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

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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 dilation 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 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, vascular 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 microbicidal 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).


The authors declare no conflict of interest.

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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. Nox 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. S3), 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 volume compared with wild-type littermates (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 littermates 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 littermates 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 [WT band: 7.2 ± 1.1; KO band: 6.7 ± 1.1; transgenic band: 6.0 ± 0.9 (nuclear) 6.9 ± 0.8 (cytosolic); 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 littermate controls 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. 4A). By contrast, Nox4-transgenic mice had significantly higher myocardial capillary density than wild-type littermates 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 angiogenesis is the release of angiogenic factors, notably VEGF that a central mechanism underpinning myocardial stress-induced angiogenesis 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 littermates 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 dichloroacetate, 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).
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 in 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. S6F). 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 indicate 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 H$_2$O$_2$ (as further confirmed in the current study) whereas Nox2 primarily generates O$_2^-$ (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 Hif1α 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 Hif1α 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 argue 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 back-crossed >10 generations onto a C57BL/6 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 isocitrate 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 mmol/L) 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.

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