NADPH oxidase-4 mediates protection against chronic load-induced stress in mouse hearts by enhancing angiogenesis

Min Zhang1,4,2, Alison C. Brewer1,4, Katrin Schröder2,1, Celio X. C. Santos3, David J. Grieve2, Minshu Wang5, Narayana Anilkumar6, Bin Yu7, Xuebin Dong7, Simon J. Walker3, Ralf P. Brandes8, and Ajay M. Shah4,2

1Cardiovascular Division, King’s College London British Heart Foundation Centre, London SE5 9PJ, United Kingdom; and 2Institut für Kardiovaskuläre Physiologie, Goethe-Universität, 60590 Frankfurt am Main, Germany

Edited* by Salvador Moncada, University College London, London, United Kingdom, and approved September 14, 2010 (received for review July 4, 2010)

Cardiac failure occurs when the heart fails to adapt to chronic stresses. Reactive oxygen species (ROS)-dependent signaling is implicated in cardiac stress responses, but the role of different ROS sources remains unclear. Here we report that NADPH oxidase-4 (Nox4) facilitates cardiac adaptation to chronic stress. Unlike other Nox proteins, Nox4 activity is regulated mainly by its expression level, which increases in cardiomyocytes under stresses such as pressure overload or hypoxia. To investigate the functional role of Nox4 during the cardiac response to stress, we generated mice with a genetic deletion of Nox4 or a cardiomyocyte-targeted overexpression of Nox4. Basal cardiac function was normal in both models, but Nox4−null animals developed exaggerated contractile dysfunction, hypertrophy, and cardiac dilatation during exposure to chronic overload whereas Nox4-transgenic mice were protected. Investigation of mechanisms underlying this protective effect revealed a significant Nox4-dependent preservation of myocardial capillary density after pressure overload. Nox4 enhanced stress-induced activation of cardiomyocyte hypoxia inducible factor 1 and the release of vascular endothelial growth factor, resulting in increased paracrine angiogenic activity. These data indicate that cardiomyocyte Nox4 is a unique inducible regulator of myocardial angiogenesis, a key determinant of cardiac adaptation to overload stress. Our results also have wider relevance to the use of nonspecific antioxidant approaches in cardiac disease and may provide an explanation for the failure of such strategies in many settings.

Cardiac remodeling | hypoxia inducible factor | reactive oxygen species

Heart failure results from disease stresses that chronically increase cardiac workload (1). The cardiac response to such insults involves remodeling of the cardiomyocytes, vasculature, and extracellular matrix that may initially be adaptive. Persistent stress, however, results in contractile dysfunction, fibrosis, ventricular dilatation, and capillary rarefaction. Specific pathways are thought to drive adaptive vs. maladaptive features of remodeling (1–3).

Increased reactive oxygen species (ROS) production is implicated in cardiac remodeling through several mechanisms, including the activation of signaling pathways that promote cardiomyocyte hypertrophy, abnormal excitation-contraction coupling, mitochondrial dysfunction, cell death, and extracellular matrix remodeling (4, 5). Clinical trials of antioxidants have yielded disappointing results, however, and effective therapies based on targeting ROS remain elusive. ROS in a stressed heart may emanate from several sources, including mitochondria, NADPH oxidases of the Nox family, uncoupled nitric oxide (NO) synthases, and xanthine oxidases, but the functional roles of individual sources remain unclear (5). It is recognized, however, that different sources may modulate distinct signaling pathways through regulated, spatially restricted ROS production (6). Indeed, some ROS-mediated effects are beneficial rather than detrimental, e.g., in cardioprotective signaling elicited by preconditioning (7).

Nox family enzymes generate ROS by catalyzing electron transfer from NADPH to molecular O2. Seven family members exist (Nox1–5 and Duox1–2), each based on a distinct catalytic subunit and with tissue-specific expression (8, 9). The prototypic Nox enzyme, Nox2, mediates microbial activity in phagocytes through generation of large amounts of superoxide (O2−). In nonphagocytic cells, however, Nox2 and other Nox proteins generate low levels of ROS that are involved in intracellular signaling (9). Previous studies using Nox2-null mice and other models showed that Nox2 in the heart is involved in the development of cardiac hypertrophy and contractile dysfunction induced by angiotensin II, pressure overload, or myocardial infarction (10–15). Nox4 differs from Nox2 and other Nox enzymes in that it is regulated mainly by its expression level and does not require agonist stimulation or association with regulatory subunits for activation (16–18). Recent studies also suggest that it generates predominantly H2O2 rather than O2− (17–20). Previous work showed that Nox4 is expressed at a low level in the adult mammalian heart and that its abundance increases during pressure overload (11), but its pathophysiological functions in vivo are unknown.

In this study, we generated a Nox4-null mouse model and a cardiomyocyte-targeted Nox4-transgenic model to elucidate the effects of Nox4 during cardiac stress. In marked contrast to the effects of Nox2 and other ROS sources, increases in cardiomyocyte Nox4 resulted in protection against pressure overload-induced adverse cardiac remodeling. Nox4 facilitated preservation of myocardial capillary density during pressure overload by regulating stress-induced cardiomyocyte hypoxia inducible factor 1 (Hif1) activation and release of vascular endothelial growth factor (VEGF), resulting in increased paracrine angiogenic activity. These data indicate that Nox4 is a unique stress-inducible regulator of myocardial angiogenesis that facilitates adaptation to cardiac overload stress.

Results

Myocardial Nox4 Expression Increases During Stress. We first analyzed changes in myocardial Nox4 expression during development and in response to various stresses. Nox4 expression increased after in vivo pressure overload, myocardial infarction, or in vitro hypoxia (Fig. 1A). Nox4 expression was significantly lower in the myocardium of healthy young or old animals compared with fetal hearts (Fig. 1B). The increase in Nox4 protein expression after pressure overload was largely in cardiomyocytes (Fig. S1A). This induction of Nox4 is similar to that of so-called fetal genes that are reactivated in the adult heart during stress (3).
Cardiac Nox4 induction during stress and generation of Nox4-null mice. (A) Changes in Nox4 expression after pressure overload (Band), myocardial infarction (MI), or in vitro hypoxia (Hyp) 24 h compared with respective controls (Ctl). **P < 0.01; n = 4–6/group. (B) Nox4 expression in 3-mo (3m)- and 12-mo (12m)-old mice compared with fetal heart. ***P < 0.01; n = 5/group. (C) Western blots showing loss of Nox4 protein in heart and kidney of homozygous Nox4-KO mice (Top) and and loss of change in cardiac Nox2 and p22phox levels (Bottom). (D) H2O2 production in heart and kidney of Nox4-KO compared with WT. *P < 0.05; n = 6/group.

Nox4-null Mice Have Exaggerated Load-Induced Cardiac Dysfunction. To evaluate the role of increases in Nox4 during cardiac stress, we generated mice deficient in Nox4 (Fig. S1B). Deletion of endogenous Nox4 resulted in a total loss of Nox4 protein and a small reduction in H2O2 production as assessed by a homovanillic acid assay (Fig. 1 C and D). Myocardial levels of Nox2 and p22phox were unaffected by Nox4 deletion (Fig. 1C). Nox4-null mice were born in the expected Mendelian ratio, bred normally, and showed no obvious abnormal baseline phenotype (Table S1).

Basal cardiac size and function assessed by echocardiography were unchanged in Nox4-null mice (Fig. 2A). To assess the effects of the absence of Nox4 during cardiac stress, we performed suprarenal aortic constriction to generate a chronic pressure overload. The degree of pressure overload was similar in Nox4-null mice and wild-type (WT) littermates, and there was no difference in mortality. As expected, 6 wk of pressure overload caused contractile impairment and ventricular dilatation in wild-type mice. Nox4-null animals, however, developed significantly greater cardiac dilatation and contractile impairment than wild type (Fig. 2A). Nox4-null mice also developed exaggerated cardiac hypertrophy after chronic pressure overload at both the whole-heart and the cardiomyocyte level, as well as increased interstitial fibrosis (Fig. 2B–D). These data therefore suggest that an increase in myocardial Nox4 expression is protective against the detrimental consequences of chronic pressure overload.

Cardiomyocyte-Targeted Nox4-Transgenic Mice Show No Basal Dysfunction. To further validate a protective role of Nox4 during cardiac overload, we generated transgenic mice with a cardiomyocyte-targeted increase in Nox4. Nox4-transgenic mice had significantly increased Nox4 protein levels (Fig. 3A and Fig. S2A). Nox4 heterodimerizes with p22phox, with the two proteins stabilizing each other (8, 16), and increased Nox4 expression was accompanied by a twofold increase in p22phox levels. Nox2 levels by contrast were similar between strains. Nox4-transgenic mice had a modest elevation of myocardial H2O2 production (Fig. 3B) but did not show increased O2•− levels as assessed by electron paramagnetic resonance spectroscopy (EPR) (Fig. S2B), which is in line with data that suggest that Nox4 generates predominantly H2O2 rather than O2•− (17–20). Consistent with this, myocardial nitrotyrosine levels—as a readout of nitrosative stress resulting from interaction of O2•− and NO—were unaltered in Nox4-transgenic mice, which also had unaltered endothelial NO synthase levels (Fig. 3A and Fig. S2A). Overexpressed Nox4 protein
in transgenic cardiomyocytes was found in a perinuclear location, similar to the location of endogenous Nox4 in normal cardiomyocytes or after myocyte transfection (Fig. S5), and as reported in other cell types (21–23). Nox4-transgenic mice were grossly normal and showed no cardiac dysfunction up to 12 mo of age, although the older animals had a slightly increased cardiac mass compared with wild-type littersates (Table S2). There was no evidence of increased fibrosis or apoptosis in the hearts of Nox4-transgenic mice (Fig. S2 C and D). These results show that an increase in myocardial Nox4 levels in the absence of stress has no significant detrimental consequences.

**Cardiomyocyte-Targeted Nox4-Transgenic Mice Are Protected Against Load-Induced Stress.** We next subjected Nox4-transgenic mice and wild-type littersates to chronic pressure overload. Quantification of in vivo left ventricular (LV) pressure-volume relations revealed that both systolic and diastolic function were better preserved in Nox4-transgenic mice than in wild type after pressure overload for 9 wk (Fig. 3C). The protective effect of Nox4 was confirmed by echocardiography (Fig. S4A) as well as in a second independent transgenic line (Fig. S5). Nox4-transgenic mice developed less cardiac hypertrophy after pressure overload than wild-type littersates and also had significantly less interstitial fibrosis (Fig. 3 D–F and Fig. S4 B–D). Taken together, the data obtained so far using both loss-of-function and gain-of-function approaches indicate that an increase in myocardial Nox4 expression is protective against chronic pressure overload-induced cardiac dysfunction.

### Nox4-Dependent Enhancement of Myocardial Capillary Density During Pressure Overload

Signaling effectors of the cardiac response to chronic pressure overload include various protein kinases that are potentially redox-sensitive (3, 5). We undertook an immunoblotting-based profiling screen (Kinexus Bioinformatics) that encompasses a broad range of signaling pathways in Nox4-transgenic and wild-type mice subjected to pressure overload. The only protein with a more than twofold difference in phosphorylation between groups was Akt1. Quantitative immunoblotting showed that phosphorylated Akt (S473) levels were modestly elevated in Nox4-transgenic hearts, but there was no difference between Nox4-null mice and wild-type mice subjected to pressure overload (Fig. S5C). Levels of myocardial apoptosis after pressure overload were also unaffected either by Nox4 deletion or by cardiomyocyte-specific overexpression of WT band: 7.2 ± 1.1; KO band: 6.7 ± 1.1; transgenic band: 6.0 ± 0.9; nuclease P40 = 6–9/group; P = NS. A key determinant of functional cardiac compensation during chronic pressure overload has recently been recognized as the extent of myocardial capillarization, with insufficient angiogenesis being a driver of heart failure (24–27). Quantification of myocardial capillary density in LV sections of Nox4-null mice, Nox4-transgenic mice, and respective wild-type littersates showed that although there were no differences between groups at baseline, after imposition of pressure overload, capillary density was significantly lower in Nox4-null mice compared with wild type (Fig. 4 A). By contrast, Nox4-transgenic mice had significantly higher myocardial capillary density than wild-type littersates after pressure overload (Fig. 4B). These results suggest that Nox4 up-regulation during cardiac stress is required to protect against load-induced cardiac dysfunction by controlling the compensatory increase in myocardial capillary density.

**Nox4 Enhances Cardiomyocyte Hif1α and VEGF.** Previous work shows that a central mechanism underpinning myocardial stress-induced cardiomyocyte dysfunction is the release of angiogenic factors, notably VEGF (25–27). We found that cardiac VEGF-A protein levels were significantly increased in Nox4-transgenic mice whereas Nox4-null mice had markedly lower levels than wild-type after pressure overload (Fig. 5A). Immunostaining for VEGF in heart demonstrated a significant increase at cardiomyocyte membranes and in vessels in Nox4-transgenic mice after aortic banding whereas Nox4-null animals showed very little staining (Fig. S6A). An important upstream transcriptional regulator of VEGF during load-induced stress is Hif1α (26), which is known to be redox-regulated (28). Hif1α protein levels were significantly higher in Nox4-transgenic hearts compared with wild type after pressure overload (Fig. 5B). By contrast, Hif1α levels in Nox4-null mice were significantly lower than in wild-type littersates after aortic banding.

To more directly investigate the relationship between cardiomyocyte Nox4, Hif1α, and VEGF, we studied cultured cardiac cells. Overexpression of Nox4 in cultured cardiomyocytes increased H2O2 levels and slightly increased Hif1α protein levels during normoxia but substantially enhanced them during hypoxia (Fig. 6A and Fig. S6 B and C). A similar Nox4-dependent augmentation of Hif1α levels was observed in H9c2 cardiomyoblasts after treatment with a chemical hypoxia mimic, a chemical hypoxia mimic (Fig. 6B). Hypoxia-induced Hif1α accumulation in control cells was significantly reduced by the potent but nonspecific Nox inhibitor, diphenylene iodonium, or by catalase (Fig. 6C). Importantly, similar effects were observed after specific down-regulation of endogenous Nox4 by siRNA-mediated knockdown (Fig. 6D).
Sham Band
Sham    Band

Fig. 4. Nox4-dependent maintenance of myocardial capillary density. (A) Representative LV sections from Nox4-null mice and WT littermates stained with isocitrate B4 to label myocardial capillaries (yellow) and WGA to outline cardiomyocytes (red). 1, WT sham; 2, KO sham; 3, WT band; 4, KO band. Mean data at the right (n = 7–12/group). (B) Increased myocardial capillary density in Nox4-transgenic mice. Representative LV sections at the left. 1, WT sham; 2, TG sham; 3, WT band; 4, TG band. Mean data to the right (n = 6/group). **P < 0.01 for band vs. respective sham; ##P < 0.01 for TG or KO band vs. WT band. Scale bars: 20 μm.

Both transcriptional and posttranslational mechanisms could potentially be involved in Hif1α up-regulation (28). We found that Hif1α mRNA expression in vivo was significantly higher in Nox4-transgenic than in wild-type mice, but there was no significant difference in levels between Nox4-null mice and wild-type littermates (Fig. S6 D and E). Notably, Hif1α protein levels in Nox4-transgenic mice were not elevated at baseline despite increased mRNA levels, suggesting that an effect at the protein level was more important. Increasing Nox4 in cultured cardiomyocytes did not affect Hif1α mRNA levels during either normoxia or hypoxia (Fig. S6 E). Paracrine Angiogenic Activity Mediated by Nox4. We found that Nox4-induced increases in cultured cardiomyocyte Hif1α during hypoxia were accompanied by an increase in VEGF levels in the cells and even more so in their conditioned medium, indicative of extracellular release (Fig. 7 A and B). To directly test the potential angiogenic effects of Nox4-dependent changes in factors secreted by cardiomyocytes, we undertook in vitro endothelial cell tube formation assays. The conditioned medium of Nox4-overexpressing cardiomyocytes subjected to hypoxia markedly enhanced endothelial tube formation as compared with that of hypoxic cardiomyocytes overexpressing a β-galactosidase (β-gal) control gene (Fig. 7C and Fig. S6 D). The angiogenic effect of cardiomyocyte-conditioned medium was almost fully inhibited by a VEGF-blocking antibody but was unaltered by nonspecific IgG. These results indicate that the paracrine release of VEGF is central to the proangiogenic effects of cardiomyocyte Nox4.

Discussion
This study provides definitive data on the in vivo function of Nox4 in the heart with the use of complementary loss-of-function and gain-of-function models. We present a previously unrecognized and unexpected protective role of an endogenous ROS-generating enzyme in the cardiac response to load-induced stress, involving an enhancement of myocardial capillary density and functional cardiac compensation. The proangiogenic role of Nox4 involves a paracrine mechanism in which Nox4 up-regulation in cardiomyocytes leads to enhanced Hif1α activation and increased release of VEGF, which in turn promotes capillarization. The beneficial effects of Nox4 contrast markedly with those of other ROS sources in the remodeling heart, such as mitochondria, which have been found to be detrimental and have formed the basis for the testing of antioxidant therapies in human heart failure (29, 30). Our findings, however, indicate that therapeutic strategies may need to be directed toward specific ROS sources and pathways rather than the nonspecific targeting of ROS.

Nox enzymes differ from most other ROS sources in that ROS generation is their primary function (8, 9). Among the Nox enzymes expressed in the cardiovascular system, Nox4 is unique in that its activity is regulated mainly by its protein level whereas Nox1/Nox2 activation are under control of posttranslational mechanisms such as agonist-dependent phosphorylation of regulatory subunits (8, 16–18). Nox4 appears to be largely stress-inducible with low expression in the healthy adult heart and most other tissues apart from kidney (31), but with significantly
increased levels after stresses such as pressure overload or hypoxia. Our findings that Nox4-null mice have no obvious abnormalities in the absence of stress are consistent with the notion of stress inducibility. Nox4 differs from Nox2 in two other important respects: First, its subcellular location in cardiomyocytes is in or associated with the perinuclear endoplasmic reticulum whereas activated Nox2 is found predominantly at the cell membrane (32). Second, several recent independent reports indicate that Nox4 generates predominantly H2O2 (as further confirmed in the current study) whereas Nox2 primarily generates O2 (refs. 17–20). These differences in regulation, activation mechanism, subcellular location, and ROS generation translate into isoform-specific actions in isolated cellular models (9, 19, 22, 33). In the heart, previous studies in mouse models of defective Nox2 activation or its deletion showed that Nox2 is detrimental during remodeling, causing increased hypertrophy, apoptosis, and contractile dysfunction (10–15). The current results on Nox4, taken together with previous data on Nox2, indicate that the two isoforms contrast markedly in their effects on cardiac remodeling, with Nox2 being detrimental and Nox4 beneficial.

The major mechanism underlying the beneficial effects of Nox4 during load-induced stress is an increase in myocardial capillaries, with capillary density being impaired in Nox4-null animals but better preserved in Nox4-transgenic mice compared with wild type. Myocardial angiogenesis is tightly coupled to cardiomyocyte growth during heart development (34) and is also an important determinant of the response to disease-causing stresses such as pressure overload (24–27). Stress-induced angiogenesis has been shown to be underpinned by the release of VEGF from cardiomyocytes to exert paracrine effects on adjacent vessels (25).

Myocardial VEGF production is under the control of transcription factors such as Hif1 and GATA4 (26, 27), but the upstream signals regulating activation of these factors during cardiac stress remain unclear. The results reported here suggest that stress-induced increase in Nox4 levels is a key mechanism that enhances cardiomyocyte Hif1α levels during overload, in turn leading to VEGF release and an increase in angiogenic capacity. This facilitates functional compensation of the heart, which is manifested as better-preserved contractile function and a reduced extent of cardiac hypertrophy, fibrosis, and dilatation. Hif1α levels are the primary regulator of Hif1 transcriptional activity through dimerization with Hif1β and the recruitment of coactivators (28). Although Hif1α levels may be influenced by changes in mRNA expression, the major regulatory mechanism is via oxygen-dependent hydroxylation of Hif1α protein by prolyl hydroxylases, which results in its targeting for proteosomal degradation. Hydroxylase activity is inhibited during hypoxia, leading to increased Hif1α levels. Although we found an increase in Hif1α mRNA levels in Nox4-transgenic mice, the dominant mechanism by which Nox4 increased Hif1α appeared to be at the protein level. Assessment of Hif1α hydroxylation indicated that Nox4 may act by inhibition of prolyl hydroxylase activity, thereby stabilizing Hif1α and increasing protein levels. Interestingly, previous studies have reported that ROS may increase Hif1α mRNA expression in vascular cells (35) as well as inhibit Hif1α-prolyl hydroxylases in tumor cells (36).

In contrast to load-induced cardiac stress or ischemia, angiogenesis is detrimental in cancer by promoting tumor growth, and anti-angiogenic therapies are considered a promising strategy. Additionally, Nox4 reportedly has prosurvival effects in certain tumors and is thought to be a suitable therapeutic target (37, 38). Our results suggest, however, that caution should be exercised in using Nox4-targeted cancer therapy in patients with cardiac overload (e.g., hypertension) to avoid cardiotoxicity. In this study, we used a mouse model with global Nox4 deficiency so that part of the detrimental effect observed in pressure overloaded Nox4-null mice could potentially be due to loss of Nox4 in non-cardiomyocytes—in, for example, other cardiac cell types or organs such as the kidneys—although the data in cardiomyocyte-specific Nox4-transgenics argues against this. Regardless of this point, the current results with global Nox4-null mice may be predictive of potential side effects with systemic Nox4-inhibitor therapy.

Despite treatments such as beta-blockers and angiotensin-converting enzyme inhibitors that decrease mortality in heart failure patients, prognosis remains poor. New therapeutic strategies that can impact on disease mechanisms are therefore needed. ROS imbalance has long been recognized as potentially important in the remodeling and failing heart, but its therapeutic targeting has proven elusive. The present study indicates that ROS have not only
detrimental but also beneficial effects in the remodeling heart depending upon the source and suggests that specific targeting of an individual ROS source linked to an adaptive and potentially disease-preventing pathway (i.e., myocardial angiogenesis) may be a useful approach. Our results also have wider relevance to the use of nonspecific antioxidant approaches in human diseases and may provide an explanation for the failure of such strategies in many settings.

Methods
Detailed methods are provided in SI Methods.

Gene-Modified Mice. Nox4-null mice were generated by targeted deletion of the translation initiation site and exons 1 and 2 of the gene (Fig. S1B). Cardiomyocyte-targeted Nox4-transgenic mice were generated using the mouse Nox4 cDNA downstream of the mouse α-myosin heavy chain promoter. All lines were backcrossed >10 generations onto a C57BL6 background.

Animal Studies. Procedures were performed in accordance with the Guidance on the Operation of the Animals (Scientific Procedures) Act, 1986 (United Kingdom). Aortic constriction was induced by suprarenal banding (11).

Histology. FITC-conjugated wheat germ agglutinin (WGA) was used to outline cardiomyocytes and Picrosirius red staining to assess fibrosis (15). Capillaries were immunostained with isocitin B4 (39).

Cell Studies. Neonatal rat cardiomyocytes were subjected to hypoxia using a 95% N2/4% CO2/1% O2 mixture. Human umbilical vein endothelial cells seeded on Matrigel-coated slides were used for tube formation assays.

Detection of ROS. H2O2 levels were detected with a homovanillic acid assay (22). EPR was used to measure O2·− generation by heart particulate fractions, using a 5,5-dimethylpyrroline-N-oxide (DMPO, 50 mM) spin trap (40).

Statistics. All data are presented as mean ± SEM. Comparisons were undertaken by Student's t test or one-way ANOVA, as appropriate, followed by a post hoc Tukey's test. P < 0.05 was considered significant.

ACKNOWLEDGMENTS. We thank M. Mayr for advice on tube formation assays. This study was supported by the British Heart Foundation (Grants RG/08/01/25922, CH/99/001, and RE/08/003), the Leducq Foundation, by EUGeneNet (EU FP6 Grant LSHM-CT-2005-018833), by the German Search Foundation (Grants SFB815 TPA1, SFB834 TPA2), and by the Excellence Cluster Cardio-Pulmonary System and Goethe University.

Supporting Information

Zhang et al. 10.1073/pnas.1009700107

SI Methods

Gene-Modified Mice. Nox4-null mice were generated by targeted deletion of the translation initiation site and exons 1 and 2 of the gene (Genoway). A 5′ murine Nox4 genomic DNA fragment was isolated from a 129sv DNA BAC library and used to generate a targeting construct containing exons 1 and 2 flanked by loxP sites, a negative-selection diphtheria toxin A cassette and a positive selection neomycin cassette flanked by Flippase Recognition Target sites. The targeting construct was electroporated into 129sv embryonic stem cells, recombinant clones identified by PCR and Southern blot analysis, and injected into C57BL/6 blastocysts. Heterozygous mice obtained from germine chimeras were bred with C57BL/6 Cre-deleter mice and Flp-deleter mice to generate heterozygous knockout (KO) mice. Nox4-null animals were obtained by intercrossing progeny and were backcrossed >10 generations with C57BL/6 mice.

To generate cardiomyocyte-targeted Nox4-transgenic mice, the mouse Nox4 cDNA was cloned downstream of the mouse α-myosin heavy chain promoter (courtesy of J. Robbins, Children’s Hospital, Cincinnati, OH). The construct was linearized and microinjected into fertilized CBA/C57BL/6 pronuclei. Positive founders were identified by PCR and were backcrossed for >10 generations onto a C57BL/6 background.

Aortic Constriction. All procedures were performed in accordance with the Guidance on the Operation of the Animals (Scientific Procedures) Act, 1986 (United Kingdom). We studied male Nox4-null mice or Nox4-transgenic mice and respective matched wild-type littersmates, all on a C57BL/6 background. Aortic constriction was induced in ≈20-g mice by suprarenal banding under 2% isoflurane anesthesia, as previously described (1). Sham constriction involved identical surgery apart from band placement. Animals were studied up to 9 wk postsurgery. Animals were anesthetized with 2% isoflurane, and a 1.4F microconductance pressure catheter (ARIA SPR-853; Millar Instruments) was inserted via the right carotid artery into the left ventricle (LV) (2). Inferior vena cava occlusions were performed to generate end-systolic pressure-volume relations. Data were collected on Chart via Powerlab (Adinstruments) and analyzed using PVAN software (Millar Instruments). Heart rates were above 400 bpm in all experiments.

Real-Time RT-PCR. An Applied Biosystems PRISM 7700 machine was used with SYBR Green and the comparative cycle threshold method, with β-actin as internal controls. Oligonucleotide primers (forward, reverse) were the following: Nox4 (mouse) TGAAC-TACAGTGAAAGATTCTTGGAC, GACACCGTCGAC-GAGGAAAT; Nox4 (rat) GCCTAGGATTGTGTTTGGACGAGA, GCGAAGTGTAAGGAGGACTGT; Hif1α (mouse) CACC-GATTTGCCATGGA, TTCGAAGTCGCAAGACTCATCTTTT; Hif1α (rat) CCTGCTCCTCAGGAAAGTTTT; CAGCGCGTG-GAGCTAGCA; and β-actin CTTGAAAGAGATGACCGAGGCA-TCA, TGTTAGCACCAGAGCCGATACAG.

Histology. Hearts that were in situ fixed in diastole were paraffin-embedded. To assess whole-heart morphology, longitudinal sections were prepared and stained with hematoxylin and eosin. For all other histology, 6-μm transverse cross-sections were used. FITC-conjugated wheat germ agglutinin (FITC-WGA, Vector RL-1022) was used to outline cardiomyocytes. Interstitial fibrosis was assessed by blinded quantitative image analysis (Openlab, Improvision) of Picrosirius red-stained sections (3). Capillaries were immunostained with isocitrate B4 (Vector B-1205) and capillary density quantified as the number of capillaries per square millimeter (4). Apoptosis was assessed by TUNEL staining (Millipore S7110 kit). VEGF in the LV was detected with an anti-VEGF primary antibody, which was visualized with Alexa 568-conjugated anti-rabbit IgG (Molecular Probes).

Western Blotting and Protein Kinase Profiling. LV homogenates or cell lysates were separated by SDS/PAGE and transferred onto nitrocellulose membranes. Nuclear proteins were extracted as described (5). For the detection of hydroxylated Hif1α, Mg132 (5 μmol/L) was added immediately before cell lysis and also included in the lysis buffer. Antibodies used were the following: Nox4 (6); Nox2 and endothelial nitric oxide synthase (eNOS) (BD Transduction); p22phox (Santa Cruz Biotechnology); HIF-1α (Thermo Scientific); HIF-1α hydroxylated-ProGluPro128 and -Pro564, nitrotyrosine, and VEGF (Abcam); and pAkt (S473), pan-Akt, and histone-H3 (Cell Signaling). Actin (Sigma) was used as a loading control. Blots were quantified by densitometry. The protein kinase activity profiling screen was performed commercially (Kinetics, Kinexus Bioinformatics).

Cultured Cardiomyocyte Studies. Primary cultures of neonatal rat cardiomyocytes (NRC) were prepared as described previously (7). Rat H9c2 cardiomyoblasts (ATCC) were cultured in DMEM high glucose supplemented with FBS (10%), streptomycin (100 μmol/L), and penicillin (100 U/mL). Adenoviral vectors expressing mouse Nox4 or β-galactosidase were generated using the AdEasy Adenoviral Vector System (Qbiogene), amplified in HEK293 cells, and isolated using the Adenopure virus purification kit (Puresyn). Cells were cultured for 24 h, infected with virus at a multiplicity of infection of 40 for 24 h, washed with PBS, and starved overnight before exposure to hypoxia. siRNA against Nox4 (sequence 5′GCGGCGAUCUUCAAGAC**Pu**TT3′) and a universal negative control were from Ambion. Serum and antibiotic-free siRNA–reduced serum minimum essential medium (OPTI-MEM, Invitrogen) complexes were incubated with cells for 24 h after which OPTI-MEM-free medium was replaced. Cells were used for experiments after an additional 24 h. To induce hypoxia, cells were placed in a hypoxia chamber (Stem Cell Technologies) flushed with a 95% N2/5% CO2/1% O2 gas mixture for 24 h. Chemical hypoxia was induced by exposure to CoCl2 (200 μmol/L). For VEGF detection in culture supernatants, equal volumes (10 μL) derived from NRC exposed to hypoxia were centrifuged (2,000 × g for 3 min) to pull down debris, and the supernatants were dried under vacuum (Speed Vac Plus model SC-110A, Thermo Savant). Pellets were resuspended in 0.1 mL water, protein content was extracted, and samples were submitted to the usual electrophoresis procedure.

Detection of ROS. H2O2 levels were detected with a homovanillic acid (HVA) assay by using the catalase-inhibitable signal (6). The assay was performed on NRC in six-well plates or using tissue minced into small pieces in 1 mL Hanks’ balanced salt solution.
with or without 750 U/mL catalase at 37 °C. HVA fluorescence was detected on a plate fluorimeter (Tecan GENios). H$_2$O$_2$ levels were estimated using a standard curve and normalized by protein content. Electron paramagnetic resonance spectroscopy (EPR) was used to measure O$_2^-$ generation by heart particulate fractions (8). Briefly, the particulate fraction (80 μg) was incubated with the 5,5-dimethylpyrroline-N-oxide (DMPO; 50 mmol/L) spin trap in PBS containing diethylene triamine penta-acetic acid (100 μmol/L), pH 7.4, for 5 min at 37 °C. NADPH (0.6 mmol/L) was added and incubated for an additional 5 min, and spectra were recorded in a Magnettech Miniscope MS2000 spectrometer. Instrument conditions were the following: microwave power—50 mW and modulation amplitude—1 Gauss (G), with a gain of 9 × 10$^2$. The four-line spectrum (solid circle in Fig. S2B) is consistent with a DMPO-hydroxy (DMPO-OH) radical adduct ($\alpha_N$ = 14.9 G and $\alpha_H$ = 14.9 G) as generated using the positive control of xanthine (0.1 mmol/L)/xanthine oxidase (0.05 U/mL) under similar conditions. The DMPO-OH adduct was quantified by assessing the height of the second peak as a linear function, using 4-hydroxy-2,2,6,6-tetramethyl-1-piperidinyloxy (Tempol) as a standard.

**Immunostaining.** Adult cardiomyocytes were isolated from adult Nox4-transgenic and wild-type mice using collagenase digestion. Cells were plated onto laminin-pretreated slides, fixed, and permeabilized. After incubation with primary antibody or nonspecific IgG, bound antibodies were visualized with Alexa 488 conjugated anti-rabbit or Alexa 568 conjugated anti-mouse IgG (Molecular Probes). Imaging was done on a Leica laser scanning confocal microscope (TCS-SP5).

**Endothelial Tube Formation Assay.** The cell culture supernatants (conditioned media) of NRC were used for in vitro endothelial tube formation assays. Human umbilical vein endothelial cells (HUVECs) were seeded on Matrigel-coated eight-well chamber slides and incubated with 0.4 mL conditioned medium for 4 h at 37 °C, with or without VEGF blocking antibody (2 μg/mL; R&D System, AF564) or nonspecific IgG. Tube formation was examined by phase-contrast microscopy, and Axiovision software (Carl Zeiss) was used to quantify tube formation (9). The length of tubes in three to four randomly selected fields in each well was measured and compared with untreated HUVECs.

**Statistics.** Data are presented as mean ± SEM. Comparisons of groups were undertaken by Student’s $t$ test or one-way ANOVA, as appropriate. A post hoc Tukey’s test was performed to isolate differences. $P < 0.05$ was considered significant.

Fig. S1. Increase in cardiac Nox4 during pressure overload and generation of Nox4-null mice. (A) Nox4 protein expression in heart after aortic banding (Band) or control surgery (Sham) shown by Western blots. LV sections show immunostaining for Nox4 (1, Sham; 2, Band; 3, Band + blocking antigenic peptide). Mean data are in bar graph. Scale bar: 20 μm. **P < 0.01; n = 4/group. (B) Targeting strategy for generation of Nox4-null mice. Southern blots, performed after excision at AvrII sites, show 9.3- and 3.2-kb bands in heterozygous knockout (Het) mice but only the 9.3-kb band in WT. The 5’ AvrII site in the knockout allele is produced by the targeted deletion.
Fig. S2. Basal phenotype of Nox4-transgenic mice (TG). (A) Quantification of protein levels of Nox4, p22phox, Nox2, eNOS, and nitrotyrosine in hearts of Nox4-TG and wild-type littermates (WT). **P < 0.01; *P < 0.05; n = 3/group. (B) EPR spectra for detection of O$_2^-$ in heart. The four-line spectrum (•) is consistent with the DMPO-OH radical adduct ($a_N = 14.9$ G and $a_H = 14.9$ G), as confirmed by a xanthine (0.1 mmol/L)/xanthine oxidase (0.05 U/mL) positive control under similar conditions. Inhibition of signal by superoxide dismutase (SOD) was used to confirm specificity. Measurements were made in the presence of NADPH (0.6 mmol/L) except where indicated. Mean yields (arbitrary units) from three independent experiments were the following: a and d, undetectable; b, WT = 1.50 ± 0.21; c, TgNox4 = 1.6 ± 0.31; P = NS. (C) Lack of increase in interstitial fibrosis in 12-mo-old Nox4-transgenic mice. Representative LV sections stained with Picrosirius red (scale bar: 50 μm) and mean data from seven to nine mice/group. (D) Quantification of TUNEL staining in LV sections of 12-mo-old Nox4-transgenic mice and wild-type littermate controls (seven to nine mice/group).
Fig. S3. Immunostaining for Nox4 in cardiomyocytes. (A) Nox4 immunostaining (green) in myocytes from Nox4-transgenic and wild-type hearts. (Lower right) A higher magnification of the boxed area in Upper right. Scale bars: 20 μm and 5 μm, respectively. Specificity of immunostaining was confirmed with a blocking antigenic peptide (Lower left). Cardiomyocyte nuclei were stained with DAPI (blue). (B) Nox4 staining in cultured cardiomyocytes. (i) Endogenous Nox4 in neonatal rat cardiomyocytes. (ii) Cells infected with a Nox4-expressing adenovirus. Cells were stained for Nox4 and for protein disulfi de isomerase (PDI) as an endoplasmic reticulum marker. Yellow color in the merged images indicates colocalization. Scale bar: 20 μm. (iii) The panel at the left shows pixel-by-pixel fluorescence intensities of the two fluorophores in the cell shown at the right, along an arbitrary line, assessed using the Leica TCS-SP5 software. A significant overlapping pattern was found between Nox4 and PDI.
**Fig. S4.** Nox4-transgenic mice are protected against pressure overload-induced cardiac dysfunction and remodeling. (A) Echocardiographic analyses. Representative M-mode traces are shown at the left. Mean data from >15 animals per group are shown at the right. IVSD, interventricular septal thickness in diastole; LVEDD, LV end-diastolic diameter; LVESD, LV end-systolic diameter; FS, % fractional shortening. **P < 0.01 and *P < 0.05 for band vs. respective sham; ##P < 0.01 for TG band vs. WT band. (B) Representative longitudinal sections of hearts of animals subjected to pressure overload or sham surgery. Scale bar: 2 mm. (C) Reduced cardiomyocyte hypertrophy after pressure overload in Nox4-transgenic mice: representative WGA-stained LV sections. Scale bar: 20 μm. (D) Reduced fibrosis in LV of Nox4-transgenic mice: representative Picosirius red-stained sections. Scale bar: 20 μm.
Fig. S5. Protection against load-induced stress in a second line of Nox4-transgenic mice. (A) Western blots showing Nox4 expression in heart of two independent transgenic mouse lines. Myocardial Nox4 protein levels were 7.7 ± 1.1-fold higher in transgenic mice of line 2 vs. wild type (n = 3). Data for line 1 are shown in the main text and in Figs. S2–S4. (B) Effect of chronic pressure overload (Band) on WT and Nox4-transgenic (TG) mice from line 2. Sham denotes control groups. HW/BW, heart weight/body weight ratio; LV/BW, left ventricular/body weight; IVSD, LV end-diastolic diameter; LVESD, LV end-systolic diameter; FS, fractional shortening. **P < 0.01 for band vs. respective sham; #P < 0.05; ##P < 0.01 for TG band vs. WT band; n ≥ 7/group. (C) Changes in phosphorylated Akt (P-Akt; S473) and total Akt (T-Akt) in LV of Nox4-transgenic (Left) and Nox4-null mice (Right) compared with respective wild-type littermates. (Upper Left and Upper Right) Representative immunoblots. (Lower Left and Lower Right) mean data. *P < 0.05 for TG Band vs. WT Band; n = 4/group.
**Fig. S6.** Regulation of VEGF and Hif1α by Nox4. (A) Immunostaining for VEGF in LV sections of WT, TG and KO mice. (Scale bar 50 μm.) (B) Mean data from experiments with Nox4 overexpression in cardiomyocytes illustrated in Fig. 6A. **P < 0.01 for Nox4 hypoxia vs. normoxia; #P < 0.05 for Nox4 vs. β-gal; n = 3/group.** (C) H2O2 generation by Nox4 compared to β-gal-overexpressing cardiomyocytes. **P < 0.01; n = 3/group.** (D, E) Hif1α mRNA levels in LV of Nox4-transgenic mice (D) or Nox4-null mice (E) compared to respective WT. *P < 0.05 for band vs. respective sham; #P < 0.05 for TG vs. respective WT; n = 4/group. (F) Effect of Nox4 or β-gal on Hif1α mRNA levels in cardiomyocytes during normoxia or hypoxia. n = 6/group. (G) Representative phase contrast micrographs showing the effect of cardiomyocyte-conditioned medium of 1, control (β-gal-expressing) myocytes; 2, Nox4-overexpressing myocytes; 3, Nox4-overexpressing myocytes plus a VEGF-blocking antibody; 4, Nox4-overexpressing myocytes plus non-specific IgG, on endothelial cell tube formation.

**Table S1.** Baseline phenotype of Nox4-null mice aged 2 mo

<table>
<thead>
<tr>
<th></th>
<th>WT (n = 23)</th>
<th>KO (n = 21)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>18.2 ± 0.3</td>
<td>18.9 ± 0.4</td>
</tr>
<tr>
<td>Heart weight (g)</td>
<td>0.112 ± 0.003</td>
<td>0.116 ± 0.004</td>
</tr>
<tr>
<td>Kidney weight (g)</td>
<td>0.237 ± 0.07</td>
<td>0.240 ± 0.01</td>
</tr>
<tr>
<td>Lung weight (g)</td>
<td>0.166 ± 0.011</td>
<td>0.154 ± 0.005</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>0.935 ± 0.030</td>
<td>0.978 ± 0.038</td>
</tr>
<tr>
<td>Spleen weight (g)</td>
<td>0.079 ± 0.003</td>
<td>0.074 ± 0.002</td>
</tr>
<tr>
<td>Blood pressure (mmHg)</td>
<td>97 ± 1</td>
<td>98 ± 1</td>
</tr>
</tbody>
</table>
Table S2. Basal characterization of Nox4-transgenic mice compared with WT littermates at ages 3 and 12 mo

<table>
<thead>
<tr>
<th></th>
<th>3 mo</th>
<th>12 mo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT (n = 13)</td>
<td>TG (n = 13)</td>
</tr>
<tr>
<td>BW (g)</td>
<td>25.8 ± 0.4</td>
<td>25.0 ± 0.3</td>
</tr>
<tr>
<td>HW/BW (mg/g)</td>
<td>4.53 ± 0.07</td>
<td>4.75 ± 0.05</td>
</tr>
<tr>
<td>LV/BW (mg/g)</td>
<td>3.47 ± 0.05</td>
<td>3.57 ± 0.05</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>426 ± 13</td>
<td>409 ± 11</td>
</tr>
<tr>
<td>IVSD (mm)</td>
<td>0.76 ± 0.01</td>
<td>0.80 ± 0.01</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>2.55 ± 0.03</td>
<td>2.34 ± 0.07</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>4.13 ± 0.03</td>
<td>4.10 ± 0.07</td>
</tr>
<tr>
<td>FS (%)</td>
<td>38.3 ± 0.5</td>
<td>42.9 ± 1.1*</td>
</tr>
</tbody>
</table>

BW, body weight; HW, heart weight; HR, heart rate; IVSD, interventricular septal thickness in diastole; LVEDD, LV end-diastolic diameter; LVESD, LV end-systolic diameter; FS, fractional shortening. Data are means ± SEM.

*P < 0.05 compared with respective WT littermates.