Integrative Physiology

Critical Role of the NAD(P)H Oxidase Subunit p47^phox for Left Ventricular Remodeling/Dysfunction and Survival After Myocardial Infarction

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Abstract—Accumulating evidence suggests a critical role of increased reactive oxygen species production for left ventricular (LV) remodeling and dysfunction after myocardial infarction (MI). An increased myocardial activity of the NAD(P)H oxidase, a major oxidant enzyme system, has been observed in human heart failure; however, the role of the NAD(P)H oxidase for LV remodeling and dysfunction after MI remains to be determined. MI was induced in wild-type (WT) mice (n = 46) and mice lacking the cytosolic NAD(P)H oxidase component p47^phox (p47^phox^-/- mice) (n = 32). Infarct size was similar among the groups. NAD(P)H oxidase activity was markedly increased in remote LV myocardium of WT mice after MI as compared with sham-operated mice (83 ± 8 versus 16.7 ± 3.5 nmol of O_2^- µg^-1 min^-1; P < 0.01) but not in p47^phox^-/- mice after MI (13.5 ± 3.6 versus 15.5 ± 3.5 nmol of O_2^- µg^-1 min^-1), as assessed by electron-spin resonance spectroscopy using the spin probe CP-H. Furthermore, increased myocardial xanthine oxidase activity was observed in WT, but not in p47^phox^-/- mice after MI, suggesting NAD(P)H oxidase–dependent xanthine oxidase activation. Myocardial reactive oxygen species production was increased in WT mice, but not in p47^phox^-/- mice, after MI. LV cavity dilatation and dysfunction 4 weeks after MI were markedly attenuated in p47^phox^-/- mice as compared with WT mice, as assessed by echocardiography (LV end-diastolic diameter: 4.5 ± 0.2 versus 6.3 ± 0.3 mm, P < 0.01; LV ejection fraction, 35.8 ± 2.5 versus 22.6 ± 4.4%, P < 0.05). Furthermore, cardiomyocyte hypertrophy, apoptosis, and interstitial fibrosis were substantially reduced in p47^phox^-/- mice as compared with WT mice. Importantly, the survival rate was markedly higher in p47^phox^-/- mice as compared with WT mice after MI (72% versus 48%; P < 0.05). These results suggest a pivotal role of NAD(P)H oxidase activation and its subunit p47^phox for LV remodeling/dysfunction and survival after MI. The NAD(P)H oxidase system represents therefore a potential novel therapeutic target to prevent cardiac failure after MI. (Circ Res. 2007;100:894-903.)

Key Words: myocardial infarction ■ remodeling ■ heart failure ■ NAD(P)H oxidase ■ superoxide anion

Left ventricular (LV) remodeling processes after myocardial infarction (MI) remain a major challenge and contribute to development and progression of heart failure.1 Reactive oxygen species (ROS) have been implicated in LV remodeling and dysfunction.2–4 Treatment with structurally different antioxidants prevented LV remodeling and dysfunction after MI.2–4 However, the pathophysiologically important enzymatic sources leading to increased myocardial ROS production after MI remain to be further characterized. Notably, a markedly increased myocardial activity of the NAD(P)H oxidase, an important oxidant enzyme system, has recently been observed in human failing as compared with nonfailing hearts, raising the question of the relevance of this enzyme system for LV remodeling and dysfunction after MI.5,6 Interestingly, NAD(P)H oxidase activity is significantly enhanced by several stimuli relevant to the pathophysiology of heart failure, such as mechanical stretch,7,8 angiotensin II,9–11 α-adrenergic agonists,12 endothelin-1,13 or tumor necrosis factor-α.14,15 We and others have recently shown that membrane translocation of the NAD(P)H oxidase subunit p47^phox is important for activation of the enzyme in response to angiotensin II.10,16,17 Moreover, recent data suggest a potential interaction of the NAD(P)H oxidase with the xanthine oxidase (XO) system,18 an oxidant enzyme that has also been shown to be activated in experimental and human heart failure.19–21

In the present study, we therefore examined the role of NAD(P)H oxidase activation for LV remodeling and dysfunction and survival after MI. Furthermore, we assessed the role of NAD(P)H oxidase for XO activation after MI.

Materials and Methods
MI was induced by permanent ligation of the left anterior descending coronary artery, as described previously,22,23 in male wild-type (WT) mice and mice lacking the cytosolic NAD(P)H oxidase component...
expression of the membrane subunits p22phox and gp91phox and the cytosolic subunit p67phox were significantly increased in both WT and p47phox−/− mice after MI (Figure 1C). The expression of the GTP protein rac1 was elevated in WT mice after MI, but not in p47phox−/− mice after MI (Figure 1C).

Myocardial Superoxide Production After MI
Myocardial superoxide production, as assessed by lucigenin-enhanced chemiluminescence and dihydroethidium fluorescence staining using confocal microscopy was markedly increased in remote LV myocardium of WT mice after MI, but not in p47phox−/− mice after MI (Figure 1E and 1F).

Echocardiographic Analysis and Hemodynamic Measurements
LV cavity dilation after MI was substantially reduced in p47phox−/− mice as compared with WT mice (LV end-diastolic diameter: p47phox−/− versus WT 4.5 ± 0.2 versus 6.3 ± 0.3 mm; P < 0.01; Figure 2A). Furthermore, LV systolic function as assessed by LV ejection fraction (Figure 2C) or LV fractional shortening (Table) was better preserved in p47phox−/− mice as compared with WT mice after MI.

LV dP/dt min was better preserved in p47phox−/− mice as compared with WT mice after MI (Table). LV dP/dt max was significantly reduced in WT and p47phox−/− mice after MI. There was a trend for a better-preserved LV dP/dt max in p47phox−/− mice as compared with WT mice after MI that, however, did not reach statistical significance. Mean arterial pressure after MI as measured invasively by the micromanometer conductance catheter was not significantly different between WT and p47phox−/− mice (Table).

LV Morphology and Morphometry Analysis
Myocardial hypertrophy after MI, as assessed by cardiomyocyte cross-sectional area, was largely prevented in p47phox−/− mice as compared with WT mice (Figure 3A). Similarly, there was a significant increase of LV weight in WT mice after MI that was largely blunted in p47phox−/− mice after MI (Figure 3C). Furthermore, mRNA expression of the cardiac hypertrophy marker skeletal muscle α-actin was only increased in WT mice after MI, but not in p47phox−/− mice after MI (Figure 3D and 3E).

The interstitial collagen volume fraction was increased to a greater extent in WT mice as compared with p47phox−/− mice after MI (Table and Figure 3G and 3H). The infarct size was similar between both groups (Table).

Apoptosis Detection in LV Myocardium After MI
The number of apoptotic nuclei as detected by TUNEL staining was significantly reduced in the LV border zone of p47phox−/− mice as compared with WT mice after MI (Figure 4A and 4B). Similarly, the analysis of cleaved caspase-3 revealed reduced levels of the large fragment (19 kDa) of activated caspase-3 in the LV border zone of p47phox−/− mice as compared with WT mice after MI (Figure 4C and 4D). There was no significant change of uncleaved caspase-3 between WT and p47phox−/− deficient mice after MI, suggesting that a NAD(P)H oxidase-dependent pathway is involved in caspase-3 activation after MI (Figure 4D).
Matrix Metalloproteinase-2 Gelatinolytic Activity After MI

The echocardiographic analysis demonstrated a markedly reduced LV dilatation after MI in p47<sup>phox</sup>−/− mice as compared with WT mice. Notably, we have previously observed an NAD(P)H oxidase-dependent activation of matrix metalloproteinase (MMP)-2 by mechanical stretch and angiotensin II in cultured vascular smooth muscle cells.27,28 Furthermore, MMP-2–deficient mice have previously been shown to have a reduced LV dilatation after MI.29 We therefore postulated that NAD(P)H oxidase is involved in MMP-2 activation after MI and determined MMP-2 gelatinolytic activity in p47<sup>phox</sup>−/− and WT mice after MI. These studies revealed a significantly reduced gelatinolytic activity of MMP-2 in the remote LV myocardium of p47<sup>phox</sup>−/− mice as compared with WT mice 5 days after MI (Figure 4E and 4F).
ESR Spectroscopic Analysis of NO Production After MI
NO production was similar in aortas of sham-operated WT and p47phox<sup>-/-</sup> mice. NO production was reduced in WT mice and p47<sup>-/-</sup> mice after MI. Importantly, however, NO production was markedly better preserved in p47<sup>-/-</sup> mice as compared with WT mice after MI (Figure 5A). Representative ESR scans of vascular NO production of WT and p47<sup>-/-</sup> mice are shown in Figure 5B.

Effect of Treatment With the XO Inhibitor Allopurinol on LV Remodeling and Dysfunction in WT and p47phox<sup>-/-</sup>-Deficient Mice After MI
To further assess the relative contribution of NAD(P)H oxidase and XO activation to LV remodeling and dysfunction after MI, both WT and p47<sup>-/-</sup> mice were treated with allopurinol or vehicle (n=6 to 10). LV cavity dilation and LV dysfunction were significantly reduced in WT mice treated with allopurinol as compared with vehicle-treated mice (Table I in the online data supplement). In contrast, allopurinol treatment had no effect on LV dilatation and dysfunction in p47<sup>-/-</sup> mice after MI (supplemental Table I). Notably, LV cavity dilation and LV systolic function were better preserved in p47<sup>-/-</sup> mice after MI as compared with allopurinol-treated WT mice, suggesting that prevention of NAD(P)H oxidase activation exerts both XO-dependent and XO-independent beneficial effects after MI (supplemental Table I).

LV weight, cardiomyocyte cross-sectional area, and interstitial collagen volume were reduced after allopurinol treatment in WT mice, but not in allopurinol treated p47<sup>-/-</sup> mice after MI (supplemental Table I). Infarct size was similar among all groups with vehicle or allopurinol treatment (supplemental Table I).

Echocardiographic and Hemodynamic Parameters and Histomorphometric Analysis in WT and p47phox<sup>-/-</sup>-Deficient Mice After Sham or MI Operation

<table>
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<tr>
<th>Echocardiography</th>
<th>Sham WT</th>
<th>MI WT</th>
<th>Sham p47phox&lt;sup&gt;-/-&lt;/sup&gt;</th>
<th>MI p47phox&lt;sup&gt;-/-&lt;/sup&gt;</th>
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<td>LV ejection fraction, %</td>
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<td>5.6±0.3†</td>
<td>2.9±0.1</td>
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<td>Mean infarct size, %</td>
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<td>258±9.5</td>
<td>550.0±34.4‡</td>
<td>346.4±22.4</td>
<td>406.6±15.5§</td>
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EDD indicates end-diastolic diameter; ESD, end-systolic diameter; MAP, mean arterial pressure; N/A, not applicable. *P<0.05 vs sham WT, †P<0.01 vs sham WT, ‡P<0.05 vs sham p47phox<sup>-/-</sup>, §P<0.01 vs sham p47phox<sup>-/-</sup>, ¶P<0.05 vs MI WT, ||P<0.01 vs MI WT.
Importantly, the survival rate was substantially higher in p47<sub>phox</sub>−/− mice as compared with WT mice after MI (72% versus 48%; P < 0.05; Figure 6).

**Discussion**

The present study provides direct evidence that a deficiency of the NAD(P)H oxidase and its subunit p47<sub>phox</sub> protects from LV remodeling and dysfunction after MI. p47<sub>phox</sub> deficiency reduced LV cavity dilatation and dysfunction as well as cardiomyocyte hypertrophy, apoptosis, and interstitial fibrosis after MI. All of these beneficial effects may have contributed to improved survival of p47<sub>phox</sub>-deficient mice after MI. Furthermore, our ESR spectroscopic analysis

**Figure 3.** A, Cardiomyocyte cross-sectional area (CSA) of sham- and MI-operated WT and p47<sub>phox</sub>−/− mice (n = 5 to 8). B, Representative high-power photomicrographs of hematoxylin/eosin-stained and wheat germ agglutinin-stained (red) LV cross-sections, with nuclei stained with Hoechst (blue). C, LV weight of sham- and MI-operated WT and p47<sub>phox</sub>−/− mice. D, Northern blot analysis of the cardiac hypertrophy marker skeletal muscle α-actin in sham- and MI-operated WT and p47<sub>phox</sub>−/− mice. E, Representative Northern blot analysis of the cardiac hypertrophy marker skeletal muscle (SkM) α-actin in sham- and MI-operated WT and p47<sub>phox</sub>−/− mice. F, Low-power photomicrographs of hematoxylin/eosin-stained LV cross-sections obtained from WT and p47<sub>phox</sub>−/− mice after MI. G, Relative interstitial collagen fraction of sham- and MI-operated WT and p47<sub>phox</sub>−/− mice (n = 10 to 12). H, Representative high-power photomicrographs of Sirius red-stained remote LV myocardium from MI-operated WT and p47<sub>phox</sub>−/− mice. *P < 0.05 vs sham WT, **P < 0.01 vs sham WT, $P < 0.05$ vs sham p47<sub>phox</sub>−/−, $$$P < 0.01$ vs sham p47<sub>phox</sub>−/−.
suggests that NAD(P)H oxidase triggers the increase of myocardial XO activity after MI that further augments myocardial oxidant stress.

Experimental and clinical studies have demonstrated increased ROS generation in failing hearts that was related to LV dysfunction and dilatation.30,31 Accumulating evidence suggests that ROS may play a major role in the development and progression of LV remodeling and dysfunction. However, the pathophysiologically relevant source of increased myocardial ROS production after MI remains to be further characterized.

Of note, recent studies have demonstrated increased cardiac NAD(P)H oxidase activity in human heart failure.5,6 Several studies have shown that NAD(P)H oxidase is expressed in vascular and myocardial cells in both rodents and humans.5,6,32–34 Notably, the NAD(P)H oxidase system is activated by stimuli relevant to the pathophysiology of heart failure, ie, angiotensin II, α-adrenergic agonists, tumor necrosis factor-α, and increased mechanical stretch.7–15,32 These studies have raised the question of the pathophysiological relevance of NAD(P)H oxidase activation for LV remodeling and dysfunction after MI. In

Figure 4. Myocardial apoptosis after MI. A, Number of TUNEL-positive nuclei in sham- and MI-operated WT and p47phox−/− mice (n=4 to 7). B, Representative LV sections demonstrating TUNEL-positive nuclei (marked by arrows) in the infarct border zone of WT and p47phox−/− mice after MI. C, Western blot analysis of cleaved caspase-3 in sham- and MI-operated WT and p47phox−/− mice (n=4 to 5). D, Representative Western blot analysis of the large fragment (19 kDa) of cleaved caspase-3 and uncleaved caspase-3 (35 kDa) in the infarct border zone of WT and p47phox−/− mice. E, Gelatin zymography analysis of MMP-2 in sham- and MI-operated WT and p47phox−/− mice (n=6 to 7). F, Representative gelatin zymography analysis of myocardial MMP-2 in sham- and MI-operated WT and p47phox−/− mice. *P<0.05 vs sham WT, **P<0.01 vs sham WT, $P<0.05$ vs sham p47phox−/−, $$$$P<0.01$ vs sham p47phox−/−.
the present study, mice deficient in the NAD(P)H oxidase subunit p47<sub>phox</sub> had a markedly reduced myocardial oxidant stress and LV remodeling and dysfunction after MI. Of note, direct exposure of cardiomyocytes to ROS has been shown to promote hypertrophy and apoptosis, so that it is conceivable that the reduction of myocardial oxidant stress in p47<sub>phox</sub>-/- mice may have directly contributed to prevention of cardiomyocyte hypertrophy and apoptosis. Moreover, exposure of cardiomyocytes to superoxide anions has been shown to induce a contractile dysfunction, suggesting that, in particular, a prevention of the increase in myocardial superoxide production after MI may have preserved LV function in p47<sub>phox</sub>-/- mice.

In addition to direct effects of ROS on LV remodeling and dysfunction, a reduced interaction of ROS with nitric oxide (NO), in particular, with endothelial NO synthase–derived NO, may contribute importantly to beneficial effects of NAD(P)H oxidase inhibition after MI. Notably, increased vascular inactivation of NO by superoxide has been observed in experimental heart failure after MI. Preserved NO availability, as observed in p47<sub>phox</sub>-/- mice after MI in the present study, therefore likely represents an additional mechanism whereby reduced NADPH oxidase activity exerts beneficial effects after MI.

Of note, recent studies have demonstrated increased cardiac and vascular XO activity in experimental and human heart failure, another major source of ROS. The present study suggests that the increase in myocardial XO activity after MI is dependent on the NAD(P)H oxidase system. In this respect, recent in vitro data have suggested that XO may be activated in a redox-sensitive manner, dependent on the NAD(P)H oxidase. This concept is strongly supported by our observations in vivo that p47<sub>phox</sub>-/- mice had no increase of myocardial XO activity after MI, as suggested by ESR spectroscopic analysis.

Notably, in recent studies, we and others have observed beneficial effects of XO inhibition on LV dysfunction and remodeling. To further assess the relative contribution of NAD(P)H oxidase and XO to LV remodeling and dysfunction after MI in the present study, both WT and p47<sub>phox</sub>-/- mice were randomized to treatment with the XO inhibitor allopurinol or vehicle. Of note, we have previously observed that this dose of allopurinol results in an
effective inhibition of myocardial XO activity in mice after MI. The results of these studies suggest that NAD(P)H oxidase activation after MI promotes LV remodeling and dysfunction both by XO-dependent and XO-independent effects. In addition to XO activation, NAD(P)H oxidase-derived superoxide may directly contribute to LV remodeling and dysfunction or may trigger activation of other sources of oxygen radicals, ie, uncoupling of endothelial NO synthase or increase mitochondrial ROS production. In this respect, it has recently been suggested that hydroxyl radicals, originated from superoxide anions, cause mitochondrial damage that may represent another mechanism whereby increased superoxide production may stimulate myocardial ROS production and augment myocardial damage. This concept is further supported by a recent study demonstrating that overexpression of the mitochondrial antioxidant peroxiredoxin-3 (Prx-3) protected mice from mitochondrial dysfunction and LV failure after MI.

Of note, in recent in vitro studies, we have demonstrated that MMP-2 activation in response to mechanical stretch or angiotensin II is prevented in vascular smooth muscle cells deficient in p47phox. In the present study, MMP-2 activation was reduced in p47phox−/− mice as compared with WT mice in vivo, which may have contributed to prevention of LV dilatation after MI in these mice. This concept is supported by recent observations in MMP-2−/− deficient mice, which had a reduced LV dilatation after MI. Theoretically, a reduction of the proteolytic activity of the gelatinase MMP-2 should lead to an accumulation of substrates, in particular, collagens. In the present study, however, there was a reduced interstitial fibrosis in p47phox−/− mice after MI. The present study is, therefore, in line with previous studies suggesting that MMP-2 is not only involved in degradation of collagens but also has additional functions in cardiac remodeling. For example, MMP-2 has been suggested to contribute to tissue invasion of inflammatory cells, which may promote interstitial fibrosis.

In contrast to the present study, a recent study by Frantz et al does not indicate a protective effect of a deficiency of the membranous NAD(P)H oxidase subunit gp91phox on LV remodeling and dysfunction after MI. Notably, however, these authors do not observe a reduced myocardial ROS production in gp91phox−/− deficient mice after MI and demonstrate that Ncx 1, a homolog of gp91phox, is upregulated in these mice. A compensation for gp91phox by gp91phox homologs, therefore, represents 1 potential explanation for the different findings as compared with the present study using p47phox−/− deficient mice. Similarly, in 2 recent studies analyzing mice with “pressure overload” hypertrophy, deficiency of gp91phox did not inhibit cardiac hypertrophy. However, gp91phox−/− mice still had an increased myocardial NAD(P)H oxidase activity after “pressure overload,” suggesting a compensation of gp91phox by other homologs (Ncx 1 and 4) of this NAD(P)H oxidase subunit.

Moreover, there are several cell types in the myocardium that express NAD(P)H oxidase and its subunit p47phox, ie, cardiomyocytes, endothelial cells, infiltrating neutrophils, and fibroblasts. A conditional knockout approach or a cell-specific transgene restoration of NADPH oxidase subunits in NAD(P)H oxidase−/− deficient mice will be required to determine the relative contribution of NAD(P)H oxidase in these different cell types to LV remodeling and dysfunction after MI.

Furthermore, Grote et al have recently suggested that young p47phox−/− deficient mice have an increased systolic blood pressure as measured by the tail cuff method. In the present study, we determined mean arterial pressure after MI that was similar in p47phox−/− deficient and WT mice, as measured by the micromanometer conductance catheter. The different methods used (ie, invasive versus noninvasive measurement) may, at least in part, explain these different findings. A higher blood pressure, however, would also be unlikely to contribute to reduced LV remodeling and dysfunction in p47phox−/− deficient mice after MI.

In summary, deficiency of the NAD(P)H oxidase subunit p47phox prevented LV remodeling and dysfunction after MI and reduced cardiomyocyte hypertrophy, apoptosis, and interstitial fibrosis, which was associated with an improved survival. Importantly, p47phox deficiency also prevented myocardial XO activation after MI, thereby providing evidence that this enzyme is activated in a redox-sensitive manner in vivo. Treatment strategies that reduce NAD(P)H oxidase activation may therefore represent a novel option to prevent cardiac failure after MI.

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Disclosures
None.

References


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Methods Supplement

**Measurement of NAD(P)H oxidase and Xanthine Oxidase Activity by Electron Spin Resonance (ESR) Spectroscopy:** Activity of NAD(P)H oxidase and xanthine oxidase were determined in remote LV myocardium (50 µg protein) by ESR spectroscopy as described previously\(^1,2\) by using the spin probe 1-hydroxy-3-carboxypyrrolidine and a MiniScope ESR spectrometer (Magnettech). The ESR settings were the following: field center: 3367.67 G; field sweep width: 108.92 G; microwave frequency: 9.82 GHz; microwave power: 20 mW; magnetic field modulation frequency: 100 kHz; modulation amplitude: 2 G. The intensity of ESR spectra was quantified after subtraction of the ESR signal of samples without NAD(P)H or xanthine (obtained for each sample).

**Measurement of Myocardial Superoxide Production:** Superoxide production was measured in remote LV myocardium using two approaches, i.e. lucigenin-enhanced chemiluminescence and dihydroethidium fluorescence staining as described previously.\(^3,4\) In brief, intact, nonhomogenized pieces of LV myocardium (2 x 2 mm) were suspended in Krebs/HEPES buffer and transferred to a scintillation counter (LS 6500, Beckman Instruments Inc.) for measurement of lucigenin (5 µM)-enhanced chemiluminescence. Photon counts were recorded and expressed per milligram of dry weight. Detection of myocardial superoxide production using dihydroethidium fluorescence staining was performed as described in detail previously.\(^4\) Matched pairs of myocardial sections from mice after sham- or MI-operation were processed simultaneously, and images were acquired with identical acquisition parameters using confocal microscopy (Leica TCS SP2).

**Echocardiographic Measurements:** Echocardiography studies were performed under light anesthesia (100 mg/kg ketamine, 1.25 mg/kg xylazine, and 0.6 mg/kg atropine i.p.) and spontaneous respiration with a commercially available ultrasound system (ATL 5000 CV).
with a linear 15-MHz, high-frequency transducer as described previously.\textsuperscript{2, 5, 6} The investigator (A.S.) was blinded to the experimental group.

**Hemodynamic Measurements:** Hemodynamic measurements were performed in mice with the use of a 1.4F micromanometer conductance catheter (SPR-719; Millar Instruments) as described previously.\textsuperscript{7} In brief, mice were anesthetized with isoflurane (1 – 2 %) and mechanically ventilated, and the catheter was inserted in the LV cavity via the right carotid artery.

**Histomorphometric Analysis:** After fixation, LV tissue slices were embedded in paraffin, cut into 4-µm sections, and stained with collagen-specific Sirius red F3BA as described previously.\textsuperscript{2, 5, 7} Interstitial collagen volume fraction was quantified by polarized light microscopy of picrosirius red-stained LV tissue sections (Axiovert 100, Zeiss; original magnification x400). Tissue morphometry was performed in a blinded fashion with the Quantimet 500MC digital image analyzer. Mean cardiomyocyte cross-sectional area and infarct size were determined in hematoxylin-eosin-stained sections or sections stained with antibodies recognizing wheat germ agglutinin (WGA; Vector) with a digital image analyzer as described previously.\textsuperscript{2, 5} The expression of skeletal muscle alpha (SkMα) actin, as a cardiac hypertrophy marker, was measured using Northern blot analysis, as described previously.\textsuperscript{8, 9}

**Detection of Myocardial Apoptosis:** Apoptotic nuclei were detected in LV myocardium by in situ terminal deoxynucleotidyl transferase (TdT)-mediated digoxigenin-conjugated dUTP nick end-labeling (TUNEL) and Hoechst 33258 staining as described previously.\textsuperscript{8}

As a second approach we examined cleaved caspase-3 levels using Western blot analysis of LV myocardium and a polyclonal cleaved caspase-3 (Asp 175) antibody (Cell Signaling). Levels of uncleaved caspase-3 were detected using a different polyclonal caspase-3 antibody
(Cell Signaling). Protein was visualized with an enhanced chemiluminescence detection system.

**Matrix Metalloproteinase (MMP-2) -2 Gelatin Zymography:** MMP-2 gelatinolytic activity was determined as described in detail previously. In brief, homogenized LV tissue samples were separated by 8.75 % SDS-PAGE supplemented with 1.5 mg/ml gelatin. Gels were renaturated by 2.5 % Triton X-100 for 60 minutes, after that by a substrate buffer incubation (50 mmol/l Tris/HCl, pH 7.5, containing 5 mmol/L CaCl₂, 0.02% Brij-35) for 48 hours. Gels were stained with 0.5% Coomassie blue (Roth). LV tissue samples were obtained five days after sham or MI operation (LV remote myocardium).

**Western Blot Analysis of Myocardial NAD(P)H Oxidase Subunit Expression:** Expression of the other NAD(P)H oxidase subunits was analysed in remote LV myocardium (50 µg protein) by western blotting as described previously using the following antibodies: rabbit polyclonal anti-p22phox and anti-p67phox antibody (Santa Cruz), mouse monoclonal anti-gp-91phox antibody (BD Transduction Laboratories), a rabbit polyclonal anti-Rac 1 antibody, a horseradish peroxidase conjugated secondary anti-mouse or an anti-rabbit antibody (Amersham Biosciences). Equal protein loading was verified by reprobing the membrane with a mouse anti-GAPDH antibody (Santa Cruz).

**Measurement of NO Production by Electron Spin Resonance (ESR) Spectroscopy:** NO production was measured by an ESR-based method using colloid Fe(II)-diethyldithiocarbamate (Fe(DETC)₂ as a spin trap. Whole mouse aortas were cut into 2 mm rings and transferred into 1,5 ml Krebs solution. Calcium ionophore (A23187, 10 µmol/l) and 500 µl of Fe (II)-diethyldithiocarbamate solution, consisting of Fe sulphate (800 µmol/l) and DETC (800 µmol/l), both solved in 0.9 % sodium chloride solution, were added. After incubation for 1 hour at 37 °C, aortic rings were frozen in liquid nitrogen. ESR recordings
were performed by using a Dewar flask. Instrument settings were the following: field center: 3300.84 G; field sweep width: 79.22 G; microwave frequency: 9.82 GHz; microwave power: 40 mW; magnetic field modulation frequency: 100 kHz; modulation amplitude: 10 G. Aortic NO production was expressed in pmol x mg aortic dry weight $^{-1}$ x 60 min $^{-1}$.

**Statistical Analysis:** All data are expressed as mean ± SEM. To compare data between groups, the Mann-Whitney-Test was used. Comparison of survival was performed using a Kaplan-Meier analysis. A value of $P < 0.05$ was considered statistically significant.
References:


**Supplemental Table 1**

Echocardiographic and Histomorphometric Analysis Comparing the Effect of Allopurinol or Vehicle Treatment in WT and p47<sup>phox</sup>/- mice after MI (n=6-10)

<table>
<thead>
<tr>
<th></th>
<th>MI WT V</th>
<th>MI WT A</th>
<th>MI p47&lt;sup&gt;phox&lt;/sup&gt;-V</th>
<th>MI p47&lt;sup&gt;phox&lt;/sup&gt;-A</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Echocardiography</strong></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>LV ejection fraction, %</td>
<td>17.6±3.6</td>
<td>27.9±2.7**</td>
<td>35.6±1.8‡</td>
<td>36.7±4.6</td>
</tr>
<tr>
<td>LV fractional shortening, %</td>
<td>7.6±2.2</td>
<td>17.3±2.3**</td>
<td>22.9±1.4‡</td>
<td>26.1±3.2</td>
</tr>
<tr>
<td>LV end-diastolic diameter, mm</td>
<td>6.8±0.4</td>
<td>5.2±0.2**</td>
<td>4.6±0.1‡</td>
<td>4.8±0.3</td>
</tr>
<tr>
<td>LV end-systolic diameter, mm</td>
<td>6.4.8±0.4</td>
<td>4.3±0.2**</td>
<td>3.5±0.1‡</td>
<td>3.6±0.3</td>
</tr>
<tr>
<td><strong>Histomorphometric analysis</strong></td>
<td></td>
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<tr>
<td>Mean infarct size, %</td>
<td>46±4.3</td>
<td>43.6±3.9</td>
<td>47.1±3.5</td>
<td>45.1±1.4</td>
</tr>
<tr>
<td>LV weight, mg/g BW</td>
<td>6.1±0.4</td>
<td>4.6±0.3*</td>
<td>4.3±0.2</td>
<td>4.2±0.3</td>
</tr>
<tr>
<td>Myocyte CSA, µm&lt;sup&gt;2&lt;/sup&gt;</td>
<td>593±34</td>
<td>400.9±19.3**</td>
<td>409.3±20</td>
<td>406.1±10</td>
</tr>
<tr>
<td>Interstitial collagen fraction, % control</td>
<td>572±45</td>
<td>328±51**</td>
<td>244±16</td>
<td>257±20</td>
</tr>
</tbody>
</table>

V, vehicle. A, allopurinol. BW, body weight. All other abbreviations are as defined in text.

*, P<0.05 vs. MI WT-V; **, P<0.01 vs. MI WT-V.

‡, P<0.05 vs. MI WT-A