Involvement of Nox2 NADPH Oxidase in Adverse Cardiac Remodeling After Myocardial Infarction

Yee H. Looi, David J. Grieve, Anjana Siva, Simon J. Walker, Narayana Anilkumar, Alison C. Cave, Michael Marber, Mark J. Monaghan, Ajay M. Shah

Abstract—Oxidative stress plays an important role in the development of cardiac remodeling after myocardial infarction (MI), but the sources of oxidative stress remain unclear. We investigated the role of Nox2-containing reduced nicotinamide-adenine dinucleotide phosphate oxidase in the development of cardiac remodeling after MI. Adult Nox2−/− and matched wild-type (WT) mice were subjected to coronary artery ligation and studied 4 weeks later. Infarct size after MI was similar in Nox2−/− and WT mice. Nox2−/− mice exhibited significantly less left ventricular (LV) cavity dilatation and dysfunction after MI than WT mice (eg, echocardiographic LV end-diastolic volume: 75.7±5.8 versus 112.4±12.3 μL; ejection fraction: 41.6±3.7 versus 32.9±3.2%; both P<0.05). Similarly, in vivo LV systolic and diastolic functions were better preserved in Nox2−/− than WT mice (eg, LV dp/dt max: 7969±385 versus 5746±234 mm Hg/s; LV end-diastolic pressure: 12.2±1.3 versus 18.0±1.8 mm Hg; both P<0.05). Nox2−/− mice exhibited less cardiomyocyte hypertrophy, apoptosis, and interstitial fibrosis; reduced increases in expression of connective tissue growth factor and procollagen 1 mRNA; and smaller increases in myocardial matrix metalloproteinase−2 activity than WT mice. These data suggest that the Nox2-containing reduced nicotinamide-adenine dinucleotide phosphate oxidase contributes significantly to the processes underlying adverse cardiac remodeling and contractile dysfunction post-MI. (Hypertension. 2008;51:319-325.)

Key Words: NADPH oxidase ■ cardiac remodeling ■ reactive oxygen species ■ fibrosis ■ hypertrophy

Myocardial infarction (MI) is the most common cause of heart failure in developed countries. Heart failure develops because of adverse post-MI cardiac remodeling, a process that involves progressive changes in structure and function of the extracellular matrix and cardiac myocytes in the noninfarcted myocardium and results in significant left ventricular (LV) cavity dilatation, interstitial fibrosis, myocyte hypertrophy, and depressed contractile function. Reactive oxygen species (ROS) production and oxidative stress are increased after both experimental and clinical MI, and growing evidence supports an important role for this in the processes underlying cardiac remodeling post-MI. ROS modulate several of the processes underlying cardiac remodeling, such as the activation and expression of matrix metalloproteinases (MMPs), interstitial fibrosis, and myocyte hypertrophy. In addition, experimental reduction in ROS levels can attenuate remodeling post-MI, eg, after chronic treatment with dimethylthiourea or transgenic overexpression of glutathione peroxidase.

The sources of ROS production and the mechanisms by which they influence cardiac remodeling post-MI remain unclear. Recent studies indicate that a major ROS source in the heart is a family of reduced nicotinamide-adenine dinucleotide phosphate (NADPH) oxidases. The prototypic NADPH oxidase is composed of a membrane-bound heterodimer consisting of a catalytic Nox2 subunit (also known as gp91phox) and a p22phox subunit and several cytosolic subunits (p47phox, p67phox, p40phox, and Rac) that associate with the heterodimer in the activated enzyme. Five Nox isoforms (Nox1 to 5) form the basis of distinct NADPH oxidases. Nox2 and Nox4 are expressed in cardiomyocytes, fibroblasts, and endothelial cells. The Nox2 NADPH oxidase was found to have an important role in angiotensin II–induced cardiac hypertrophy and interstitial fibrosis, with both of these processes being inhibited in Nox2−/− mice subjected to short-term angiotensin II infusion. Furthermore, angiotensin II–induced hypertrophy was inhibited in mice with cardiomyocyte-specific deletion of Rac1, an essential subunit for Nox2 activation, suggesting that Nox2 in cardiomyocytes may be involved in the hypertrophic response. However, Nox2−/− mice subjected to chronic pressure overload by aortic constriction developed a similar extent of hypertrophy to matched wild-type (WT) mice, indicating that Nox2 is dispensable for the response to pressure overload. Nevertheless, the Nox2−/− mice in the latter study had reduced interstitial fibrosis after aortic constriction, suggest-
ing that Nox2 may exert specific effects on the extracellular matrix.\textsuperscript{17} Taken together, these studies suggest that the involvement of Nox2 in cardiac hypertrophy may vary depending on the precise stimulus for hypertrophy.

The possible role of Nox2 NADPH oxidase in cardiac remodeling post-MI has not been investigated. However, Nox2 expression was reportedly increased in human cardiomyocytes in myocardial samples from patients who died of MI,\textsuperscript{13} as well as in the myocardium of rats after MI.\textsuperscript{18} Increased NADPH oxidase activity was also documented in the myocardium of patients with end-stage heart failure.\textsuperscript{12} The current study investigated the role of Nox2 oxidase in cardiac remodeling post-MI.

**Methods**

A detailed Methods section is provided in the online data supplement (available at http://hyper.ahajournals.org). In brief, MI was achieved by permanent left coronary ligation in female Nox2\textsuperscript{-/-} mice and matched WT controls. “Sham” groups underwent similar surgery apart from left anterior descending ligation. Experimental analyses were undertaken at 4 weeks postsurgery. Cardiac structure and function were assessed by echocardiography, cardiac catheterization, and histology. Apoptosis was determined by TUNEL staining. Gene expression was analyzed by real-time PCR using β-actin mRNA for normalization. NADPH-dependent superoxide production was determined in noninfarcted LV homogenates using lucigenin (5 μmol/L)-enhanced chemiluminescence.\textsuperscript{9} MMP gelatinolytic activity was assessed by gelatin zymography.\textsuperscript{7}

**Results**

**Infarct Size and Survival**

Infarct size 4 weeks after MI was not significantly different between groups (WT MI, 39.9±1.5% versus Nox2\textsuperscript{-/-} MI, 42.6±0.9%; n=6 per group; P value not significant). Similarly, infarct size measured 24 hours after MI by triphenyl-tetrazolium chloride staining, before any significant remodeling had occurred, was also not different between groups (WT MI, 44.6±2.0% versus Nox2\textsuperscript{-/-} MI, 48.6±2.5%; n=6 per group; P value not significant). Area at risk was also similar between groups (data not shown). Survival rate at 4 weeks post-MI was slightly higher in Nox2\textsuperscript{-/-} mice compared with WT mice (92% versus 79%; n=38 per group) but was not statistically significant (P=0.098). No deaths were observed in sham-operated groups.

**Cardiac Volumes and Function by Echocardiography**

The Table provides data for echocardiographic parameters measured 4 weeks post-MI. LV dilatation after MI as measured by LV end-diastolic diameter or volume was significantly reduced in Nox2\textsuperscript{-/-} mice compared with WT mice. Similarly, Nox2\textsuperscript{-/-} mice had better systolic function after MI compared with WT, reflected in a higher percentage of fractional shortening and ejection fraction and a lower LV end-diastolic diameter and volume. Heart rate was similar among groups. Figure 1 shows representative M-mode recordings illustrating the reduced LV remodeling in Nox2\textsuperscript{-/-} mice.

**Cardiac Catheterization**

Cardiac systolic dysfunction post-MI was significantly less in Nox2\textsuperscript{-/-} mice than WT mice, as indexed by LV dp/dt\textsubscript{max} or LV dp/dt\textsubscript{min} normalized for LV end-diastolic volume (Table). Similarly, LV dp/dt\textsubscript{min} and the isovolumic relaxation time

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT Sham</th>
<th>Nox2\textsuperscript{-/-} Sham</th>
<th>WT MI</th>
<th>Nox2\textsuperscript{-/-} MI</th>
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<tr>
<td>n</td>
<td>24</td>
<td>24</td>
<td>29</td>
<td>33</td>
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<tr>
<td>Heart rate, bpm</td>
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<td>439±10</td>
<td>469±10</td>
<td>461±10</td>
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<td>LVESD, mm</td>
<td>3.77±0.04</td>
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<td>5.22±0.11*</td>
<td>4.81±0.09†</td>
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<tr>
<td>LVEDD, mm</td>
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<td>2.45±0.06</td>
<td>4.17±0.12*</td>
<td>3.67±0.10†</td>
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<td>LVEDV, μL</td>
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<td>42.0±2.4</td>
<td>112.4±12.3*</td>
<td>75.7±5.8†</td>
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<tr>
<td>LVEDV, μL</td>
<td>20.4±1.4</td>
<td>16.0±1.2</td>
<td>79.1±11.7*</td>
<td>46.2±6.0†</td>
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<td>Fractional shortening, %</td>
<td>32.3±1.5</td>
<td>35.4±1.3</td>
<td>20.1±1.1*</td>
<td>24.0±1.0†</td>
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<tr>
<td>Ejection fraction, %</td>
<td>57.7±2.4</td>
<td>61.5±2.1</td>
<td>32.9±3.2*</td>
<td>41.6±3.7†</td>
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</tbody>
</table>

LVESD indicates LV end-systolic diameter; LVEDD, LV end-diastolic diameter; LVEDV, LV end-diastolic volume; LVEDV, LV end-systolic volume; MABP, mean arterial blood pressure; LVEDP, LV end-diastolic pressure.

\*P<0.05 vs appropriate sham control.

†P<0.05 Nox2\textsuperscript{-/-} MI vs WT MI.
constant, τ, were significantly less impaired in Nox2−/− mice, indicating better preserved LV relaxation. LV end-diastolic pressure increased post-MI but to a significantly lesser extent in Nox2−/− mice than WT mice (Table). Mean arterial blood pressure was reduced only in WT mice after MI.

**Myocardial Hypertrophy**

MI resulted in a significant increase in the heart/body weight ratio in WT but not Nox2−/− mice (13.8±2.9% versus 3.6±3.1%, respectively; Figure 2A). Consistent with this, myocyte cross-sectional area increased in the noninfarcted LV sections of WT MI mice to a greater extent than in Nox2−/− MI mice (Figure 2B). Atrial natriuretic factor mRNA expression in the noninfarcted left ventricle also increased significantly more in WT MI than Nox2−/− MI mice (Figure 2C), but no changes were found in brain-type natriuretic peptide expression (data not shown). MI caused significantly increased lung/body weight ratio in WT, which was attenuated in Nox2−/− mice (WT MI, 9.4±0.8 mg/g versus WT sham, 6.3±0.1 mg/g; n=19; P<0.05; Nox2−/− MI, 8.8±0.6 mg/g versus Nox2−/− sham, 7.6±0.2 mg/g; n=18; P value not significant).

**Apoptosis**

In heart sections examined 4 weeks post-MI, there was a significant increase in TUNEL-positive cells in the noninfarcted remodeling myocardium of WT mice, which was significantly blunted in the Nox2−/− mice after MI (Figure 2D).

**Cardiac Fibrosis**

Four weeks after MI, the level of interstitial fibrosis in the noninfarcted LV was significantly lower in Nox2−/− mice compared with WT mice (Figure 3 and Figure 4A). Consistent with this, mRNA expression of procollagen I, fibronectin, and connective tissue growth factor was significantly attenuated in Nox2−/− MI compared with WT MI mice (Figure 4B through 4D). Procollagen III mRNA level was similar in Nox2−/− and WT MI groups (data not shown). Perivascular fibrosis was significantly increased after MI, but the levels were not significantly different between WT and Nox2−/− groups (WT sham, 0.29±0.04% fibrosis/vessel area versus WT MI, 0.61±0.06%; n=6; P<0.05; Nox2−/− sham, 0.27±0.02% versus Nox2−/− MI, 0.59±0.06%; n=6; P value not significant).

**Myocardial MMP Activity**

Myocardial MMP-2 activity was significantly increased 4 weeks post-MI in WT animals. However, there was no significant increase in MMP-2 activity in Nox2−/− MI mice (Figure 4E and Figure S1, available in the online supplement). Expression of MMP-2 mRNA in WT mice after MI was also significantly higher than in Nox2−/− MI mice (Figure 4F). Expression of tissue inhibitor of metalloproteinase 2 mRNA was similar in the WT and Nox2−/− MI groups (Figure 4G). MMP-9 activity was found to be low in all of the groups (data not shown).

**Figure 1.** Representative M-mode echocardiograms from Nox2−/− and WT mice subjected to MI or sham coronary ligation.

**Figure 2.** Markers of cardiac hypertrophy in WT and Nox2−/− mice. A, Heart weight/body weight ratio (HW/BW); n=18. B, Myocyte cross-sectional area. C, Atrial natriuretic factor mRNA expression in the noninfarcted left ventricle also increased significantly more in WT MI than Nox2−/− MI mice (Figure 2C), but no changes were found in brain-type natriuretic peptide expression (data not shown). MI caused significantly increased lung/body weight ratio in WT, which was attenuated in Nox2−/− mice (WT MI, 9.4±0.8 mg/g versus WT sham, 6.3±0.1 mg/g; n=19; P<0.05; Nox2−/− MI, 8.8±0.6 mg/g versus Nox2−/− sham, 7.6±0.2 mg/g; n=18; P value not significant).
Expression and NADPH Oxidase Activity
Nox2 mRNA expression increased significantly in the non-infarcted left ventricle of WT animals post-MI but was not detected in Nox2\(^{-/-}\) animals (Figure 5A). On the other hand, Nox4 mRNA expression increased significantly after MI in both WT and Nox2\(^{-/-}\) groups (Figure 5B). NADPH-dependent superoxide production in myocardial homogenates was significantly increased after MI to a similar level in both WT and Nox2\(^{-/-}\) groups (Figure 5C). Superoxide generation was virtually abolished by diphenyleneiodonium or Tiron but was not inhibited by the NO synthase inhibitor N\(^{\text{G}}\)-nitro-L-arginine methyl ester hydrochloride, allopurinol, or rotenone in any group (data not shown).

3-Nitrotyrosine Staining and Levels
We assessed 3-nitrotyrosine levels as an in situ marker of myocardial oxidative/nitrosative stress. Immunohistochemistry demonstrated increased 3-nitrotyrosine staining in non-infarcted LV sections of WT hearts post-MI compared with shams (Figure 6A and 6B). The increase in 3-nitrotyrosine staining post-MI was significantly attenuated in Nox2\(^{-/-}\) mice (Figure 6C and 6D). Analyses of tyrosine nitrosylated proteins by immunoblot confirmed higher levels in WT MI than Nox2\(^{-/-}\) MI mice (Figure 7).

Discussion
The major novel finding of this study is that the Nox2 NADPH oxidase contributes significantly to adverse cardiac remodeling after MI. Nox2\(^{-/-}\) mice demonstrated reduced LV dilatation, cardiomyocyte hypertrophy, apoptosis, interstitial fibrosis, and MMP-2 activity and had better preserved contractile function after MI compared with WT littermates. These differences were not attributable to differences in infarct size, which were similar in WT and Nox2\(^{-/-}\) mice, consistent with previous data reporting no differences in infarct size in p47phox\(^{-/-}\) mice (which are also deficient in Nox2 NADPH oxidase activity).\(^{19}\)

Increasing evidence suggests that ROS contribute to the development and progression of LV remodeling post-MI, but the sources of ROS production and their effects on the mechanisms underlying the remodeling process remain unclear. Potential cardiac ROS sources include mitochondria, xanthine oxidase, uncoupled NO synthases, and NADPH oxidases\(^{20}\) and in the early stages immediately post-MI, also infiltrating inflammatory cells.\(^{21}\) NADPH oxidases are of particular interest, because ROS production by these enzymes...
is specifically triggered by agonists and seems especially important in modulating downstream redox signaling.\textsuperscript{14,15} The recognition that there are 5 oxidase isoenzymes (Nox1 through 5) with distinct patterns of tissue expression and regulation\textsuperscript{14} also suggests that there may be isoform-specific actions. Recent studies from several groups showed that Nox2-derived ROS are involved in the development of cardiac hypertrophy depending on the stimulus; whereas Nox2 is pivotal in short-term angiotensin II–induced hypertrophy, it is dispensable for pressure overload hypertrophy.\textsuperscript{9–11,17} The current study clearly implicates Nox2 oxidase in the development of cardiac hypertrophy and other aspects of adverse remodeling post-MI, because genetic deletion of Nox2 significantly attenuated these effects. Although investigation of the possible contribution of other ROS sources, such as xanthine oxidase or uncoupled NO synthases, was beyond the scope of the current study, it is noteworthy that NADPH oxidase–derived ROS can act as a trigger for ROS generation by these sources.\textsuperscript{22} However, uncoupled NO synthases (which require NADPH as a cofactor) were probably not a major contributor in the current study, because NADPH-dependent ROS generation was not reduced by an NO synthase inhibitor.

Nox2 deletion resulted in less adverse LV remodeling after MI by influencing several components of the cardiac phenotype, including ventricular dilatation, interstitial fibrosis, cardiomyocyte hypertrophy, and apoptosis. MMPs are the major enzymes responsible for matrix turnover, and an increase in MMP activity relative to tissue inhibitor of metalloproteinase is recognized to be an important driver of ventricular dilatation post-MI.\textsuperscript{23} Furthermore, MMP activation is highly redox sensitive and, in the vasculature, MMP-2 activation by mechanical stress was reported to be NADPH

Figure 5. Myocardial Nox isoform expression and NADPH oxidase activity in WT and Nox2\textsuperscript{−/−} mice. A and B, Nox2 and Nox4 mRNA expression. C, NADPH oxidase activity measured by lucigenin chemiluminescence (CL); RLU indicates relative light units. Values are mean±SEM, n=6 per group. *P<0.05 vs appropriate sham control; ND indicates not detected.

Figure 6. Representative left ventricular sections stained for 3-nitrotyrosine. A, WT sham. B, WT MI. C, Nox2\textsuperscript{−/−} sham. D, Nox2\textsuperscript{−/−} MI. E, Semiquantification data (mean±SEM), n=4 per group. *P<0.05 vs appropriate sham control; †P<0.05 vs WT MI.

Figure 7. Western blot analysis of 3-nitrotyrosine protein expression. A, Representative blot. B, Mean data±SEM, n=4 per group. *P<0.05 vs appropriate sham control; †P<0.05 vs WT MI.
oxidase–dependent, being inhibited in p47phox−/− mice. In the current study, the Nox2-dependent increase in MMP-2 expression without a corresponding change in tissue inhibitor of metalloproteinase 2 expression is likely to have been important for LV dilatation, although this does not exclude the involvement of other MMPs. An increase in interstitial fibrosis associated with dysregulation of normal matrix turnover is also a prominent feature of the remodeled heart. Oxidative stress promotes fibrosis by modulating fibroblast proliferation, collagen synthesis, and MMP activation. We found that interstitial fibrosis accompanying remodeling was significantly attenuated in Nox2−/− mice together with a blunting of increases in procollagen I and fibronectin expression in the noninfarcted myocardium. This is a similar mechanism to previous data demonstrating Nox2-dependent cardiac profibrotic effects in response to angiotensin II infusion or pressure overload, as well as Nox2-dependent vascular fibrosis. The observed increase in connective tissue growth factor expression may be an important contributor to the Nox2-dependent increase in fibrosis.

Adverse remodeling post-MI is generally accompanied by significant contractile dysfunction. In vivo comparison of cavity size and contractile function in Nox2−/− and WT mice by echocardiography and cardiac catheterization showed that LV dilation indexed by LV end-diastolic diameter and volume was significantly reduced post-MI in Nox2−/− mice. In addition, systolic dysfunction, as evidenced by decreases in echocardiographic ejection fraction and fractional shortening and reduction in LV dp/dt max or in LV dp/dt min normalized for end-diastolic volume (to correct for the preload-dependence of this index), was significantly reduced in Nox2−/− mice. Hearts of Nox2−/− mice also had better isotropic properties (higher LV dp/dt max and lower τ) and less diastolic dysfunction as assessed by LV end-diastolic pressure. At least part of the reason for better preserved contractile function in Nox2−/− mice may be as a secondary effect of reduced matrix remodeling and fibrosis. However, we also found that myocyte apoptosis in the noninfarcted myocardium was significantly reduced in post-MI Nox2−/− hearts, which may contribute to better overall function. In addition, ROS could potentially impact on contractile function through effects on excitation–contraction coupling, myofilament properties, and/or cellular energetics.

An interesting finding in the current study was that the expression of Nox4 increased significantly and to a similar extent in the WT and Nox2−/− groups. As expected, Nox2 was only expressed in WT hearts, and the levels increased modestly in the remodeling heart. Total NADPH oxidase activity in ex vivo myocardial homogenates was increased to a similar extent in WT and Nox2−/− hearts post-MI; this is most likely to have been because of the significant increase in Nox4 expression in both groups, because Nox4 is known to have much higher constitutive basal activity than Nox2. On the other hand, 3-nitrotyrosine staining and immunoblotting as a marker of in situ oxidative/nitrosative stress were significantly greater in WT compared with Nox2−/− mice after MI. The reasons for the discrepancy between ex vivo oxidase activity and in situ nitrotyrosine staining are not clear, but an explanation may be that the in situ marker better reflects chronic Nox2-derived ROS generation, because this Nox isoform requires specific activation to generate ROS. Several recent studies report that different Nox isoforms may have distinct effects in pathological settings where either Nox1 or Nox2 is coexpressed. The finding in the current study that Nox2-deficient animals had better preserved LV function after MI supports the idea that the presence of Nox4, per se, cannot compensate for the absence of Nox2 in this setting. However, this does not exclude the possibility of cross-talk between the 2 isoforms; indeed, the observation that Nox4 levels increased in both WT and Nox2−/− groups suggests that this isoform may have a distinct role.

The current study does not establish which Nox2-expressing cell types are responsible for the differences in post-MI remodeling found between WT and global Nox2 knockout mice. Nox2 is known to be expressed in cardiomyocytes, endothelial cells, fibroblasts, and inflammatory cells; all cell types that could potentially contribute to adverse remodeling. Previous studies in isolated cells convincingly demonstrated that Nox2 may contribute to hypertrophy of cardiomyocytes, activation of endothelial cells, proliferation of vascular adventitial fibroblasts, and phagocytosis of leukocytes. At the cardiomyocyte level, activation of extracellular signal–regulated kinase 1/2 and the transcription factor nuclear factor kB are both implicated in angiotensin II–induced hypertrophy, and an involvement of cardiomyocyte Nox2 in vivo is suggested by a recent study in mice with cardiomyocyte-specific deletion of Rac1. Altered signaling in the cardiomyocyte readily induces changes in the extracellular matrix and fibroblasts, eg, through paracrine effects of secreted growth factors. Alternatively, an influx of inflammatory cells may also influence remodeling post-MI, secondary to increases in infarct size and through the generation of cytokines and other factors that activate MMPs and modify the extracellular matrix. Interestingly, although Nox2 oxidase is important for leukocyte phagocytosis, it has been shown in several studies that Nox2−/− mice demonstrate similar inflammatory cell recruitment to WT animals and, in fact, demonstrate relatively normal resistance to most microbes, which is thought to be because of compensation by other ROS-generating pathways in these cells. In the current study, it was notable that Nox2−/− mice had similar infarct sizes and perivascular fibrosis to their WT littermates and that the majority of differences between the strains were in the remote noninfarcted myocardium, suggesting that these changes may not be accounted for merely by altered inflammation. Nevertheless, definitive analyses of the roles of different Nox2-expressing cell types in this setting will require the development of appropriate new cell-specific genetic mouse models.

**Perspectives**

This study demonstrates that Nox2 oxidase significantly contributes to the processes underlying adverse cardiac remodeling and contractile dysfunction after MI, in large part through alterations in the remote noninfarcted myocardium. These effects are Nox isoform-specific and suggest that targeting specific sources of ROS production in the remodeling heart could have therapeutic potential.
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