Pivotal Role of a gp91phox-Containing NADPH Oxidase in Angiotensin II–Induced Cardiac Hypertrophy in Mice

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Background—Angiotensin II induces both cardiac and vascular smooth muscle (VSM) hypertrophy. Recent studies suggest a central role for a phagocyte-type NADPH oxidase in angiotensin II–induced VSM hypertrophy. The possible involvement of an NADPH oxidase in the development of cardiac hypertrophy has not been studied.

Methods and Results—Mice with targeted disruption of the NADPH oxidase subunit gp91phox (gp91phox−/−) and matched wild-type mice were subjected to subcutaneous angiotensin II infusion at a subpressor dose (0.3 mg/kg/day) for 2 weeks. Systolic blood pressure was unaltered by angiotensin II in either group. Angiotensin II significantly increased heart/body weight ratio, atrial natriuretic factor and β-myosin heavy chain mRNA expression, myocyte area, and cardiac collagen content in wild-type but not gp91phox−/− mice. Angiotensin II treatment increased myocardial NADPH oxidase activity in wild-type but not gp91phox−/− mice.

Conclusions—A gp91phox-containing NADPH oxidase plays an important role in the development of angiotensin II–induced cardiac hypertrophy, independent of changes in blood pressure. (Circulation. 2002;105:293-296.)

Key Words: hypertrophy ■ angiotensin ■ free radicals ■ myocardium

Angiotensin II (Ang II) plays an important role in the development and progression of cardiac hypertrophy.1 ACE inhibitors and Ang II receptor antagonists attenuate cardiac hypertrophy, both experimentally and in hypertensive patients.1 Multiple signaling pathways, eg, protein kinase C, mitogen-activated protein kinases (MAPKs), and others, are implicated in Ang II–induced cardiac hypertrophy.2 Recent studies suggest that Ang II mediates vascular smooth muscle (VSM) hypertrophy via production of intracellular reactive oxygen species (ROS), which activate crucial signaling cascades and have mitogenic effects.3,4 ROS production has also been implicated in cardiomyocyte hypertrophy.2,5

Phagocyte-type NADPH oxidases, a major source of ROS in cardiovascular cells, are implicated in Ang II–induced VSM hypertrophy4 and hypertension.6 These oxidases are expressed in endothelium,7,8 VSM,4 adventitial fibroblasts,9 and cardiomyocytes.10 In endothelium and fibroblasts, gp91phox is the major subunit responsible for enzyme activity, whereas in VSM, homologues such as nox1 may be more important.11 To date, the potential role of a gp91phox-containing NADPH oxidase in the development of cardiac hypertrophy has not been studied.

Methods

Experiments were performed in accordance with the Guidance on the Operation of Animals (Scientific Procedures) Act, 1986 (UK) on male gp91phox−/− mice12 (The Jackson Laboratory, Maine) and matched wild-type controls with the same genetic background (C57BL/6J). gp91phox mRNA expression was analyzed by reverse transcription–polymerase chain reaction (RT-PCR) in left ventricle (LV) homogenates to confirm the absence of cardiomycocytes, using specific primers targeted to exon 3, which is disrupted in gp91phox−/− mice.12

NADPH Oxidase Activity

Isolated gp91phox−/− and control hearts (n=4/group) were perfused with Ang II (0.1 to 1 μmol/L) for 10 minutes and then snap-frozen. NADPH-dependent superoxide production was measured in LV homogenates using lucigenin (5 μmol/L)-enhanced chemiluminescence (NADPH 300 μmol/L; 100 μg protein; 37°C).4,7 Some experiments were performed in the presence of a cell-permeable O2 scavenger 4,5-dihydroxy-1,3-benzene-disulfonic acid (Tiron, 20 mmol/L), the flavoprotein inhibitor diphenyleneiodonium (DPI, 10 μmol/L), or a nitric oxide synthase inhibitor Nω-Nitro-L-arginine methyl ester hydrochloride (L-NNAME, 100 μmol/L).

Animal Model

Wild-type and gp91phox−/− mice (4±2 days old) were anesthetized by inhalation of 2% isoflurane, 98% oxygen. Osmotic minipumps (Alzet Model 1002; Alza Corp) containing either Ang II (infusion rate 0.3 mg/kg/day) or vehicle were implanted in the midscapular region. Blood pressure was monitored by tail cuff plethysmography (World Precision Instruments, UK) in conscious mice (≥9/group) following ≥3 training periods.

Assessment of Hypertrophy

Mice (n=6/group) were euthanized and body and heart weights recorded. Additional hearts (n=3/group) were fixed, sectioned (5 μm), and labeled with anti-laminin B2 antibody and counterstained...
with hematoxylin. Myocyte areas (>50 cells/section) were measured from transverse sections, by a blinded observer, using a digital image analyzer (Openlab 3.3.3, Improvision, UK). Collagen content was assessed by quantifying the blue pixel content from LV cryosections (6 μm) (n=3/group) stained with Mason’s trichrome.

Atrial natriuretic factor (ANF) and β-myosin heavy chain (β-MHC) mRNA expression was measured by semiquantitative RT-PCR, with normalization to GAPDH expression.

Statistics
Data are presented as mean±SEM. Comparisons between groups were made by 1-way ANOVA, followed by Fischer’s least significance post hoc test or Student’s unpaired t test. A value of P<0.05 was considered significant.

Results
Baseline Characteristics
gp91phox mRNA was expressed in LV of wild-type but not gp91phox−/− mice (Figure 1A). Similar results were obtained using pure isolated cardiomyocytes (not shown). There was no significant difference in body weight among the 4 groups (in grams): sham wild-type, 25.0±0.7; Ang II−infused wild-type, 24.4±0.7; sham gp91phox−/−, 25.1±0.5; Ang II−infused gp91phox−/−, 24.4±0.6.

NADPH-Dependent Superoxide Production
Ang II treatment induced significant dose-dependent increases in NADPH oxidase activity in wild-type hearts but had no effect in gp91phox−/− hearts (Figure 1B). NADPH-dependent superoxide production was abolished by DPI and Tiron in all groups, but was unaffected by L-NNAME (data not shown).

Blood Pressure
Basal systolic blood pressure was significantly lower in gp91phox−/− compared with wild-type mice (127±2.2 mm Hg versus 139±2.3 mm Hg). Ang II infusion (0.3 mg/kg/day) did not increase systolic blood pressure in either group (Figure 1C).

Cardiac Hypertrophic Response
Ang II infusion caused a significant increase in heart/body weight ratio in wild-type mice. This effect was substantially blunted and nonsignificant in gp91phox−/− mice (≈8% increase versus 20% in wild-type) (Figure 1D). Expression of ANF and β-MHC mRNA, 2 molecular markers of cardiac hypertrophy, was significantly increased after Ang II infusion in wild-type but not gp91phox−/− mice (Figures 2A and 2B). LV myocyte area was significantly increased in myocardial sections of wild-type mice treated with Ang II but not in corresponding gp91phox−/− animals (Figure 2C). Interstitial cardiac fibrosis was also significantly increased by Ang II infusion in wild-type mice, whereas no increase was observed in gp91phox−/− animals (Figure 2D).

Discussion
The principal novel finding of this study is that a gp91phox-containing NADPH oxidase, present in cardiomyocytes, is centrally involved in the direct cardiac hypertrophic response to Ang II. Although Ang II infusion could theoretically also cause cardiac hypertrophy indirectly through changes in hemodynamic load (due to vasoconstriction), this possibility was excluded in the present study by the use of a subpressor
Ang II dose. A marked attenuation of Ang II–induced cardiac hypertrophy in gp91phox−/− mice was demonstrated not only by morphometric assessment of heart/body weight ratio but also by measurements of myocyte area in LV sections and by mRNA expression of 2 standard molecular markers of cardiac hypertrophy, ANF and β-MHC. Accumulation of interstitial collagen is an important feature of cardiac hypertrophy, contributing both to ventricular remodeling and diastolic dysfunction.13 AT1-overexpressing mice display a significant increase in collagen deposition in the myocardium.14 Interestingly, the marked increase in interstitial collagen content observed with Ang II infusion in wild-type mice was virtually abolished in gp91phox−/− mice. Taken together, these data provide the first convincing evidence that several aspects of Ang II–induced cardiac hypertrophy (ie, myocyte hypertrophy and interstitial fibrosis) are dependent on the presence of a gp91phox–containing NADPH oxidase. The relative contributions of different cell types (eg, cardiomyocytes, endothelial cells, fibroblasts) to this Ang II–induced, gp91phox-dependent hypertrophic response warrants further investigation.

In VSM, Ang II increases NADPH oxidase-dependent ROS production, which is thought to activate signaling pathways involved in the hypertrophic response.5 In the present study, we found that NADPH oxidase activity was increased by Ang II in wild-type mice but that Ang II had no effect in gp91phox−/− animals. This finding is consistent with the hypothesis that ROS produced by a gp91phox–containing NADPH oxidase are involved in Ang II–mediated cardiac hypertrophy. The downstream pathways modulated by Ang II–stimulated ROS production in cardiac myocytes will require further study. In preliminary studies, we found no differences in the activation of extracellular signal regulated kinase (ERK1/2), c-Jun N-terminal kinase, or p38 MAPK between wild-type and gp91phox−/− mice treated with Ang II (data not shown).

An interesting observation in this study was that baseline systolic blood pressure was significantly lower in conscious gp91phox−/− mice compared with wild-type controls. Whereas gp91phox homologues, such as nox1, are thought to be more important than gp91phox itself for VSM function,11 the present data suggest that gp91phox is also involved in the regulation of VSM tone. Very recently, Wang et al15 also reported a reduced systolic blood pressure in gp91phox−/− mice. These authors also found that infusion of pressor doses of Ang II did increase blood pressure in gp91phox−/− mice. In the present study, it is unlikely that the lower blood pressure in gp91phox−/− mice accounted for the attenuated cardiac hypertrophic response to Ang II because we studied subpressor doses. Furthermore, in pilot studies, infusion of a pressor dose of Ang II failed to induce hypertrophy in these animals. The gp91phox−/− mice tended to have very slightly higher baseline heart/body weight ratios than wild-types (P=NS); however, this is unlikely to have limited hypertrophy because maximal hypertrophy in wild-type animals can exceed 100% (eg, after aortic banding; data not shown).

The present results add significantly to the growing body of evidence in support of an important role for NADPH oxidase in cardiovascular physiology and pathophysiology.16 Previous studies using the same mice with targeted disruption of gp91phox demonstrated an involvement of gp91phox in Ang II–induced vascular hypertrophy in vivo13 and in the regulation of vascular tone2 and basal systemic blood pressure.15 The present study is the first to suggest a direct functional role for a gp91phox–containing NADPH oxidase in the pathogenesis of Ang II–induced cardiac hypertrophy.

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