PRECLINICAL STUDIES

Involvement of the Nicotinamide Adenosine Dinucleotide Phosphate Oxidase Isoform Nox2 in Cardiac Contractile Dysfunction Occurring in Response to Pressure Overload

David J. Grieve, PhD, Jonathan A. Byrne, PhD, MRCP, Anjana Siva, PhD, MRCP, Joanne Layland, PhD, Sofian Johar, MRCP, Alison C. Cave, PhD, Ajay M. Shah, MD, FAHA

London, England

OBJECTIVES
This study sought to examine the role of Nox2 in the contractile dysfunction associated with pressure-overload left ventricular hypertrophy (LVH).

BACKGROUND
Reactive oxygen species (ROS) production is implicated in the pathophysiology of LVH. The nicotinamide adenosine dinucleotide phosphate oxidase isoform, Nox2, is pivotaly involved in angiotensin II-induced hypertrophy but is not essential for development of pressure-overload LVH. Its possible impact on contractile function is unknown.

METHODS
The effects of aortic banding or sham surgery on cardiac contractile function and interstitial fibrosis were compared in adult Nox2−/− and matched wild-type (WT) mice.

RESULTS
Banding induced similar increases in left ventricular (LV) mass in both groups. Banded Nox2−/− mice had better LV function than WT by echocardiography (e.g., fractional shortening 33.6 ± 2.5% vs. 21.4 ± 2.2%, p < 0.05). Comprehensive LV pressure-volume analyses also showed significant contractile dysfunction in banded WT compared with sham, whereas banded Nox2−/− mice had preserved function (e.g., maximum rate of rise of LV pressure: banded WT, 4,879 ± 213; vs. banded Nox2−/−, 5,913 ± 259 mm Hg/s; p < 0.05). Similar preservation of function was observed in isolated cardiomyocytes. The 24-h to 36-h treatment of banded WT mice with N-acetylcysteine resulted in recovery of contractile function. Cardiac interstitial fibrosis was significantly increased in banded WT but not Nox2−/− mice, together with greater increases in procollagen I and III mRNA expression.

CONCLUSIONS
The Nox2 oxidase contributes to the development of cardiac contractile dysfunction and interstitial fibrosis during pressure overload, although it is not essential for development of morphologic hypertrophy per se. These data suggest divergent downstream effects of Nox2 on different components of the overall response to pressure overload. (J Am Coll Cardiol 2006; 47:817–26) © 2006 by the American College of Cardiology Foundation

An increase in oxidative stress is implicated in the pathophysiology of left ventricular hypertrophy (LVH) (1). Increased reactive oxygen species (ROS) production occurs both in experimental models of cardiomyocyte hypertrophy and with in vivo pressure-overload LVH, whereas antioxidants may exert beneficial effects in experimental in vivo LVH and heart failure (2–6). The ROS influence LVH pathophysiology through several mechanisms. Increased ROS production contributes to development of cardiomyocyte hypertrophy induced by G-protein-coupled receptor agonists, cytokines, and mechanical stretch, at least in part through activation of redox-sensitive protein kinases and transcription factors (3–6). Oxidative stress promotes myocyte apoptosis and necrosis, especially in advanced hypertrophy and heart failure (1,3,7). The ROS may also alter cardiac contractile properties, for example by modulating the function of sarcomemmal ion channels/exchangers, sarcoplasmic reticulum proteins, myofilaments, and/or enzymes involved in energy metabolism (8–10). Finally, ROS contribute to endothelial dysfunction through inactivation of nitric oxide, and also promote interstitial fibrosis and extracellular matrix remodeling (1,11).

Given these effects of oxidative stress, elucidation of the roles of different ROS sources (e.g., mitochondria, xanthine oxidase, uncoupled nitric oxide synthases) is important. In this regard, recent studies from several laboratories, including our own, have addressed the potential involvement of nicotinamide adenosine dinucleotide phosphate (NADPH) oxidases in LVH pathophysiology. These superoxide-generating enzymes are major cardiovascular ROS sources, particularly in the vasculature, where they play important roles in hypertension, atherosclerosis, angiogenesis, and other conditions (12). The prototypic NADPH oxidase (first characterized in neutrophils) comprises a membrane-bound heterodimer consisting of one gp91phox (or Nox2) and one p22phox subunit, and several regulatory subunits (p47phox, p67phox, p40phox, and Rac) that
associate with the heterodimer in the activated enzyme complex (12,13). The Nox subunit is the catalytic core of the oxidase and, recently, several isoforms (Nox1 to 5) were identified that have specific tissue distributions and may subserve distinct (patho)physiological functions (13). The major isoforms expressed in heart seem to be Nox2 and Nox4, with the Nox2 oxidase found in cardiomyocytes, fibroblasts, and endothelial cells (12,14–16).

The NADPH oxidases are implicated in cardiomyocyte hypertrophic signaling in response to angiotensin II (AngII) or alpha-adrenergic agonists (16,17), whereas Rac1 (which is involved in oxidase activation) induces cardiomyocyte hypertrophy (18,19). Increased cardiac NADPH oxidase expression and activity was shown in experimental pressure-overload LVH, with evidence that ROS derived from the oxidase may contribute to contractile dysfunction in this setting (14,20,21). Similarly, increased NADPH oxidase activity was also documented in failing human hearts (22,23). Recently, we began to specifically investigate the role of the Nox2 oxidase in the development of cardiac hypertrophy (15,24). In Nox2-deficient mice (Nox2−/−) (25), LVH induced by short-term subpressor AngII infusion was substantially inhibited together with abolition of AngII-induced NADPH oxidase activation (24). In marked contrast, increases in LV mass and myocyte size during pressure overload induced by aortic banding were similar in Nox2−/− and wild-type (WT) mice, indicating that Nox2 is not essential for development of pressure-overload LVH (15). The latter findings were confirmed by Maytin et al. (26). Because ROS potentially affect several aspects of cardiac structure and function, however, these studies do not address the role of Nox2 in other components of the overall response to pressure overload. In the present study, we examined the role of Nox2 in the contractile dysfunction and fibrosis associated with pressure-overload LVH.

**METHODS**

**Experimental LVH.** Adult male Nox2−/−and matched WT littermate controls underwent suprarenal abdominal aortic banding or sham surgery (15). Banding caused similar increases in invasively measured systolic blood pressure in WT (sham, 90 ± 3 mm Hg; banded, 135 ± 5 mm Hg) and Nox2−/−mice (sham, 86 ± 3 mm Hg; banded, 130 ± 8 mm Hg). Some animals were pretreated with N-acetylcysteine (500 mg/kg/day in drinking water) for 24 to 36 h before being killed. All procedures were performed in accordance with

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**Figure 1.** Effect of pressure overload on cardiac fibrosis. (A) Interstitial collagen assessed in left ventricular (LV) sections stained with Picrosirius red. (B to D) messenger ribonucleic acid expression of procollagen I, procollagen III, and fibronectin. Data are mean ± SEM from six animals. *p < 0.05 versus corresponding sham; †p < 0.05 versus banded Nox2−/−. WT = wild type.
Development of LVH. Consistent with our previous study (15), two weeks of pressure overload caused similar increases in the LV/body weight ratio in banded WT (4.57 ± 0.29 mg/g vs. 3.21 ± 0.07 mg/g, p < 0.05) and Nox2−/− (4.87 ± 0.18 mg/g vs. 3.40 ± 0.07 mg/g, p < 0.05) versus shams. Atrial, right ventricular, lung, and body weights were similar between groups. The ANF mRNA expression increased to a similar extent in banded WT and Nox2−/− mice (Fig. 1A). Procollagen I mRNA occurred in banded Nox2−/− mice (Fig. 1B). In contrast, no significant increase in procollagen I/III mRNA was observed in banded Nox2−/− mice (Fig. 1C). These results suggest that Nox2 may contribute to the development of LVH in pressure-overloaded WT mice.

Table 1. Echocardiographic Parameters in Sham-Operated and Banded WT and Nox2−/− Mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sham WT</th>
<th>Banded WT</th>
<th>Sham Nox2−/−</th>
<th>Banded Nox2−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate (beats/min)</td>
<td>431 ± 18</td>
<td>451 ± 18</td>
<td>411 ± 11</td>
<td>437 ± 17</td>
</tr>
<tr>
<td>IVSD (mm)</td>
<td>0.77 ± 0.02</td>
<td>1.13 ± 0.04*</td>
<td>0.75 ± 0.04</td>
<td>1.06 ± 0.02*</td>
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<tr>
<td>LVEDD (mm)</td>
<td>3.92 ± 0.05</td>
<td>3.82 ± 0.08</td>
<td>3.94 ± 0.15</td>
<td>3.93 ± 0.08</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>2.48 ± 0.10</td>
<td>3.01 ± 0.12*</td>
<td>2.64 ± 0.19</td>
<td>2.61 ± 0.10</td>
</tr>
<tr>
<td>FS (%)</td>
<td>36.9 ± 2.4</td>
<td>21.4 ± 2.2*</td>
<td>33.6 ± 3.1</td>
<td>33.6 ± 2.5</td>
</tr>
</tbody>
</table>

Data are mean ± SEM from 8 to 10 animals. *p < 0.05 versus appropriate sham control.

FS = fractional shortening; IVSD = interventricular septal thickness in diastole; LVEDD = left ventricular end-diastolic dimension; LVESD = left ventricular end-systolic dimension; WT = wild-type.
the increase in procollagen III mRNA was significantly lower than in banded WT. Fibronectin mRNA did not increase in any group (Fig. 1D).

**Echocardiography.** Consistent with the morphometric data, banded WT and Nox2−/− mice showed similar increases in IVSD after two weeks (Table 1). Fractional shortening was significantly reduced in banded WT mice compared with sham, with a significant increase in LVESD. In contrast, neither LVESD nor fractional shortening were significantly altered in banded Nox2−/− mice. Heart rate and LVEDD were similar among groups.

**LV pressure-volume analyses.** Table 2 shows parameters of steady-state cardiac function in WT and Nox2−/− mice, studied two weeks after surgery. Banded WT mice showed significant reductions in LVdP/dt max, end-systolic pressure, and ejection fraction, and an increase in end-systolic volume.

![Figure 2](image_url)

Figure 2. Left ventricular (LV) function in isolated ejecting hearts from wild-type (WT) and Nox2−/− mice across a range of LV end-diastolic volumes (EDV). Changes in A and D: maximum rate of rise of LV pressure (LVdP/dt max); changes in B and E: maximum rate of fall of LV pressure (LVdP/dt min); changes in C and F: stroke work (SW). Data are mean ± SEM from eight animals. *p < 0.05 banded (solid squares) versus sham (open squares).
compared with sham, indicating impaired systolic function. LVdP/dtmin was also significantly reduced, and isovolumic relaxation time-constant (Tau) increased in banded WT mice, indicating impaired relaxation. None of these parameters were significantly altered in banded Nox2−/− mice.

Figure 2 shows LVdP/dtmax, LVdP/dtmin, and stroke work measured across a range of LV volumes and confirms significant reductions in these parameters in banded WT mice but preservation of function in banded Nox2−/− mice. The differences in stroke work between WT and Nox2−/− mice are further appreciated from the representative LV pressure-volume loops shown in Figures 3A and 3D, in which stroke work is the area of the loop.

Figure 3 shows representative examples of data obtained during transient aortic occlusion. The slope of the ESPVR was significantly decreased in banded WT mice, indicating reduced contractility, but was unaltered in banded Nox2−/− mice (Figs. 3B and 3E). The slope of the EDPVR was significantly increased in banded WT mice, indicating increased diastolic stiffness, but was unaltered in banded Nox2−/− mice (Figs. 3C and 3E). Mean data are shown in Table 2.

Cardiomyocyte function. To assess whether differences in contractile function found at the whole-heart level were also present at the cellular level, we studied isolated cardiomyocytes. Myocytes from banded WT mice had significantly reduced twitch shortening (by ~20%) compared with sham (Figs. 4A and 4C). In contrast, the shortening of myocytes from banded Nox2−/− mice was non-significantly reduced (Figs. 4B and 4D).

Effect of N-acetylcysteine. Treatment with the antioxidant N-acetylcysteine for 36 h before killing at the two-week post-surgical time point resulted in normalization of echocardiographic fractional shortening and LVESD in banded WT mice (Figs. 5A and 5C); heart rate, IVSD, and LVEDD (Fig. 5B) were unaffected. N-acetylcysteine also increased LVdP/dtmax, stroke work, ESPVR (Figs. 5D to 5F), ejection fraction, and LVdP/dtmin (data not shown) in isolated ejecting hearts of banded WT mice. Furthermore, in vivo N-acetylcysteine treatment for 24 h significantly improved contraction of cardiomyocytes isolated from banded WT hearts (e.g., shortening velocity, untreated, −2.0 ± 0.07 μms⁻¹; treated, −2.9 ± 0.16 μms⁻¹; p < 0.05), but had minimal effects in the sham group (untreated, −2.4 ± 0.07 μms⁻¹; treated, −2.6 ± 0.11 μms⁻¹; p = NS). A similar improvement of contractility was found with addition of N-acetylcysteine to isolated hearts or myocytes for 15 to 20 min, but this effect did not stabilize over this time period (i.e., continued to increase).

To assess whether the effects of N-acetylcysteine were attributable to reduced oxidative stress, we assessed 3-nitrotyrosine staining in LV sections. Banded WT hearts showed increased 3-nitrotyrosine staining, but this was
significantly reduced in hearts from banded animals treated with N-acetylcysteine for 36 h (Fig. 6).

**Contractile function after eight weeks of pressure overload.** To assess whether the differences in contractile function between WT and Nox2^{-/-} mice persisted after longer durations of pressure overload, animals were studied at eight weeks after surgery. Figure 7 shows cardiac functional parameters measured in isolated ejecting hearts. Banded WT mice showed substantial reductions in LVdP/dt\text{max}, LVdP/dt\text{min}, and cardiac work compared with shams, which were greater than those observed after two weeks (Fig. 3). Banded Nox2^{-/-} mice now also showed significant reductions in LVdP/dt\text{max}, LVdP/dt\text{min}, and cardiac work, but the degree of impairment remained much less than in banded WT mice.

**Figure 4.** Effect of pressure overload on cell shortening (CS) in cardiomyocytes from wild-type (WT) and Nox2^{-/-} mice. (A and B) Representative traces of myocyte contraction. (C and D) Mean ± SEM from ≥60 cells from four hearts. Dark columns = banded mice; lighter columns = shams. *p < 0.05 versus sham.

**Figure 5.** Effect of N-acetylcysteine on cardiac function assessed (A to C) by echocardiography and (D to F) in isolated ejecting hearts. Light columns = control animals; darker columns = N-acetylcysteine-treated animals. Data are mean ± SEM from eight animals. *p < 0.05 versus corresponding control.
DISCUSSION

The major novel finding of this study is that the Nox2 NADPH oxidase contributes to development of cardiac contractile dysfunction and interstitial fibrosis in response to pressure overload, even though it is not essential for development of LVH per se. The Nox2 oxidase therefore influences specific components of the overall response to pressure overload, indicating that related but distinct components of the overall cardiac hypertrophic phenotype may be subject to independent regulation.

Oxidative stress is recognized to be involved in LVH pathophysiology, but the precise roles of different ROS sources remains unclear (1–6). Furthermore, the possibility of divergent effects on contractile function or interstitial fibrosis as opposed to increases in LV mass has not been widely addressed. Recent work suggests a role for NADPH oxidases in the development of cardiac hypertrophy and interstitial fibrosis (14,21,24). A relatively unique attribute of NADPH oxidases in contrast to other ROS sources (e.g., mitochondria, xanthine oxidase) is that ROS generation in response to specific stimuli and subsequent modulation of downstream signal transduction seems to be a primary function of these enzymes (12,13). In addition, the presence of at least two cardiac Nox isoforms (Nox2 and Nox4) raises the possibility of not only stimulus-specific but also isoform-specific roles (12,13,15). Our previous findings that LVH induced by subpressor AngII was inhibited in Nox2−/− mice (24) indicated that Nox2-derived ROS are specifically involved in cardiac AngII signaling. Consistent with this, AngII-induced cardiac hypertrophy and fibrosis were inhibited in mice lacking apoptosis signal-regulating kinase 1, which is known to be redox-sensitive (32). However, Nox2−/− mice developed a similar degree of LVH to WT animals in response to pressure overload (15,26), indicating that even though Nox2 mediates hypertrophy in response to AngII, other pathways may be more important or may substitute for Nox2 when the stimulus is pressure overload. Nevertheless, the current study shows that Nox2 plays a
major role in the development of interstitial fibrosis and contractile dysfunction in response to pressure overload. Interestingly, pressure overload was found to increase NADPH oxidase activity and ROS production even in Nox2/−/− mice, secondary to upregulation of Nox4 in these animals (15). This finding, together with the results of the present study, indicates that the downstream effects of Nox2 during pressure overload are highly specific; i.e., neither Nox4 nor other ROS sources apparently promotes fibrosis or contractile dysfunction in Nox2/−/− mice.

Previous seminal studies have established that although cardiomyocyte hypertrophy and interstitial fibrosis often co-exist, they are to a significant extent independently regulated (33). Oxidative stress is pro-fibrotic both in the heart and in other organs, by stimulating fibroblast proliferation, collagen deposition, and activation of matrix metalloproteinases (1,33–35). The novel finding of the current study is the demonstration of a specific role for the Nox2 oxidase in promoting interstitial cardiac fibrosis during pressure overload. Inhibition of interstitial fibrosis in banded Nox2/−/− mice was shown both by histology and real-time reverse transcriptase-polymerase chain reaction. Taken together with previous data indicating an involvement of Nox2 in AngII-induced cardiac, vascular, and hepatic fibrosis (24,28,36), these results may suggest a broader role of Nox2 in mediating pathological fibrosis.

The other major effect of Nox2 was found to be in the development of contractile dysfunction during pressure overload. We undertook detailed characterization of contractile function by both echocardiography in vivo and LV pressure-volume analyses in isolated ejecting hearts. The WT mice had significant systolic dysfunction after two weeks of pressure overload as evidenced by reduced echocardiographic fractional shortening, increased LVESD, and reduction in pressure (LVdP/dtmax) and volume-based indices (LV end-systolic volume and ejection fraction) as well as the slope of the ESPVR. Banded WT mice also had impaired relaxation (reduced LVdP/dtmin, prolonged tau) and evidence of diastolic dysfunction (increased slope of EDPVR). In marked contrast, none of these parameters were significantly altered in banded Nox2/−/− mice. Furthermore, brief (24-h to 36-h) treatment with the antioxidant N-acetylcysteine improved contractile function and reduced LV 3-nitrotyrosine staining in WT bands, suggesting that the dysfunction was indeed mediated via ROS. Whereas we previously showed that chronic (two-week) treatment with N-acetylcysteine inhibited the development of pressure-overload hypertrophy (15), the current brief (24-h to 36-h) protocol was specifically chosen to investigate effects on contractile function independent of hypertrophy. With more prolonged pressure overload (eight weeks), we found that contractile dysfunction did develop in
Nox2\textsuperscript{−/−} mice but the magnitude of dysfunction remained less than with banded WT. These data suggest that Nox2 may be particularly important for contractile dysfunction in the early stages of pressure overload. With more prolonged overload, it is likely that other mechanisms, such as ROS-independent abnormalities of excitation-contraction coupling and energetic deficit, may also become relevant (37). Our results contrast with a recently published study in which no differences in cardiac function were found between Nox2\textsuperscript{−/−} and WT mice subjected to aortic constriction (26). However, in that study, the pressure overload was probably much more severe than in the present study because it resulted in \(>50\%\) LVH after just one week and \(~50\%\) mortality, compared with \(<20\%\) mortality in our study. These data leave open the possibility that the contribution of Nox2 to contractile dysfunction during pressure overload may depend on the degree of overload, a question that warrants further investigation.

Increased ROS production may lead to contractile dysfunction through several mechanisms, including alterations in cellular energetics, changes in excitation-contraction coupling, and/or alterations in myofilament calcium responsiveness (1,8–10,38). At the whole-heart level, contractile function may also be influenced by alterations in the extracellular matrix (11). In the current study, we found significant differences in function of cardiomyocytes isolated from banded Nox2\textsuperscript{−/−} and WT, suggesting that a significant proportion of the contractile dysfunction in whole hearts may be attributable to intrinsic myocyte dysfunction. However, the differences in diastolic properties observed between banded Nox2\textsuperscript{−/−} and WT may be related to the observed changes in interstitial fibrosis. The precise mechanisms underlying Nox2-dependent contractile dysfunction require further investigation, but our preliminary studies do not show significant differences in expression of SERCA2a or phospholamban between banded Nox2\textsuperscript{−/−} and WT (data not shown).

In summary, this study shows that the Nox2 NADPH oxidase specifically contributes to the development of both contractile dysfunction and interstitial fibrosis in response to pressure overload, despite not being essential for the development of LVH per se. This divergent regulation of LV hypertrophy and LV function is analogous to a number of other recent reports of experimental models in which these aspects of cardiac hypertrophy have been found to be independently regulated, e.g., by the PI3K-PTEN pathway (39,40) and the MEKK1-JNK pathway (41). Also analogous to these reports, the Nox2 oxidase in the heart not only seems to be activated in an isoform-specific manner, but also exerts isoform-specific downstream effects on distinct components of the overall cardiac hypertrophic phenotype. These data underscore the likely importance of independent redox regulation of different aspects of the cardiac response to pressure overload.

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