Activation of NADPH Oxidase During Progression of Cardiac Hypertrophy to Failure

Jian-Mei Li, Nick P. Gall, David J. Grieve, Mingyou Chen, Ajay M. Shah

Abstract—Increased reactive oxygen species (ROS) production is implicated in the pathophysiology of left ventricular (LV) hypertrophy and heart failure. However, the enzymatic sources of myocardial ROS production are unclear. We examined the expression and activity of phagocyte-type NADPH oxidase in LV myocardium in an experimental guinea pig model of progressive pressure-overload LV hypertrophy. Concomitant with the development of LV hypertrophy, NADPH-dependent $O_2^-$ production in LV homogenates, measured by lucigenin (5 μmol/L) chemiluminescence or cytochrome c reduction assays, significantly and progressively increased (by $\approx 40\%$ at the stage of LV decompensation; $P<0.05$). $O_2^-$ production was fully inhibited by diphenyleneiodonium (100 μmol/L). Immunoblotting revealed a progressive increase in expression of the NADPH oxidase subunits p22phox, gp91phox, p67phox, and p47phox in the LV hypertrophy group, whereas immunolabeling studies indicated the presence of oxidase subunits in cardiomyocytes and endothelial cells. In parallel with the increase in $O_2^-$ production, there was a significant increase in activation of extracellular signal–regulated kinase 1/2, extracellular signal–regulated kinase 5, c-Jun NH2-terminal kinase 1/2, and p38 mitogen-activated protein kinase. These data indicate that an NADPH oxidase expressed in cardiomyocytes is a major source of ROS generation in pressure overload LV hypertrophy and may contribute to pathophysiological changes such as the activation of redox-sensitive kinases and progression to heart failure. (Hypertension. 2002;40:477–484.)

Key Words: hypertrophy ■ free radicals ■ heart failure ■ myocardium ■ reactive oxygen species

An increase in oxidative stress resulting from increased cardiac generation of reactive oxygen species (ROS) is implicated in the pathophysiology of pressure-overload left ventricular hypertrophy (LVH) and congestive heart failure, both experimentally and in clinical studies.1-3 Increased ROS production is implicated in the development of cellular hypertrophy and remodeling, at least in part through activation of redox-sensitive protein kinases such as the mitogen-activated protein kinase (MAPK) superfamily. The transition from compensated pressure-overload LVH to heart failure is associated with increased oxidative stress, which may promote myocyte apoptosis and necrosis. Several key proteins involved in excitation-contraction coupling, such as sarcoplasmic reticulum calcium release channels, can undergo redox-sensitive alterations in activity, which contributes to myocardial contractile dysfunction. ROS also has indirect effects resulting from increased inactivation of NO and consequent generation of peroxynitrite, eg, coronary vascular endothelial dysfunction and peroxynitrite-induced inhibition of myocardial respiration.4

The sources of ROS generation that contribute to these effects in pressure-overload LVH and heart failure remain poorly defined. Potential sources include the mitochondrial electron transport chain, xanthine oxidase, cytochrome P450–based enzymes, dysfunctional NO synthases (NOSs), and infiltrating inflammatory cells such as neutrophils. Recently, a major source of ROS production in cardiovascular cells such as vascular smooth muscle, endothelium, and adventitial fibroblasts has been shown to be a phagocyte-type NADPH oxidase.5-8 The prototypic NADPH oxidase complex consists of a core heterodimer comprising 1 p22phox and 1 gp91phox subunit, and 4 regulatory subunits—p40phox, p47phox, p67phox, and rac1.9 In vascular smooth muscle, the gp91phox subunit is replaced by 1 of 2 homologs termed Nox1 and Nox4.5 The ROS-generating activity of vascular smooth muscle and endothelial NADPH oxidases is increased by stimuli such as angiotensin II, tumor necrosis factor-α, growth factors and cyclical load. Vascular NADPH oxidases are implicated in the development of angiotensin II–induced vascular smooth muscle hypertrophy, hypertension, endothelial dysfunction, and atherosclerosis.5

Whether a similar NADPH oxidase is expressed in the heart, particularly in cardiomyocytes, and whether it plays a role in the pathophysiology of LVH and heart failure have received little attention. Some studies have provided biochemical evidence for the presence of a phagocyte-type NADPH oxidase in cardiomyocytes, but the molecular nature

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of the enzyme was not addressed. Recently, we reported that experimental angiotensin II–induced cardiac hypertrophy was inhibited in gene-modified mice lacking the gp91phox subunit of NADPH oxidase. However, the response of NADPH oxidase to the more clinically relevant stimulus of chronic pressure overload is unknown. Furthermore, it is unclear what cardiac cell types the oxidase is expressed in or what changes occur in oxidase expression and activity during development of hypertrophy.

The aims of the present study were to investigate the following in an experimental model of progressive pressure-overload LVH: (1) whether alterations in expression and activity of NADPH oxidase contribute to increased ROS generation during progression to decompensated LVH and failure, (2) whether the oxidase is expressed in cardiomyocytes, and (3) the relationship between ROS generation and potential functional consequences such as activation of redox-sensitive MAPKs.

**Methods**

**Experimental Hypertrophy**

All procedures were performed in accordance with the Guidance on the Operation of the Animals (Scientific Procedures) Act 1986 (Her Majesty’s Stationery Office, London, United Kingdom). Male Dunkin-Hartley guinea pigs (200 to 250 g; Harlan UK Ltd, Bicester, UK) underwent suprarenal abdominal aortic banding or sham banding. Aortic banding in guinea pigs is a well-established model of pressure-overload LVH that has been preferred over small rodent models in several studies because guinea pig LV more closely resembles human myocardium with respect to myosin heavy-chain isoform expression and electrophysiological characteristics. In this model, compensated LVH is present from 2 weeks after surgery followed by a transition phase from 4 to 6 weeks and then overt LV decompensation at 8 to 10 weeks. Animals from the banded and sham groups were euthanized at 1, 3, 6, and 10 weeks postoperatively for harvesting of heart tissue. LV and right ventricular weights, lung weight, and body weight were determined, and tissue for immunoblotting, immunohistochemistry, or biochemical analysis was snap-frozen in liquid nitrogen and stored at −70°C until study.

**Protein extraction and Immunoblotting**

LV protein samples were prepared as described previously without Triton X-100. The antibodies against p22phox, CD31, phospho-extracellular signal–regulated kinase (ERK) 1/2, phospho-c-Jun NH2-terminal kinase (JNK), and ERK5 were from Santa Cruz Biotechnology; antibodies to gp91phox, p47phox, p47phox, and rac1 were kind gifts from Dr F Wientjes (University College London, United Kingdom); the monoclonal anti-p67phox was a kind gift from Dr M Quinn (Montana State University, Bozeman, Mont). Human neutrophil membrane protein (kindly provided by Dr F Wientjes) was the positive control for p22phox and gp91phox, and the protein extract from human phagocytic U937 cells after phorbol 12-myristate 13-acetate stimulation was the positive control for the other subunits. An anti-cardiac troponin I monoclonal antibody was kindly provided by Dr I Trayer (University of Birmingham, United Kingdom). The anti-a-tubulin monoclonal was from Sigma; and the anti-phospho-p38 MAPK antibody was from Cell Signaling Technology.

**Immunohistochemistry**

Samples were prepared as described previously. Frozen sections were first treated with a Biotin Blocking kit (DAKO) according to manufacturer instructions. Primary antibodies were used at 1:50 to 1:250 dilution in 0.1% BSA/PBS for 30 minutes at room temperature. Biotin-conjugated anti-rabbit, anti-goat, or anti-mouse IgG (1:500 dilution) was used as a secondary and was detected by a StreptABCComplex/HRP kit (DAKO). For immunofluorescence staining, specific binding was detected by fluorescein isothiocyanate– or tetramethyl rhodamine isothiocyanate (TRITC)-labeled extravidin (green and red fluorescence, respectively). Normal rabbit or mouse IgG (5 μg/mL) was used instead of primary antibody as a negative control. Images were acquired on a Zeiss microscope coupled to a digital imaging system (Improvision, United Kingdom).

**Lucigenin Chemiluminescence**

O2− production by LV tissue homogenate (n=6 hearts per group at each time point) was measured using lucigenin-enhanced chemiluminescence in a microplate luminometer (Anthos Lucy 1, Austria). A low lucigenin concentration (5 μmol/L) was employed to minimize artifactual O2− production owing to redox cycling. Briefly, proteins were diluted in modified HEPES buffer and distributed (100 μg/well) onto a 96-well microplate. NADPH (100 μmol/L) and dark-adapted lucigenin were added to wells just before reading. O2− production was expressed as arbitrary light units over 20 minutes. Some experiments were performed in the presence of superoxide dismutase (SOD, 200 U/mL) or a cell-permeable superoxide scavenger, tiron (10 mmol/L). The following agents were used in some experiments to assess potential sources of O2− production: diphenyleneiodonium (100 μmol/L), a flavoprotein inhibitor that blocks NADPH oxidase; the NOS inhibitor N-nitro-L-arginine methyl ester (100 μmol/L); an inhibitor of the mitochondrial electron chain, rotenone (50 μmol/L); and a xanthine oxidase inhibitor, oxyquinol (100 μmol/L). All studies were performed in triplicate.

**Cytochrome c Reduction Assay**

NADPH-dependent O2− production was also examined using SOD-inhibitable cytochrome c reduction. LV tissue homogenate (final concentration 1 mg/mL) diluted in Dulbecco’s modified Eagle’s medium without phenol red was distributed in 96-well plates (final volume, 200 μL/well). Cytochrome c (500 μmol/L) and NADPH (100 μmol/L) were added in the presence or absence of SOD (200 U/mL) and incubated at room temperature for 30 minutes. Cytochrome c reduction was measured by reading absorbance at 550 nm on a microplate reader.

**Statistics**

All data are presented as mean±SD from ≥6 animals in each group at each time point after the operation (except anywhere mentioned in the text). Comparisons were made by unpaired t test, with Bonferroni correction for multiple testing, or by 1-way ANOVA as appropriate. P<0.05 was considered significant.

**Results**

**NADPH-Dependent ROS Generation During Progression of LVH**

As reported previously, LV/body weight ratio was significantly higher by 3 weeks after surgery in the banded group compared with the sham group. Lung/body weight ratio was increased in the banded group by 10 weeks after surgery (Figure 1A and 1B). Body weights were similar in bands and shams at each stage (data not shown).

NADPH-dependent O2− production by LV homogenates assessed by lucigenin chemiluminescence was significantly increased in the banded group by 3 weeks after surgery, concomitant with development of LVH, and had increased further by 6 weeks after surgery (Figure 1C). No further increase in ROS production was observed between 6 and 10 weeks.

Similar results were obtained with the cytochrome c reduction and lucigenin assays. Figure 2A shows a comparison of ROS production measured by cytochrome c reduction or lucigenin chemiluminescence at the 10-week stage.
Enzymatic sources of NADPH-dependent ROS production were examined using specific inhibitors in the lucigenin assay. Figure 2B shows results for the 10-week stage (similar results were obtained at 3 and 6 weeks). ROS production was abolished either by tiron or diphenyleneiodonium, and was substantially reduced by SOD, both in the banded and sham groups. Rotenone and oxypurinol had no effect in either group. Interestingly, there was a slight (17 ± 11%) but significant (P < 0.05) reduction in NADPH-dependent ROS production in the presence of Nω-nitro-arginine methyl ester in the banded group.

**Protein Expression of NADPH Oxidase Subunits**

LV tissue homogenates from the same hearts as used for measuring ROS production were used for immunoblotting. All 5 subunits of NADPH oxidase—ie, p22phox, gp91phox, p67phox, p47phox, and p40phox, and rac1—were detectable in both groups using anti-human neutrophil antibodies (Figure 3). gp91phox in guinea pig LV tissue migrated as a band at 75 kDa and 2 bands of higher apparent molecular weight (mw) at ~90 to 100 kDa, which was broadly similar to the pattern for neutrophil membrane protein. p47phox was detected as a doublet band, both in guinea pig myocardium and U937 cells. The expression of p22phox, gp91phox, p67phox, and p47phox became progressively higher in the LVH group compared with shams with increasing LVH (Figures 3 and 4). Interestingly, p22phox, gp91phox, and p67phox expression appeared to increase earlier than did p47phox, which did not increase until 6 weeks after surgery. The expression level of p40phox and rac1 was similar in the 2 groups.

To assess which cell types in the heart expressed NADPH oxidase subunits, we undertook immunohistochemistry on LV sections from sham and LVH groups. Figure 5A shows the expression of gp91phox detected by peroxidase histostaining in sham (top) and LVH (middle) at 10 weeks after surgery. It is evident that gp91phox was expressed within the cardiac myocytes and that the level of expression was higher in LVH tissue compared with shams. Similar results were obtained with immunofluorescence staining (data not shown). p67phox and p47phox were also expressed in cardiomyocytes (data not shown). NADPH oxidase subunits were also expressed in endothelial cells, albeit at a lower level. Figure 5B
shows LV sections double immunolabeled with a goat polyclonal anti-CD31 antibody as a specific endothelial cell marker \(^1\) (red fluorescence) and either a monoclonal anti-p67\(_{\text{phox}}\) antibody (green fluorescence; Figure 5B, left) or a rabbit polyclonal anti-p22\(_{\text{phox}}\) antibody (green fluorescence; Figure 5B, right). Superimposition of the images indicates that p67\(_{\text{phox}}\) and p22\(_{\text{phox}}\) were expressed both in coronary endothelium and cardiomyocytes.

Figure 3. Representative immunoblots showing changes in expression of NADPH oxidase subunits in guinea pig LV tissue during LVH progression. Cardiac troponin I expression was used as a loading control. Neutrophil membrane extract was used as a positive control for p22\(_{\text{phox}}\) and gp91\(_{\text{phox}}\), and phorbol 12-myristate 13-acetate-stimulated U937 cell protein was used for the other subunits.

Figure 4. Changes in protein expression of p22\(_{\text{phox}}\), gp91\(_{\text{phox}}\), p67\(_{\text{phox}}\), p47\(_{\text{phox}}\), p40\(_{\text{phox}}\), and rac1 during LVH progression. Immunoblots from 3 independent sets of experiments (n=9) were densitometrically scanned, and the results were expressed as mean±SD. All 3 bands were scanned for gp91\(_{\text{phox}}\). Cardiac troponin I detected in the same sample was used as a loading control. *P<0.05 vs respective sham controls by unpaired t test with Bonferroni correction.
Changes in Expression and Phosphorylation of p38-MAPK, ERK1/2, JNK, and ERK5 During the Development of LVH and Heart Failure

To investigate the possible relationship between NADPH oxidase activation and the activation of redox-sensitive kinases, we measured the phosphorylation of ERK1/2, p38-MAPK, JNK, and ERK5 (Figure 6). The levels of phosphorylated ERK1/2 increased significantly in LV myocardium of the banded group compared with shams by 3 weeks after surgery and continued to increase further with progression of LVH and heart failure. The expression of ERK5 and phospho-p38 MAPK was significantly increased in LVH samples at 6 and 10 weeks after surgery. The levels of phosphorylated JNK increased significantly during the early stages of LVH but declined during the phase of LV decompensation (10 weeks).

Discussion

The major findings of the present study are that (1) all the main components of a functional phagocyte-type NADPH oxidase are expressed in guinea pig myocardium, notably in cardiomyocytes; (2) NADPH oxidase–derived ROS generation increases significantly in pressure overload LVH, at least partly as a result of increased expression of oxidase components; and (3) the increase in myocardial NADPH oxidase activity is paralleled by activation of the redox-sensitive kinases ERK1/2, ERK5, and p38 MAPK and by progression of cardiac hypertrophy. Collectively, these findings suggest that NADPH oxidase activation may play an important pathophysiological role in progressive pressure-overload LVH and heart failure.

Although a phagocyte-type NADPH oxidase has been demonstrated in many cardiovascular cell types, previous data for its expression in cardiomyocytes were mainly bio-
In a recent study, cardiac mRNA expression of p22phox and gp91phox was reportedly increased after myocardial infarction; however, this was probably related to increased neutrophil infiltration, whereas evidence of cardiomyocyte expression was not reported. In the current study, we show that all the main subunits of the phagocyte-type NADPH oxidase (including gp91phox) are expressed at protein level in guinea pig myocardium, and that the cell types in which the oxidase is expressed include the cardiomyocyte.

Although increased ROS production during progressive pressure-overload LVH is well recognized, the sources of these ROS have been unclear. In this study, we clearly demonstrated that a phagocyte-type NADPH oxidase was a major ROS source. Interestingly, a small but significant proportion (∼17%) of NADPH-dependent ROS production could be inhibited by an NOS inhibitor, suggesting that dysfunctional NOS activity may also partly contribute to ROS production. This is not surprising because it has been suggested that ROS production from any source may induce dysfunctional O2−-generating NOS activity, as a consequence of ROS-dependent degradation of the essential NOS cofactor tetrahydrobiopterin. Potential mechanisms through which NADPH oxidase activity is increased include transcriptional upregulation of oxidase components and posttranslational modifications, such as p47phox phosphorylation and rac1 translocation. In the present study, at least part of the mechanism underlying the increased NADPH oxidase activity was an increase in expression of p22phox, gp91phox, p67phox, and p47phox. Stimuli that augment NADPH oxidase activity in the peripheral vasculature and may be relevant to pressure-overload LVH include angiotensin II, tumor necrosis factor-α, and mechanical stretch. The relative contribution of these stimuli to the upregulation observed in the present study requires further investigation.

An increase in ROS production may have several potential effects in the hypertrophying heart. In isolated cardiomyocytes, a moderate increase in ROS induces hypertrophy and apoptosis. Angiotensin II- and tumor necrosis factor-α-induced hypertrophy of cultured cardiomyocytes is abrogated by antioxidants. Recently, we reported that angiotensin II induced in vivo cardiac hypertrophy was inhibited in gp91phox-null mice. The mitogenic effects of ROS may involve modulation of redox-sensitive signaling pathways such as Src kinases and MAPKs (including ERK1/2, ERK5, and p38).
MAPK), which leads to activation of transcriptional factors.\textsuperscript{1,24,25} The subsequent changes in gene expression may account for many of the changes in LVH. Activation of redox-sensitive signaling pathways may also be implicated in the transition of compensated LVH to heart failure. In the present study, increased myocardial NADPH oxidase activity was accompanied by activation of ERK1/2, ERK5, and p38 MAPK. ERK1/2 has been implicated in cardiac hypertrophy,\textsuperscript{1,25–27} being induced by increased pressure\textsuperscript{28} and G\textsubscript{q}-coupled receptor agonists.\textsuperscript{29} ERK1/2 and ERK5 are significantly activated by H\textsubscript{2}O\textsubscript{2} perfusion in the isolated guinea pig heart.\textsuperscript{30} Although many studies have proposed that ROS mediates cardiomyocyte hypertrophic response to agonist stimulation via the ERK cascade,\textsuperscript{3,26,27} the mechanisms that couple hypertrophic signals to activation of ERK signaling and gene expression remain unclear. The current results suggest that agonist-induced activation of NADPH oxidase may be an important upstream component of the pathway leading to activation of redox-sensitive MAPKs such as ERK1/2 and ERK5. Interestingly, in the present study, ERK1/2 activation was observed quite early at the stage of compensated LVH, whereas activation of p38 MAPK and ERK5 occurred at the transition stage between compensated and decompensated LVH. This could reflect a differential involvement of these kinases in hypertrophy versus transition to heart failure.\textsuperscript{31} In addition, we found that while activation of JNK was observed early during LVH, during the development of failure the levels of phospho-JNK declined despite maintained NADPH oxidase activity. This finding suggests dissociation between NADPH oxidase–derived ROS production and the activation of specific MAPKs.

Another important consequence of increased ROS production is endothelial dysfunction resulting from increased inactivation of NO.\textsuperscript{19} It is of interest that we found NADPH oxidase expression not only in cardiomyocytes but also in coronary microvascular endothelium. Indeed, it is well recognized that endothelial cells express NADPH oxidase and produce O\textsubscript{2}–,\textsuperscript{5,7,19,32} and we have recently reported that increased endothelial ROS production may contribute to impaired LV relaxation in pressure-overload LVH.\textsuperscript{33}

**Perspectives**

This is the first study to report a progressive activation of NADPH oxidase in the myocardium in pressure overload LVH and suggests that this enzyme system may play a role in myocardial pathophysiology analogous to reports of its importance in the vasculature. NADPH oxidase–derived ROS may potentially contribute to several aspects of the pathophysiology of LVH and heart failure. In particular, activation of redox-sensitive signaling molecules such as ERK1/2, ERK5, and p38 MAPK may be involved in the development of hypertrophy and/or transition to heart failure.

To define the precise contribution of NADPH oxidase–derived in LVH and the mechanisms underlying upregulation of NADPH oxidase activity after imposition of pressure overload, it will be necessary to undertake appropriate studies in experimental models, including gene-modified animals. The possibility that targeting myocardial NADPH oxidase expression and activity may provide a means of modifying development of LVH and heart failure merits investigation.

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