of these potential downstream signaling pathways. We explored whether Nox4 activates Akt signaling pathways. LV tissue from Tg mice showed significant increase in activating phosphorylation of Akt similar to that found in AngII-infused CTL mice (Figure 5A through 5C). Note that AngII and hNox4 had additive effects on Akt phosphorylation at S473, whereas Akt phosphorylation at T308 seemed to be already maximal with AngII or hNox4 alone (Figure 5A and 5B). Next, we examined activation of mTOR because this enzyme is a downstream effector of activated Akt. Phosphorylation of S6 kinase and 4EBP-1 were used as indices of mTORC1 activation. Tg mice showed increased phosphorylation of S6K and 4EBP-1 that was enhanced by AngII infusion (Figure 5A, 5D through 5F). Note that increased phosphorylation of Akt at S473 indicates the activation of mTORC2. Similarly, phosphorylation of mTOR was enhanced in AngII-infused CTL and Tg+AngII mice. These results demonstrate the activation of mTOR signal transduction downstream of Akt kinase in the LV of Nox4-overexpressing mice.

Next, we determined the stimulation of another effector of Akt activation, the transcription factor NFκB. The p65 subunit
of NFκB undergoes activating phosphorylation at S536 downstream of Akt activation. Therefore, we examined phosphorylation of p65 in the Tg mice. As shown in Figure 5A and 5G, a marked increase in p65 phosphorylation was found in hNox4-overexpressing mice. AngII infusion into the Tg mice significantly increased this phosphorylation in comparison with CTL or Tg mice alone.

**Effect of Nox4 Inhibitor GKT137831 on Cardiac Remodeling**

Our results described above demonstrate that overexpression of hNox4 transgene in mouse heart leads to ROS production, cardiomyocyte apoptosis, and fibrosis, and it amplifies the same changes induced by AngII infusion. We subsequently verified that remodeling changes induced by hNox4 were indeed due to increased Nox4 activity, and, more importantly, we sought to determine whether Nox4 mediates AngII effects. To this end, we used a pharmacological inhibition strategy in our Nox4-overexpressing Tg mice. GKT137831 (Genkyotex, Geneva, Switzerland) is the first-in-class selective and potent Nox4 and Nox1 small-molecule inhibitor with good oral bioavailability that has been shown to be well tolerated in healthy human subjects.20,21 GKT137831 inhibits human and mouse NOX4 with similar potency, and should therefore inhibit

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**Figure 5.** Overexpression of hNox4 transgene and angiotensin II activate Akt-mTOR and NFκB signaling by enhancing the phosphorylation of components of the signaling pathways. A, Representative immunoblot showing phosphorylation of Akt, mTOR complex 1, S6K, 4EBP1, and NFκB in LV tissue. B through E, Quantitative densitometric analyses of the immunoblot data. GAPDH was used as the internal control. n=6. *P<0.01 vs the rest groups. #P<0.01 vs the Tg or CTL+AngII. No significant difference between Tg and CTL+AngII. AngII indicates angiotensin II; ANP, atrial natriuretic peptide; CTL, control; MyH7, β-myosin heavy chain; hNox4, human NADPH oxidase 4; PAI-1, plasminogen activator inhibitor 1; and Tg, transgene.

**Figure 6.** Inhibition of Nox4 attenuates fibrotic change induced by hNox4 overexpression and angiotensin II. A, Representative microphotographs of LV tissue with Masson trichrome staining. Collagens stain in blue, muscle in red, and nuclei in black. B, The quantification of fibrotic area (blue-stained collagen). Data are shown as percentage of section areas. C through E, Protein expression of markers of fibrosis fibronectin and TGF-β1 detected by the Western blotting (C) and the quantification analyses (D and E). n=6. *P<0.001. AngII indicates angiotensin II; ANP, atrial natriuretic peptide; CTL, control; GKT, GKT137831; LV, left ventricle; Nox4, NADPH oxidase 4; Tg, transgene; and TGF-β1, transforming growth factor-beta1.
hNox4 activity in Tg mice, and in endogenous mouse Nox4 in CTL mice infused with AngII, as well. Treatment of AngII-infused Nox4 Tg mice with GKT137831 decreased ROS production and Nox activity in the LV (Figure IIA through IIC in the online-only Data Supplement). GKT137831 significantly prevented Nox4 and AngII-induced cardiac fibrosis and expression of fibrotic markers fibronectin and TGF-β1 (Figure 6A through 6E). Additionally, the inhibition of Nox4 by GKT137831 attenuated myocardial hypertrophy (Figure 7A, 7C, and 7D). The increased expression of hypertrophic markers ANP, tropomyosin, and myocardin was also prevented (P<0.01), whereas MyH7 was not altered (Figure 7E through 7H). Myocardin is a member of the fetal cardiac genes and an essential component of a molecular switch for the expression of fetal contractile genes in cardiac muscle cells.22 This protein was reported to induce cardiac hypertrophy.19 Immunoblotting analysis showed that GKT137831 treatment decreased the expression of myocardin protein (Figure 7B). In addition, GKT137831 robustly decreased the phosphorylation of Akt at Thr308, and the activation of mTORC1 (phosphorylation of S6K and 4EBP1), and mTORC2 (phosphorylation of Akt at S473) in AngII-treated Nox4 Tg mice (Figure 8A through 8E). Similarly, activating phosphorylation of p65 subunit of NFκB transcription factor was decreased by GKT137831 (Figure 8A and 8F). Treatment with the GKT compound had no effect on cardiac ROS production and did not alter profibrotic markers and cardiac fetal genes in CTL mice (Figure IIB and IID in the online-only Data Supplement). These data confirm that increased Nox4 activity is responsible for the LV changes observed in Tg mice, and identify Nox4 as a key mediator of AngII effects in the heart. Additionally, these results suggest that Akt-mTOR and NFκB are downstream of Nox4-derived ROS in mediating cardiac injury and remodeling.

Inhibition of mTOR or NFκB Suppresses Expression of Markers of Cardiac Remodeling

To verify the implication of mTOR or NFκB signaling in our animal model, we performed tissue explant culture of LVs from hNox4 Tg mice infused with AngII and treated with 0.5 μmol/L rapamycin, an inhibitor of mTOR, or 0.5 μmol/L pyrrolidine dithiocarbamate, an inhibitor of NFκB, for 24 hours. Western blot analysis showed that these 2 inhibitors significantly suppress expression of fibronectin, TGF-β, ANP, and tropomyosin but not MyH7. Interestingly, myocardin was downregulated by rapamycin but not by pyrrolidine dithiocarbamate, indicating that mTOR signaling but not NFκB regulates myocardin expression enhanced by Nox4 and AngII in our animal model (Figure X in the online-only Data Supplement). To validate the explant culture approach, cell death and Nox4 expression in the tissue explants were measured after 24 hours of culture and compared with freshly isolated tissues. LV explant cultures are histologically similar to freshly isolated tissue, and the cell death rate in the cultured explants is slightly increased, but this increase does not achieve statistical significance in comparison with freshly isolated tissue (Figure XI in the online-only Data Supplement).

Discussion

This study provides strong evidence demonstrating that increased expression/activation of Nox4 in the heart, either through Tg overexpression or through the induction of
endogenous Nox4 by AngII, induces cardiac remodeling including fibrosis and cardiomyocyte apoptosis. These pathological changes were associated with increased ROS production, the expression of profibrotic cytokines, enhanced matrix accumulation, and the reactivation of the fetal gene program. Overexpression of hNox4 in the heart was not sufficient to cause LV and cardiomyocyte hypertrophy. However, cardiac hNox4 overexpression was associated with increased expression of fetal genes generally associated with cardiomyocyte hypertrophy, and with the activation of the Akt/mTOR and NFκB signal transduction pathways that have been shown to be implicated in cardiac remodeling. The reasons for this discrepancy are not fully understood, but it is important to note that hNox4 Tg mice were normotensive. In contrast, AngII exerted similar effects in the heart but also increased blood pressure and cardiac hypertrophy. It is therefore possible that pressure overload is necessary for the development of frank LV hypertrophy. Alternatively, expression of the transgene for longer than 14 days may be required for the development of LV hypertrophy. AngII infusion markedly potentiated the effects of the Nox4 transgene, hNox4 overexpression conversely enhanced cardiac hypertrophy induced by AngII (Figure 2), suggesting that AngII plays a permissive role for Nox4 effects on these endpoints. Inhibition of Nox4 activity by GKT137831 decreases ROS levels, prevents the activation of the Akt-mTOR and NFκB and reactivation of the fetal gene program, and markedly attenuates cardiac remodeling.

Nox4 is ubiquitously expressed in various tissues and cell types, including renal cells, cardiac/smooth muscle, adipocytes, and macrophages. Distinct from other members of the Nox family, Nox4 is constitutively active, and increased expression invariably results in enhanced activity and increased ROS production. Nox4 is upregulated by vasoactive agonists such as AngII, endothelin-1, and phenylephrine and in response to cardiac injury and pathological conditions that result in cardiac remodeling. Most recently, whole-body and cardiac-specific mouse Nox4 Tg and knockout (KO) mice were generated and used to investigate the role of Nox4 in cardiovascular diseases. Collectively, the data derived from such gene-manipulated mouse models raised a growing debate on whether Nox4 exerts adverse or protective actions in cardiovascular disease. To add to the complexity, discrepant results were reported in seemingly very similar experimental settings. For instance, Tg mice overexpressing mouse Nox4 in a cardiac-specific manner demonstrated that Nox4 is either protective or deleterious in models of the LV pressure overload. Interestingly, Matsushima et al recently showed that Nox4 global KO and Nox4 tissue-specific KO animals give rise to 2 opposite phenotypes when assessed in the same model. Cardiac-specific knockout of Nox4 protects against pressure overload–induced cardiac injury, whereas the global Nox4 KO showed no protection when subjected to pressure overload. This suggests that the group of proteins or signaling intermediates affected by Nox4 may have cell-specific effects. Also, some of these pathways may protect cell function basally or in pathological conditions. Thus, the global deletion of Nox4 may be deleterious independent of the disease induced. We generated tamoxifen-inducible myocardium-specific hNox4 Tg mice and examined the cardiac phenotype of these mice. Because the systemic and local renin angiotensin system is activated in cardiac disease, we also evaluated the role of Nox4 in mediating the effects of AngII in the myocardium. We also deployed a pharmacological inhibition strategy by using the Nox4 and Nox1 inhibitor GKT137831 to establish the direct effect of Nox4 on cardiac remodeling in Tg mice overexpressing human Nox4 and infused with AngII. Nox4 was expressed, albeit at a relatively low level, in CTL mice, suggesting that Nox4 may be required for normal cellular physiological processes (Figures 1A and 2A). Interestingly, a 10-fold increase in Nox4 expression and interstitial fibrosis was observed in the LV of hNox4 Tg mice 14 days after induction of the transgene by tamoxifen. Although markers of

Figure 8. Inhibition of Nox4 suppresses activation of Akt-mTOR and NFκB signaling. A, Representative immunoblot showing phosphorylation of Akt, mTOR complex 1 activity readout, and p65 in LV tissue. B through F, Quantitative densitometric analyses of the immunoblot data. *P<0.001 between the 2 groups. **P<0.05 between the 2 groups. n=6. AngII indicates angiotensin II; CTL, control; GKT, GKT137831; Nox4, NADPH oxidase 4; and Tg, transgene.
Cardiomyocyte hypertrophy were also induced, no significant hypertrophy was present. Whether the prolonged overexpression of Nox4 results in hypertrophy remains to be determined. Studies by Ago et al. showed that overexpression of mouse Nox4 in Tg mice does not induce cardiac hypertrophy in young mice, but does cause cardiac myocyte hypertrophy as the Tg mice advance to middle age, suggesting that long-term overexpression of Nox4 may be required for the development of cardiomyocyte hypertrophy.

CTL mice infused with AngII for 2 weeks exhibited levels of Nox4 expression in the LV similar to the hNox4 Tg mice, and displayed cardiac fibrosis and hypertrophy, as well, suggesting that AngII-induced cardiac hypertrophy involves additional mechanisms besides Nox4 expression (Figure 2A, 2C, and 2D). For instance, the presence of high blood pressure in these mice may have contributed in the development of hypertrophy. The hNox4 Tg mice infused with AngII exhibited the highest expression of Nox4 and severe cardiac remodeling including both fibrosis and hypertrophy. It should be noted that the infusion of AngII in hNox4 Tg mice did not alter the expression of other Nox enzymes (Figure 2B, 2E through 2I). However, in some conditions, the manipulation of Nox4 gene may result in alteration of other Nox enzymes. For example, cardiac-specific Nox4 KO mice have reduced cardiomyocyte dysfunction in a mouse model of transverse aortic constriction, whereas systemic Nox4 KO mice exhibited more cardiac fibrosis and diastolic dysfunction than wild-type mice. Systemic deletion of Nox4 may change the status of other Nox enzymes, not only in myocytes, but also in other cellular cells. In addition, the global knockout of Nox4 may induce as-yet-unknown compensatory mechanisms during embryogenesis and development, resulting in changes in the expression of other Nox isoforms. Also, paracrine and endocrine metabolism may be regulated by whole-body Nox4 deletion to affect the physiology/pathology of the disease process. Therefore, results from tissue-specific KO and systemic KO mice are not always comparable.

Cardiac myocytes proliferate rapidly during embryogenesis but lose their proliferative capacity soon after birth.33,34 However, adult cardiac myocytes retain the ability to respond to mechanical, hemodynamic, hormonal, and pathological stimuli and contribute to cardiac remodeling, including hypertrophic growth and interstitial expansion.25 Cardiac remodeling is accompanied by the activation of a set of fetal cardiac genes that are normally expressed in the embryonic heart.18,26 The reactivation of cardiac fetal genes in postnatal cardiomyocytes in response to stress signals is a useful marker for cardiac fibrosis and hypertrophy. The administration of GKT137831 to hNox4 Tg mice infused with AngII blocked the expression of cardiac fetal genes, providing direct evidence that Nox4 mediates the enhanced expression of the fetal gene program (Figures 3, 4, 6 and 7 and Figure II in the online-only Data Supplement). Myocardin is a member of the fetal gene program and also represents a molecular switch that acts as a coactivator of the transcription factor SRF (serum response factor) which regulates contractile genes in cardiac muscle cells. This protein binds to the serum response element in the promoter region of target genes. It controls the activity of many immediate-early genes, thereby regulating cell cycle, apoptosis, cell growth, and cell differentiation. Myocardin transactivates the CArG box of the ANP gene and induces the expression of ANP, one of the most sensitive markers of hypertrophy.29,30 Furthermore, myocardin has been implicated in the differentiation of myofibroblasts.31 Mouse myocardin gene encodes 2 alternatively spliced protein isoforms of 935 and 856 amino acids.32,33 The inhibition of Nox4 by GKT137831 not only downregulated myocardin protein expression, but also altered its molecular weight (Figure 7B and 7G), likely because of the alternatively spliced transcript variants of myocardin.

In comparison with the LVs from wild-type mice, the LVs from hNox4 Tg mice and mice infused with AngII exhibited an increase in the phosphorylation of Akt at S473 and T308, and the activation of mTOR and S6K pathway, as well, as evident by enhanced phosphorylation of 4EBP1 and S6 ribosomal protein. Both kinases were further activated in the LV of Nox4 Tg mice that received AngII infusion. Treatment with GKT137831 blocked the activation of the Akt and mTOR/S6K pathways (Figures 5A through 5C and 8A through 8C). Akt has been shown to be directly involved in physiological adaptive cardiac hypertrophy in response to insulin-like growth factor I and exercise.34 Moreover, Tg mice that overexpress Akt display spontaneous cardiac hypertrophy and prolonged Akt activation in the myocardium results in dilatation and cardiac dysfunction.35 mTOR is a highly conserved serine/threonine kinase involved in vital cellular processes, including growth, gene transcription, and protein synthesis.36 Among the best-studied properties of mTOR kinase is its involvement in protein translation and cell growth through phosphorylation of S6K and 4E-BP1. Although cardiac-specific mTOR-deficient mice develop a fatal, dilated cardiomyopathy,37 the partial loss of mTOR activity37 and inhibition of mTOR by rapamycin38 significantly prevents cardiac hypertrophy. The administration of everolimus, a rapamycin analogue, decreases ventricle hypertrophy in patients who have undergone kidney transplants.39 The activation of Akt-mTOR signaling likely contributes to cardiac fibrosis and hypertrophy by enhancing protein synthesis. We provide in vivo evidence of Nox4-derived ROS generation in mTOR activation in the course of cardiac remodeling.

Overexpression of Nox4 also results in the activation of NFkB (Figures 5, 8F, and 8G). NFkB exists as an inactive dimeric complex, comprising p65 and p50 protein subunits bound to the inhibitor protein IxB. Activation of NFkB requires the phosphorylation-dependent degradation of IxB, which is mediated by the IxB kinase complex (IKK), IKKα, IKKβ, and IKKγ (NFkB essential modulator) subunits. The loss of IxB exposes the nuclear localization motif on p65 NFkB subunit permitting its phosphorylation and nuclear targeting. NFkB activation is involved in the hypertrophic response of cultured cardiomyocytes and is required for the development of cardiac hypertrophy in vivo.42,43 Moreover, there is evidence that NFkB and mTOR interact in a positive feedback loop.44,45 Our in vitro results further verified that the inhibition of mTOR or NFkB signaling significantly suppresses expression of cardiac remodeling markers enhanced by overexpressed Nox4 in our mouse models (Figure X in the online-only Data Supplement). It is likely that the Akt-mTOR-NFkB
axis might work as a central signaling pathway and bidirectionally mediates cardiac remodeling. However, little is known about the role of Nox4 or Nox4-derived ROS in the activation of Akt/mTOR and NFκB signaling pathways. Although it is known that Nox4 is required for Akt activation in renal cells, the data in the heart are very sparse. To the best of our knowledge, the present study is the first to demonstrate that Nox4 directly targets and activates mTOR, particularly the mTORC1 complex, signaling. Interestingly, our observation that Nox4 overexpression increases phosphorylation of Akt on S473, an activating site targeted by mTORC2, implies that the ROS generated by Nox4 may also modulate the mTORC2 complex. Although numerous studies have described NFκB as an upstream regulator of Nox4 expression in vascular cells, few reports positioning Nox4 upstream of NFκB are available and are all related to toll-like receptor-4 signaling. Together, our findings suggest that mTOR and NFκB pathways may integrate Nox4-dependent ROS generation to the initiation of cardiac remodeling.

Data in mice treated with GKT137831 confirm the role of Nox4 in the development of cardiac remodeling. The fact that Nox4/Nox1 inhibitor GKT137831 attenuated cardiac damage in cardiac-specific Nox4-overexpressing mice treated with AngII strongly supports the idea that Nox4 is responsible for the pathologies observed. Although Nox2, Nox1, and Nox3 are also detected in the LV, with Nox1 apparently being expressed at a higher level than Nox2 and Nox3 in LV of our mouse models, Nox4 overexpression and AngII treatment did not change their basal expression. However, because the Nox2 or Nox1 activity is regulated by the recruitment of cytosolic factors, not via the control of its expression levels like Nox4, the role of Nox2 or Nox1 cannot be excluded. Thus, the protective effect of GKT137831 treatment most likely results largely from the inhibition of Nox4 activity, but does not definitively exclude a partial contribution of Nox1. In addition to its primary localization in the plasma membrane, Nox4 localizes to other subcellular compartments. In hNox4 Tg mice compared with CTL mice, we find that Nox4 is highly expressed in endoplasmic reticulum, mitochondria, and nuclei (Figure XII in the online-only Data Supplement). These results may suggest the importance of subcellular translocation and localization in the regulation of Nox4 action in myocardium.

The dose of AngII for infusion used in this study is based on our previous studies. Smaller doses of AngII infusion were tested, but they were not sufficient to induce detectable cardiac morphological changes within the short-term (2-week) treatment of these mice, maybe suggesting a difference of sensitivity to AngII either among different mouse species or between human subjects and mice. The renin-angiotensin system is upregulated or activated in diabetes mellitus and heart failure. Actually, AngII is synthesized locally in the heart, and the concentrations of AngII in the cardiac myocytes that are available to bind to AngII receptors far exceed blood circulating concentrations. Even in studies of cultured cardiomyocytes, concentrations of AngII in the cells are 2 orders of magnitude higher than the concentrations in the medium. Furthermore, various AngII levels in the myocardium may be induced in many different forms of cardiac injury. Therefore, the concentrations of AngII that may be achieved locally in pathological states may far exceed its circulating levels, and this may explain why we did not find detectable morphological pathological changes in the LV of our mice when using lower doses of AngII infusion for 2 weeks.

Results from our second line of cardiac-specific Nox4 Tg mice showed that the Tg mice displayed a 3.5-fold Nox4 protein expression and obvious fibrosis in the LV versus CTL mice, but no myocardial hypertrophy, indicating a similarity in cardiac pathology between the 2 lines of Nox4 Tg mice we generated by using the same protocol, despite the differences in Nox4 expression level (Figure IX in the online-only Data Supplement). Our data have shown that Nox4 expression in the LV of Tg mice predominantly was located in the endoplasmic reticulum, mitochondria, and nuclei (Figure XII in the online-only Data Supplement), but we cannot exclude the possibility of expression of the transgene, if any, in unphysiological subcellular locations.

In summary, specific local up-regulation of Nox4 in the heart enhances oxidative stress and induces cardiac remodeling, associated with activation of the Akt-mTOR and NFκB signaling pathways, and the activation of cardiac fetal contractile genes, as well. Inhibition of Nox4 oxidase blocks the activation of these signaling pathways and attenuates cardiac remodeling, suggesting that NADPH oxidase inhibitors have the therapeutic potential to prevent/treat cardiac remodeling and potentially delay the development and reduce the severity of established heart failure (Figure XIII in the online-only Data Supplement).

Acknowledgments
We thank Drs Goutam Ghosh-Choudhury and Yves Gorin, at the University of Texas Health Science Center at San Antonio, and Philippe Wiesel at GenkyoTex for their valuable comments on the manuscript. We thank Dr Yidong Chen, Professor of Epidemiology and Biostatistics at the University of Texas Health Science Center at San Antonio for a review on the statistical data and processes.

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Disclosures
None.

References


33. Dan HC, Cooper MJ, Cogswell PC, Duncan JA, Ting JP, Baldwin AS. Akt dependent regulation of NF-κB by mTOR and Raptor

**CLINICAL PERSPECTIVE**

Left ventricle remodeling is a common response to acute and chronic cardiac injury that eventuates in heart failure. Activation of the renin-angiotensin system and enhanced generation of reactive oxygen species by NADPH oxidases have been implicated in cardiac remodeling. The role of NADPH oxidase 4 (Nox4) in cardiac remodeling has been controversial. Moreover, little is known about the downstream effector signaling pathways activated by Nox4-derived reactive oxygen species. In this study, we demonstrate that myocardial-specific overexpression of human Nox4 increases reactive oxygen species production and induces cardiac remodeling through activation of the Akt-mTOR and NFκB pathways. The effects of the Nox4 transgene were potentiated by angiotensin II. Treatment of angiotensin II-infused myocardial Nox4 transgenic mice with Nox4/Nox1 inhibitor GKT137831 abolishes the increase in oxidative stress, suppresses Akt-mTOR and NFκB pathways, and markedly attenuates cardiac remodeling. In addition, the mTOR inhibitor, rapamycin, or an inhibitor of NFκB, significantly inhibits the expression of cardiac remodeling markers in tissue explant cultures of left ventricles of Nox4 transgenic mice infused with angiotensin II. Because other mechanisms besides angiotensin II may increase Nox4 and reactive oxygen species production, and because Nox4 is upstream of the mTOR/NFκB signaling pathway, inhibition of Nox4 may afford superior protection to blockade of angiotensin II. GKT137831 has passed phase 1 clinical trials and is currently being evaluated in a phase 2 clinical trial in patients with type 2 diabetes mellitus and albuminuria (clinical trials.gov unique identifier NCT02020242). Clinical trials of Nox4 inhibitors to prevent cardiac remodeling and the progression of heart failure are warranted.
NADPH Oxidase 4 Induces Cardiac Fibrosis and Hypertrophy Through Activating Akt/mTOR and NF κB Signaling Pathways
Qingwei David Zhao, Suryavathi Viswanadhapalli, Paul Williams, Qian Shi, Chunyan Tan, Xiaolan Yi, Basant Bhandari and Hanna E. Abboud

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Online Supplemental Data

Supplemental Methods

Rabbit monoclonal antibody against Nox4

Nox4 antibody was raised against a peptide sequence within the NADPH binding domain of Nox4 that is unique to human and mouse NADPH oxidase sequences (the sequence is conserved between human and mouse Nox4 proteins). The antibodies were generated in collaboration with and are now available through Epitomics (Burlingame, Calif). Hybridoma clones were generated from B cells from one of the immunized rabbits and screened. Protein-G Sepharose-purified monoclonal antibodies were used to perform Western blotting, immunoprecipitation and immunohistochemistry experiments. The other antibodies used in this study are listed in Supplemental Table S3.

Generation and identification of cardiac-specific human Nox4 transgenic (c-hNox4Tg) mice

The Cre-lox system was used to generate the c-hNox4Tg mice. Lox-Stop-lox-human Nox4 transgenic mice (LSL-hNox4Tg) on a mixed genetic background of 129 and C57BL/6 were created in collaboration with Taconic Farm, Inc. In these transgenic mice, the transgene construct contains loxP-flanked DNA STOP sequence, driven by a beta-actin promoter, preventing expression of the downstream hNox4 (human Nox4) gene. When the LSL-hNox4Tg mice are crossed with alpha-MHC-MerCreMer transgenic mice (Jackson Laboratory, Stock #005650), the tamoxifen-inducible Cre-mediated recombination is expected to result in deletion of the floxed STOP sequence leading to expression of hNox4 specifically in myocardium of the offspring (Supplemental Figure S1A).

Tail tissue of the offspring were harvested and the polymerase chain reaction (PCR) assays were performed to identify c-hNox4 Tg pups using the following primers: αMHC-Cre forward 5'-AGG TGG ACC TGA TCA TGG AG-3', αMHC-Cre reverse 5'- ATA CCG GAG ATC ATG CAA GC-3'; LSL forward CGG ATC CTC GGG GAC ACC AAA TAT G, LSL reverse AAG GCA TTC CAC TGC TCC C; hNox4 forward 5'-ACC TTT GTG CCT GTA CTG TGC C-3', hNox4 reverse 5'-TGT TGC TTT GGT TTC AGT TGG AC-3'; Mouse Nox4 forward 5'-TCC AAG CTC ATT TCC CAC AGA CC-3', mouse Nox4 reverse 5'-GTG GAC ACC AAA TGT TG C T-3'. Reverse transcription polymerase chain reaction (RT-PCR) assays were carried out to identify the expression of transgenic hNox4 in myocardium and liver (Figure 1A and 1B).

Blood pressure measurement

Blood pressure of mice was measured using The CODA 8-Channel High Throughput Non-Invasive Blood Pressure system (Kent Scientific Co.). Measurements were made on conscious mice. Systolic and diastolic blood pressure, heart rate, mean blood pressure, tail blood flow, and tail blood volume were recorded.

Immunoblot analysis

Homogenates of left ventricle tissue were prepared in RIPA lysis buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM Na3VO4, 1 mM PMSF, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 1% NP-40. Protein concentrations of samples were determined by the Bio-Rad protein assay. Samples were subjected to SDS-PAGE. Proteins were transferred onto polyvinylidene fluoride microporous membranes (Bio-Rad). The membranes were blocked with 5% low-fat milk in Tris-buffered saline and then probed with primary antibodies (1:1000 dilution). The primary antibodies used in this study included antibodies against Nox4 (monoclonal
antibody from Epitomics or polyclonal antibody from Santa Cruz), Mox1 (Abcam), alpha-SM-actin (Abcam), and the remaining antibodies were purchased from Cell Signaling Tech. The appropriate HRP-conjugated secondary antibodies were added (1:3000) and bands were visualized by enhanced chemiluminescence. Densitometric analysis was performed using NIH Image/ImageJ software.3

Quantitative real-time reverse transcription polymerase chain assay (qPCR)

The assay of qPCR was performed to quantify expression of the following genes: mouse Nox isoforms 1 to 4 and transgenic hNox4, transforming growth factor beta (TGF-β), collagen-1 alpha 1, collagen-3 alpha 1, connective tissue growth factor (CTGF), plasminogen activator inhibitor-1 (PAI-1). GAPDH was used as the internal control. Sequences of primers used in this study were summarized in Supplemental Table S4. SYBR green I dye method was used for qPCR assay. In brief, after preparing total RNA, first-strand cDNA was synthesized by reverse transcription. Real-time PCR was conducted on a 7500 Fast Real-Time PCR System (Applied Biosystems) using the Power SYBR® Green Master Mix (Applied Biosystems). Comparative Ct (ΔΔCt) method was utilized to rapidly and accurately calculate relative gene expression across all the tests.

Dihydroethidium staining

Dihydroethidium (DHE), relatively specific for superoxide anion measurement, is an oxidative fluorescent dye that undergoes a two-electron oxidation to form the DNA-binding fluorophore ethidium bromide. The DHE (Calbiochem, Darmstadt, Germany) staining for superoxide was carried out as previously described.3, 4 Briefly, unfixed left ventricular frozen samples were cut into 10-μm-thick sections and placed on glass slides. DHE (10 μmol/l) was applied to each tissue section, and the slides were incubated in a light-protected humidified chamber at 37°C for 15 min. Fluorescent images of ethidium-stained tissue were obtained with a laser-scanning confocal microscope (Zeiss 510 NLO). Ethidium bromide was excited at 488 nm, and fluorescence was detected at 560 nm long-pass filter. Mean fluorescence intensity of the digitized image was measured with ImageJ software [version 1.35, National Institutes of Health (NIH), Bethesda, MD] for quantification. Generation of superoxide was demonstrated by red fluorescent labeling. Nonstained left ventricular sections were used as background control. The average of five sections stained with DHE was taken as the value for each animal. Ethidium fluorescence (excitation at 490 nm, emission at 610 nm) was examined by fluorescent microscopy.

Hydrogen peroxide assay

Intracellular hydrogen peroxide was evaluated using a genetically encoded fluorescent sensor-HyPer (Cat # FP941, Evrogen). In brief, freshly frozen sections of the left ventricles were transfected with pHyPer-cyto vector using OptiFect transfection reagent. 24 hrs post transfection incubation, the green fluorescence (530/30 nm bandpass filter) in transfected sections after excitation at 488 and 405 nm was measured using a BD FACSia II SORP flow cytometer. The production of intracellular H2O2 was measured by calculating the ratio of green fluorescence excited at 488 and 405 nm.

Detection of apoptosis

To detect apoptosis of cardiomyocytes of LV, TUNEL staining was conducted as described before.5 TUNEL staining using the TUNEL Apoptosis Detection Kit (Upstate) was performed according to the manufacturer's instructions. The sections were examined using light microscopy. Sections incubated with PBS, instead of TDT enzyme solution, served as the negative controls. The number of TUNEL-positive cells was counted in five randomly selected fields under 400x magnification for each animal. Four animals were studied per group.
**Masson's trichrome staining**

Masson's trichrome is a three-color staining assay used to recognize collagen composition of tissues in histology. Masson's trichrome staining was performed as previously described.\(^6\) Frozen sections embedded in OCT compound were used in this study. Cryosections were first fixed in 4% paraformaldehyde for 1 hour and then re-fixed in Bouin's solution overnight at room temperature. The collagen fibers were stained blue and the nuclei stained black and cytosol stained red.

**Heamatoxylin-Eosin staining**

Heamatoxylin-Eosin (HE) staining method, involves application of hemalum, which is a complex formed from aluminum ions and oxidized haematoxylin. This colors nuclei of cells (and a few other objects, such as keratohyalin granules) blue. The nuclear staining was followed by counterstaining with an aqueous or alcoholic solution of eosin, which colors other, eosinophilic structures in various shades of red, pink and orange.

**Quantitative morphometric analysis**

At sacrifice, the mice were perfused with 1XPBS containing potassium chloride to arrest the hearts in diastole. Morphometric analyses were carried out to relatively quantify myocyte size, interstitial fibrotic area, number of apoptotic cells, and intensity of DHE staining in cross-sections of LV tissues chemically stained by the protocols described above. Each LV was cross-sectionally cut into three parts, apex, the middle portion and the bottom. The bottom and apex parts were saved at – 80°C for analyses of protein and mRNA expression. The middle portions were embed in OCT compound and saved at – 80°C. Three serial cross-cryosections of the middle portion at a 300-µm-interval were used for the morphological analyses. Images of 16 fields (views with 400x magnification) of each cross-section along each of 8 radial lines (2 fields on each radial line) were visualized, captured under a microscope Zeiss Fluorescent Axio Scope-A1 with an AxioCam MRc5 camera. The 48 images of one LV were used to manually count apoptotic cells (20 cells in each field were counted), or quantify cell size and fibrotic area by using Image-Pro software. Using Image Pro and after calibration with a stage micrometer, the areas of interests can be circled by tracing the edge of the interested target and the values of quantified areas were exported to a spreadsheet in square micrometers. This is repeated for each image of LV tissue section. One mean value of cell sizes or fibrotic areas averaged from 16 images was averaged for one section. All the measurements (total 48) of 3 serial sections of a LV were averaged into a mean value representing the result for one mouse (Supplemental Figure S14).

**NADPH oxidase assay**

NADPH oxidase activity was measured by the lucigenin-enhanced chemiluminescence method. Left ventricles of the mouse hearts (a small piece) were homogenized in 400 µl of ice-cold lysis buffer (20 mM KHPO4, pH 7.0) containing a mixture of protease inhibitors using ultrasonic cell disruptor tissue homogenates were allowed to stand for 10 min. on ice. Total protein content was measured using the Bio-Rad protein assay reagent. To start the assay, 150 µg of total protein from each homogenate were added to assay buffer (50 mM phosphate buffer, pH 7.0, containing 1 mM EGTA and 150 mM sucrose), 5 µM lucigenin, and 100 µM NADPH in 500ul of reaction. Photon emission in terms of relative light units was measured every 30 sec. for 5 min. using a luminometer. Superoxide production was expressed as relative chemiluminescence (light) units/min/mg of protein.

**Tissue explant culture of left ventricle and treatments**
Under sterile conditions, Tg mice infused with AngII (Tg+AngII) were decapitated and hearts were excised. The hearts were rinsed 3 times in cold Hank’s balanced salt solution containing 2× strength antibiotics (200 U/mL of penicillin, 200 μg/mL of streptomycin). Atria and right ventricle walls were removed. Left ventricles including septum were cut into small pieces of tissue (3 to 5 mm). Explants were placed onto the bottom of 100-mm culture dishes with small amount of culture medium. Explant cultures were left untreated for 4 hours to allow for attachment to bottom of dishes in an incubator at 37°C and 5% CO2. Then complete M199 medium (Gibco) was added containing (mM) NaCl 117, KCl 5.3, CaCl₂ 1.8, MgSO₄ 0.8, NaHCO₃ 26.2, and Na₂HPO₄ 1.0, supplemented with 5% fetal bovine serum, 1% penicillin/streptomycin, 5 mg/ml insulin, 5 mg/ml transferrin, 5 ng/ml selenium, and 2 mM L-glutamine. Explant cultures were separated into three groups for treatments: Tg+AngII mice treated with vehicle (saline), 0.5 μM rapamycin or 0.5 μM NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC). The treatment lasted for 24 hours. Explants were harvested and subjected to western blot analyses.

References
Supplemental Table S1: Characteristics of mice treated with vehicle or tamoxifen treatment

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<tr>
<td></td>
<td>C57BL/6J Cre LSL-hNox4</td>
<td>C57BL/6J Cre LSL-hNox4</td>
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<td>19</td>
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<tr>
<td>Body weight (g)</td>
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<tr>
<td>Mean BP (mmHg)</td>
<td>103 ± 8</td>
<td>104 ± 9</td>
<td>106 ± 10</td>
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<tr>
<td>Heart rate</td>
<td>508 ± 23</td>
<td>490 ± 28</td>
<td>510 ± 35</td>
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<tr>
<td>LV weight (mg)</td>
<td>109 ± 6</td>
<td>105 ± 5</td>
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<td>ROS (fold in intensity)</td>
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<td>1.1 ± 0.2</td>
<td>0.9 ± 0.5</td>
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<tr>
<td>Fibrosis (% of section)</td>
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<td>0.5 ± 0.3</td>
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<tr>
<td>Myocyte size (µm²)</td>
<td>310 ± 11</td>
<td>296 ± 16</td>
<td>303 ± 10</td>
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<tr>
<td>Dead cells (/section)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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</tbody>
</table>

Note: Data shown in the table were recorded after 7 days of vehicle or tamoxifen treatment followed by 14 days of vehicle. ROS production was measured with fluorescent sensor HyPer. Fibrosis is shown as area percentage in one cross-section. ND indicates not detectable.
### Supplemental Table S2: The systemic cardiac indices of mice

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<td><strong>Body weight</strong></td>
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<td><strong>Heart weight</strong></td>
<td>0.119 ± 0.009</td>
<td>0.121 ± 0.011</td>
<td>0.147 ± 0.010*</td>
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<tr>
<td><strong>Heart/body (%)</strong></td>
<td>0.417 ± 0.034</td>
<td>0.432 ± 0.032</td>
<td>0.511 ± 0.020*</td>
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<tr>
<td><strong>Mean blood pressure</strong></td>
<td>105 ± 10</td>
<td>106 ± 8</td>
<td>130 ± 15*</td>
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<tr>
<td><strong>Heart rate</strong></td>
<td>400 ± 51</td>
<td>418 ± 73</td>
<td>397 ± 60</td>
<td>436 ± 82</td>
</tr>
</tbody>
</table>

Note: Characteristics are measured/recorded at the end of AngII or vehicle infusion. CTL indicates control mice; Tg, cardiac specific human Nox4 transgenic mice; CTL+AngII and Tg+AngII, angiotensin II-infused mice. *P < 0.05 vs WT or Tg. # P < 0.01 vs WT or Tg. n = 6.
**Supplemental Table S3**: List of antibodies used in the immunoblot assays.

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### Supplemental Table S4: List and sequence of primers used in real-time RT-PCR

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<th>Gene</th>
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Supplemental Figure Legends

Supplemental Figure S1: A, The Cre-Lox Technology for targeted homologous recombination of transgene hNox4. B, Timeframe of animal treatments. Mice at age of 16 weeks were treated with tamoxifen at a dose of 1 mg/ml in the drinking water for 7 days. Tamoxifen treatment was stopped and vehicle or Ang II were infused by i.p. Alzet micro-osmotic pumps (Model 1004) filled with angiotensin II at a concentration of 1 µg/kg/min for 14 days. For GKT treated group, GKT137831 treatment started at the same time as tamoxifen in the drinking water and lasted 3 weeks. Mice were sacrificed and tissues were harvested at the end of AngII infusion. C. RT-PCR blotting confirming specificity of primers used for detecting human (hNox4) or mouse Nox4 RNA (mNox4). There is no cross-reaction between the species. Lane M: a 100-bp ladder. Lane 1: Mouse heart RNA with mouse Nox4 primers; Lane 2: Mouse heart RNA with human Nox4 primers; Lane 3: Human cardiomyocytes RNA with mouse Nox4 primers; Lane 4: Human cardiomyocytes RNA with human Nox4 primers; Lane 5: mouse heart RNA with actin primers; Lane 6: Human cardiomyocytes RNA with actin primers.

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Supplemental Figure S3. P22phox participates in angiotensin II (AngII) upregulation of Nox4 activity in the LV. A. The qPCR analysis revealed that AngII increases p22phox mRNA expression in the LV. B. Immuno-precipitation followed by immunoblotting revealed that p22phox-Nox4 complex is also increased in the left ventricles of angiotensinII-infused mice compared to control mice. *$P < 0.01$ vs CTL in panels A to E. **$P < 0.01$ vs CTL.

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Supplemental Figure S14: Radial distribution of 16 fields of a cross-section of LV wall chosen for quantitative morphometrics. Each of 8 radial lines from the center of the LV lumen has two circles depicting the loci where images at 400× magnification were captured under a microscope Zeiss Fluorescent Axio Scope-A1 with AxioCam MRc5 camera. LV tissue was cut into three parts, apex, middle and bottom, horizontally. 3 serial cross-cryosections of the middle part at a 300-µm-interval were used for morphological analyses including quantification of fibrotic area, evaluation of cardiac myocyte size, evaluation of DHE stain intensity, alpha-SM-actin positive fibroblast area, apoptotic cell counting. Total 48 images (views) were captured for one left ventricle.
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