Pressure Overload–Induced Myocardial Hypertrophy in Mice Does Not Require gp91phox

Melanie Maytin, MD; Deborah A. Siwik, PhD; Masahiro Ito, MD; Lei Xiao, MD, PhD; Douglas B. Sawyer, MD, PhD; Ronglih Liao, PhD; Wilson S. Colucci, MD

Background—Reactive oxygen species (ROS) may mediate pressure overload–induced myocardial hypertrophy. NADPH oxidase may be involved in this process, because its expression and activity are upregulated by pressure overload and because myocardial hypertrophy caused by a subpressor infusion of angiotensin is attenuated in mice deficient in the gp91phox catalytic subunit of NADPH oxidase.

Methods and Results—To test the role of NADPH oxidase–dependent ROS in mediating pressure overload–induced myocardial hypertrophy, we subjected transgenic mice lacking gp91phox to chronic pressure overload caused by constriction of the ascending aorta. Contrary to our hypothesis, neither myocardial hypertrophy nor NADPH-dependent superoxide generation was decreased in gp91phox-deficient mice after aortic constriction. Aortic constriction caused an exaggerated increase in p22phox and p47phox mRNA in gp91phox-deficient mice.

Conclusions—These results indicate that gp91phox is not necessary for pressure overload–induced hypertrophy in the mouse and suggest the involvement of another source of ROS, possibly an NADPH oxidase that does not require the gp91phox subunit. (Circulation. 2004;109:1168-1171.)

Key Words: pressure ■ hypertrophy ■ aorta

Reactive oxygen species (ROS) have been shown to cause hypertrophy in cardiac myocytes in vitro and to mediate the hypertrophic effects of several stimuli, including mechanical strain, α-adrenergic receptor stimulation, angiotensin, endothelin, and tumor necrosis factor-α. Recently, Date et al showed that treatment with the antioxidant N-2-mercaptopyrrolionyl glycine attenuates myocardial hypertrophy caused by transverse aortic constriction in mice, leading to the conclusion that ROS play a key role in pressure overload–induced myocardial hypertrophy.

The sources of ROS that mediate myocyte hypertrophy remain to be determined. Among the possibilities are leakage from mitochondrial electron transport, xanthine oxidase, NADPH oxidase, NO synthase, and monoamine oxidase. There is evidence that NADPH oxidase is present in cardiac myocytes. Pressure overload results in increased NADPH oxidase activity and upregulated expression of several subunits of the NADPH oxidase complex in the myocardium, including gp91phox, p47phox, p67phox, and p22phox.

These observations have implicated NADPH oxidase as a likely source of ROS involved in mediating myocardial hypertrophy. Transgenic mice deficient in gp91phox catalytic subunit were developed as a model for chronic granulomatous disease, a condition in which defective NADPH oxidase activity in leukocytes leads to recurrent infections. It was shown that myocardial hypertrophy caused by a subpressor infusion of angiotensin is attenuated in mice deficient in gp91phox, suggesting the involvement of this isoform in myocardial hypertrophy. To test the role of NADPH oxidase–dependent ROS in mediating hemodynamic overload-induced myocardial hypertrophy, we subjected transgenic mice lacking gp91phox to chronic pressure overload caused by ascending aortic constriction (AAC). AAC was created as previously described in 8- to 10-week-old gp91phox-deficient (n = 27) and age-matched wild-type (WT; n = 27) C57Bl/6 mice (Jackson Laboratories, Bar Harbor, Me). At 1, 3, and 10 weeks after AAC, echocardiography was performed, and some animals from each group were euthanized. Morphometric measurements were made, a cross-sectional slice of the left ventricle was obtained, and the rest of the myocardium was snap frozen. Left ventricular (LV) systolic pressure measured before and after AAC was in WT mice (before AAC, 78 ± 8 mm Hg; after AAC, 136 ± 10 mm Hg) and gp91phox-deficient mice (before AAC, 68 ± 4 mm Hg; after AAC, 126 ± 15 mm Hg; P = 0.33 versus WT). The animal protocol was approved by the Institutional Care and Use Committee at Boston University Medical Center.

Echocardiography
LV wall thickness and chamber dimensions were measured by echocardiography performed with an Acuson Sequoia C-256 machine using a 15-MHz probe using 1% to 2% isofluorane, as previously described.

Protocol
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**Histology**
Ventricular myocardium was fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with Trichrome-Masson. Fibrosis was quantified using Bioquant Image Analysis Software.

**Cellular Morphometry**
Myocytes were isolated from WT and gp91phox-deficient mice 1 week after AAC and sham operation, as previously described, and fixed in formalin. Cellular dimensions were measured using Bioquant Image Analysis and ProScan Software.

**Lucigenin Chemiluminescence**
Lucigenin chemiluminescence was determined as per Pagano et al. Diphenylene iodonium (100 μM/L), a flavoprotein inhibitor that blocks NADPH oxidase, was used to assess the source of O₂.

**GSH/GSSG**
The ratio of reduced to oxidized glutathione in LV tissue homogenate was measured using an Oxis GSH/GSSG-412 kit.

**Northern Hybridization**
Total RNA was extracted and subjected to Northern hybridization using a full-length cDNA for rat p22phox (a generous gift from K.K. Griendling, Emory University, Atlanta, Ga) and a 570-bp cDNA fragment for rat p47phox.

**Statistical Analysis**
All data are expressed as mean ± SEM. Differences across multiple conditions were tested by 1-way ANOVA for repeated measures. Comparisons between conditions were tested by Student’s unpaired t test using the Bonferroni correction for multiple comparisons.

**Results**

**General Characteristics**
Survival in the first 2 weeks after AAC was similar in WT (47%) and gp91phox-deficient (47%) mice. Heart weight tended to be higher at 1, 3, and 10 weeks after AAC in gp91phox-deficient mice, as did HW/BW, although neither reached statistical significance (Table 1).

**NADPH Oxidase and Glutathione Concentrations**
Wall thickness increased to a similar extent in WT and gp91phox-deficient mice 1, 3, and 10 weeks after AAC and tended to be greater at 3 and 10 weeks after AAC in gp91phox-deficient mice (Figure 1A). LV end-diastolic dimension did not change 1 or 3 weeks after AAC and increased to a similar extent in WT and gp91phox-deficient mice at 10 weeks (Figure 1B). Fractional shortening decreased progressively from 1 to 10 weeks after AAC and to a similar extent in WT and gp91phox-deficient mice (Figure 1C).

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**Histology**
Myocyte dimensions and fibrosis were assessed 1 week after AAC. With sham operation, myocyte width and area were slightly smaller in gp91phox-deficient mice, whereas with AAC, all dimensions (length, width, and area) were larger in gp91phox-deficient mice (Table 2). Myocardial fibrosis after AAC was increased to a similar extent in WT and gp91phox-deficient mice.

**NADPH Oxidase and Glutathione Concentrations**
At 10 weeks, NADPH-dependent, diphenylene iodonium-inhibitable superoxide generation was similar in myocardium from sham-operated WT and gp91phox-deficient mice. NADPH oxidase activity was not increased in WT mice after AAC but was increased 2.4-fold in gp91phox-deficient mice (Figure 2A). The p22phox and p47phox mRNA levels were similar in myocardium from sham-operated WT and gp91phox-deficient mice. At 10 weeks after AAC, the expression of p22phox increased ~6-fold in WT mice and ~17-fold in gp91phox-deficient mice (Figure 2B). After AAC, the expression of p47phox did not increase in WT mice but increased ~2-fold in gp91phox-deficient mice (Figure 2B).

The ratio of reduced to oxidized glutathione at 10 weeks tended to be lower in gp91phox-deficient mice both with sham operation and after AAC (Figure 2C).

**Discussion**
In mice with genetic deficiency of gp91phox, there was no attenuation of the hypertrophic response to chronic pressure overload. Surpris-

### Table 1. Morphometric Measurements

<table>
<thead>
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<th>SHAM</th>
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<tr>
<td></td>
<td>Weeks</td>
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<td>gp91phox</td>
<td>WT</td>
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<td>Body weight (mg)</td>
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<td>Heart weight (mg)</td>
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<td>118.8±4.3</td>
<td>108.9±1.7</td>
<td>165.6±10.5†</td>
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<td>127.2±2.1</td>
<td>128.0±3.7</td>
<td>171.4±26.5†</td>
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<tr>
<td>Heart weight:body weight (mg:g)</td>
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<td>3.6±0.6</td>
<td>3.8±0.3</td>
<td>5.5±0.7†</td>
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<tr>
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<td>6.2±1.1†</td>
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<td>3.8±0.4</td>
<td>3.9±0.3</td>
<td>5.4±1.9†</td>
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<tr>
<td>Wet: dry lung ratio</td>
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<tr>
<td>Wet: dry liver ratio</td>
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<td>3.2±0.09</td>
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<td>3.1±0.2</td>
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</table>

*Data are mean ± SEM for 3–7 animals in each group.*

P < 0.05 vs WT-AAC; †P < 0.05 vs respective sham; ‡P < 0.01 vs respective sham; §P < 0.01 vs WT-AAC.
ingly, after AAC, myocardial hypertrophy tended to be greater in gp91 phox -deficient mice. Likewise, the magnitude of NADPH-dependent superoxide generation in the gp91 phox -deficient mice was not reduced and, on the contrary, tended to be higher in association with increased expression of mRNA for the p22phox and p47 phox subunits.

Although NADPH oxidase was initially identified in leukocytes, where it is the source of antibacterial levels of O₂⁻/H₂O₂, this oxidase has now been identified in several cell types in the cardiovascular system. In vascular smooth muscle cells, Griendling et al. have shown that NADPH oxidase mediates the growth effects of angiotensin. We found that several subunits of NADPH oxidase, including p22phox, gp91phox, p47phox, and p67phox, are expressed in cardiac myocytes cultured from adult rat hearts. Li et al. likewise found that several subunits of NADPH oxidase are present in guinea pig myocardium and that pressure overload-induced hypertrophy and failure are associated with increased NADPH oxidase–dependent superoxide-generating activity and upregulated expression of the p22phox, gp91phox, p67phox, and p47phox subunits. Bendall et al. additionally demonstrated in mice that a subpressor infusion of angiotensin for 2 weeks caused myocardial hypertrophy and increased NADPH oxidase activity, both of which were attenuated in gp91phox-deficient mice.

Thus, there was a strong rationale for our hypothesis that myocardial hypertrophy in response to pressure overload would be decreased in gp91phox-deficient mice attributable to decreased NADPH oxidase activity. On the contrary, neither myocardial hypertrophy nor NADPH oxidase activity was decreased in gp91phox-deficient mice after AAC. Both heart weight and anterior wall thickness tended to be greater in gp91phox-deficient mice after AAC. This tendency for increased myocardial hypertrophy was
associated with increased myocyte dimensions in gp91phox-deficient mice after AAC. Thus, our results indicate that gp91phox is not necessary for pressure overload–induced myocardial hypertrophy.

Because gp91phox seems necessary for angiotensin-stimulated myocardial hypertrophy9 and pressure overload–induced hypertrophy was inhibited by an antioxidant,6 our data suggest that multiple sources of ROS may mediate myocardial hypertrophy in a stimulus-specific manner. Although these results exclude a critical role for the gp91phox-dependent isoform of NADPH oxidase, they do not exclude a role for other isoforms that use homologs of the gp91phox catalytic subunit.17–19 It is also noteworthy that after aortic constriction NADPH oxidase activity in gp91phox-deficient mice tended to be higher than in gp91phox-deficient mice and was associated with increased expression of mRNA for the p22phox and p47phox subunits. This latter finding raises the possibility that there is cross-regulation of NADPH oxidase subunits, such that loss of gp91phox led to increased expression of other subunits that may have mediated the hypertrophic response.

Oxidative stress and the production of ROS are increased in failing myocardium.6 Possible sources of oxidative stress in failing myocardium include mitochondria20 and xanthine oxidase.21 Recent reports indicate that NADPH oxidase activity is increased in myocardium from patients with heart failure22,23 and thus may contribute to oxidative stress in this setting. More information about the role of NADPH oxidase in the myocardium and the mechanisms that regulate its expression and activity should improve our understanding of the pathophysiology of myocardial hypertrophy and failure.

Note Added in Proof

Our data are in agreement with the recent report of Byrne et al.24 who found that pressure overload–induced cardiac hypertrophy was not attenuated in mice with gp91phox deficiency and was associated with increased expression of Nox4 mRNA and protein, leading to the suggestion that Nox4 and/or other isoforms of NADPH oxidase participate in pressure overload–induced myocardial hypertrophy.

Acknowledgments

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References


Table 2. Myocyte Dimensions

<table>
<thead>
<tr>
<th>Table 2. Myocyte Dimensions</th>
<th>SHAM</th>
<th>WT</th>
<th>AAC</th>
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<tbody>
<tr>
<td></td>
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<td></td>
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<tr>
<td>Weeks</td>
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</tbody>
</table>
| Length, μm                 | 132±1| 135±1|156±1*
|                            | 135±1| 164±1†|        |
| Width, μm                  | 35±1 | 32±1†|37±1* |
|                           | 45±1†|        |        |
| Area, μm²                  | 1827±20| 1633±12†|1923±15* |
|                           | 2281±18†|        |        |
| % fibrosis                 | 3.0±0.08| 0.09±0.04|3.7±0.4* |
|                            | 4.2±0.3*|        |        |

Data are mean±SEM from 300 to 400 myocytes obtained from 1 to 2 mice per group.

*P<0.001 vs respective sham; †P<0.005 vs respective WT.
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