Myocardial Ischemia/Reperfusion Injury in NADPH Oxidase–Deficient Mice

Michaela R. Hoffmeyer, Steven P. Jones, Christopher R. Ross, Brent Sharp, Matthew B. Grisham, F. Stephen Laroux, Timothy J. Stalker, Rosario Scalia, David J. Lefer

Abstract—Previous studies have suggested that oxygen-derived free radicals are involved in the pathophysiology of myocardial ischemia/reperfusion (MI/R) injury. Specifically, neutrophils have been shown to mediate postischemic ventricular arrhythmias and myocardial necrosis. We hypothesized that MI/R injury would be reduced in the absence (−/−) of NADPH oxidase. Heterozygous control mice (n = 23) and NADPH oxidase−/− mice (n = 24) were subjected to 30 minutes of coronary artery occlusion and 24 hours of reperfusion. Myocardial area at risk per left ventricle was similar in heterozygous control hearts (55 ± 3%) and NADPH oxidase−/− hearts (61 ± 4%). Contrary to our hypothesis, the size of infarct area at risk was similar in the heterozygous control mice (42 ± 4%) and NADPH oxidase−/− mice (34 ± 5%) (P = not significant). In addition, echocardiographic examination of both groups revealed that left ventricle fractional shortening was similar in NADPH oxidase−/− mice (n = 8; 27 ± 2.5%) and heterozygous control mice (n = 10; 23.3 ± 3.3%) after MI/R. Superoxide production, as detected by cytochrome c reduction, was significantly impaired (P < 0.01) in NADPH oxidase−/− mice (n = 6) compared with heterozygous mice (n = 7) (0.04 ± 0.03 versus 2.2 ± 0.08 nmol O2−·min−1·106 cells−1). Intravital microscopy of the inflamed mesenteric microcirculation demonstrated that leukocyte rolling and adhesion were unaffected by the absence of NADPH oxidase. Oyster glycogen-stimulated neutrophil transmigration into the peritoneum was also similar in both the heterozygous control mice and NADPH oxidase−/− mice (P = not significant). These findings suggest that NADPH oxidase does not contribute to the development of myocardial injury and dysfunction after MI/R. (Circ Res 2000;87:812–817.)

Key Words: murine ■ infarct ■ oxygen free radicals ■ neutrophils ■ echocardiography

Neutrophil (PMN) accumulation into a previously ischemic zone is thought to be a hallmark of reperfusion injury.1–5 Neutrophils can produce an arsenal of damaging, proinflammatory compounds, including arachidonic acid metabolites, platelet-activating factor, proteolytic enzymes, and reactive oxygen species such as the superoxide radical. NADPH oxidase is a multisubunit enzyme that oxidizes the soluble coenzyme NADPH, resulting in the formation of a superoxide anion. The overproduction of reactive oxygen species via NADPH oxidase and degranulation of proteolytic enzymes by activated neutrophils is initiated by blood-borne chemotactic factors released after an inflammatory event such as myocardial ischemia/reperfusion (MI/R).6 Reductions in myocardial necrosis and endothelial injury have been reported in studies of MI/R injury in neutropenic animals.7–9 The role of neutrophils in ischemia/reperfusion injury has also been demonstrated in multiple studies using monoclonal antibodies to block PMN-endothelial cell adhesion molecules, thus preventing leukocyte infiltration and reducing infarct size.10–14 We have previously demonstrated that P-selectin–deficient,15 intracellular adhesion molecule-1–deficient,16 and CD-18–deficient16 mice exhibit significantly attenuated neutrophil accumulation and myocardial necrosis after MI/R. Despite the abundance of positive findings, several other reports17–20 failed to demonstrate a role for neutrophils in the pathobiology of MI/R. Consequently, the present study was designed to gain important insights into the role of PMN-derived reactive oxygen species in the pathobiology of MI/R injury.

Previous efforts have focused on the role of reactive oxygen species in myocardial reperfusion injury. A burst of reactive oxygen metabolites after reestablishing blood flow to a previously ischemic zone has been observed by several investigators in various models of MI/R.21–24 The extreme reactivity of these oxidants results in irreversible damage to vital cell components, such as membrane phospholipids, membrane ion transport proteins, and other enzymatic proteins.25 Experiments involving oxygen free radical scavengers have demonstrated reductions in infarct size and emphasized the role of reactive oxygen species in reperfusion injury.26–28
Recently, a transgenic mouse was generated containing a mutation in the gene coding for the p47\textsuperscript{phox} subunit of NADPH oxidase, rendering the enzyme nonfunctional in homozygous mutants.\cite{29} This mouse model grants unique opportunities to investigate the pathophysiology of reactive oxygen species formed by NADPH oxidase. Because of the highly reactive nature of toxic oxidant species, we hypothesized that oxidants such as the superoxide anion are a major contributor to MI/R injury and contractile dysfunction. To test this hypothesis, mice lacking NADPH oxidase and their littermate heterozygous controls were subjected to acute coronary artery occlusion and reperfusion. Additional experiments to examine the role of this enzyme in the recruitment of leukocytes to the inflamed mesentery were also performed using intravital microscopy.

Materials and Methods

Mice

p47\textsuperscript{phox}−/− deficient (−/−) mice (mean body weight=25.9±0.4 g) and heterozygous littermate control mice (mean body weight=26.5±0.6 g) were generated by Jackson et al.\cite{29} as previously described. These animals were used in a blinded fashion, and genotypes were revealed by PCR after all experimental procedures. All animal experiments complied with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 86-23, revised 1985), were approved by the Council of the American Physiology Society, and complied with state and federal regulations. All experimental procedures were approved by the Louisiana State University Health Sciences Center Animal Care and Use Committee.

Superoxide Assay

Superoxide production rates were determined as previously described,\cite{30} with minor modifications. Briefly, 10\textsuperscript{6} extravasated neutrophils were incubated in the presence of 2 µg/mL phorbol myristate acetate (PMA), 1 mg/mL cytochrome c, 30 µg/mL catalase, ±100 µg/mL superoxide dismutase in D-glucose phosphate-buffered saline (D-PBS). The cells were incubated for 30 minutes at 37°C. The cells were then removed, and the absorbance of reduced cytochrome c was measured at 550 nm. The rate of superoxide production was calculated as follows: O\textsubscript{2−} (nmol·min\textsuperscript{−1}·10\textsuperscript{6} cells\textsuperscript{−1})=(47.7×(A\textsubscript{550}−A\textsubscript{550}t))/30 minutes.

Hematology of Peripheral Blood

Total leukocyte, neutrophil, and platelet counts were performed on fresh, whole blood samples from NADPH oxidase−/− mice (n=4) and heterozygous control mice (n=4) by the clinical hematology laboratory at Louisiana State University Health Sciences Center.

Neutrophil Transmigration Assay

Heterozygous control mice (n=6) and NADPH oxidase−/− mice (n=6) were injected with 25 mg of oyster glycogen suspended in 0.5 mL PBS 6 hours before peritoneal lavage with 5 mL PBS containing 10 mmol/L D-PBS. Extravasated leukocytes were pelleted, resuspended in 1 mL D-PBS, and quantitated using a Coulter counter.

MI/R Protocol

Heterozygous control (n=23) mice and NADPH oxidase−/− deficient mice (n=24) were used for the in vivo MI/R experiments. The surgical protocol and infarct size determination were performed similar to methods described previously,\cite{31} with several modifications because of the longer period of reperfusion in the present study. Briefly, the mice were orally intubated with polyethylene-90 (PE-90) tubing and connected to a rodent ventilator (model 683, Harvard Apparatus). Body temperature was maintained at 37°C using a rectal thermometer and infrared heating lamp. The left anterior descending coronary artery (LAD) was visualized and ligated with 7-0 silk suture. Ischemia was confirmed by the appearance of myocardial hypokinesis and pallor. After 30 minutes of LAD occlusion, the ligature was removed and reperfusion was visually confirmed. The chest wall was closed and the mice were given butorphanol tartrate (0.1 mg/kg IP) for analgesia and allowed to recover in a temperature-controlled area.

At 24 hours of reperfusion, the LAD was religated, and Evans blue (1.5 mL of 1% solution) was retrogradely infused into the carotid artery to delineate the ischemic from the nonischemic zones. Ex vivo incubation in 2,3,5-triphenyltetrazolium chloride for 5 minutes at 37°C allowed differentiation between the viable and necrotic areas of the previously ischemic myocardium.

Echocardiographic Assessment of LV Function

We performed in vivo transthoracic echocardiography of the left ventricle (LV) using a 15-MHz linear array transducer (15L8) interfaced with a Sequoia C256 (Acuson). Ventricular parameters were measured according to the leading-edge technique.\cite{32} M-mode (frame rate=30 Hz; sweep speed=200 mm/s) echocardiograms were captured from short-axis views of the LV at the mid-papillary level. LV end diastolic diameters (LVEDD) and LV end systolic diameters (LVESD) were assessed in heterozygous control mice (n=10) and NADPH oxidase−/− mice (n=8) before ischemia and at 24 hours of reperfusion. LV fractional shortening (LVFS) was calculated according to the following equation: LVFS=-(LVEDD−LVESD)/LVEDD×100.

Myocardial Histology

Routine histological staining (hematoxylin and eosin) was performed on multiple sections of ischemic/reperfused myocardium to determine the extent of PMN infiltration. Neutrophils were identified by nuclear morphology in 4 fields per sample by a blinded observer and are presented as PMNs/mm\textsuperscript{2}.

Intravital Microscopy of Mouse Mesenteric Venules

The abdominal cavity was opened via a midline laparotomy, as described previously.\cite{33} Briefly, a loop of ileal mesentery was exteriorized through the midline incision and placed in a temperature-controlled superfusion chamber. A Microphot microscope (Nikon) was used to visualize the mesenteric microcirculation and the mesenteric tissue, as previously described.\cite{33} Red blood cell velocity was determined on-line using an optical Doppler velocimeter obtained from the Microcirculation Research Institute. Video recordings were made before and 60 minutes after initiation of superfusion (25 µmol/L NG-nitro-L-arginine methyl ester) for quantification of leukocyte rolling and adherence. The number of rolling and adhered leukocytes was determined off-line by playback analysis of the videotape, as previously reported.\cite{33}

Statistical Analysis

All findings were analyzed with a 2-tailed unpaired t test calculated with StatView 4.5 (Abacus Concepts). Values are reported as mean±SE, with significance set at P<0.05.

Results

Hematology

The circulating levels of leukocytes, neutrophils, and platelets from heterozygous control mice (n=4) and NADPH oxidase−/− mice (n=4) under baseline conditions are presented in
Table 1. In agreement with a previous report, the number of total leukocytes, neutrophils, and platelets was not significantly different between the 2 groups.

Superoxide Production
Graphic interpretation of superoxide production by neutrophils from NADPH oxidase−/− (n=6) and heterozygous control (n=7) mice is displayed in Figure 1. NADPH oxidase−/− neutrophils displayed a significant impairment (P<0.01) in superoxide radical production (0.04±0.03 nmol O2·min−1·106 cells−1) compared with heterozygous control animals (2.20±0.08 nmol O2·min−1·106 cells−1).

Neutrophil Transmigration
Heterozygous control mice (n=7) and NADPH oxidase−/− mice (n=6) were intraperitoneally injected with oyster glycogen to assess possible differences in neutrophil transmigration. As shown in Figure 2, the number of transmigrated neutrophils (×106/mL) was not significantly different between the control mice (5.22±1.86) and NADPH oxidase−/− mice (4.51±0.66).

Myocardial Area at Risk and Infarct Size
Both heterozygous control mice (n=23) and NADPH oxidase−/− mice (n=24) displayed similar-sized areas at risk and infarction after MI/R (Figure 3). The area at risk (AAR/LV) in heterozygous control mice was 55±3% and in NADPH oxidase−/− mice was 61±4%. Infarct/AAR (Inf/ARR) was similar in heterozygous control mice (42±4%) and NADPH oxidase−/− animals (34±5%). The area of infarct/LV (Inf/LV) was also similar between the 2 groups.

Myocardial PMN Accumulation
Infiltration of PMNs into the ischemic/reperfused myocardium of mice exposed to 30 minutes of LAD occlusion and 24 hours of reperfusion is presented in Figure 4. NADPH oxidase−/− hearts (n=4) contained significantly more (P<0.05) infiltrated PMNs than heterozygous littermate control hearts (n=4) (62±3 versus 51±1 PMNs/mm2).

Echocardiographic Assessment of LV Function
Table 2 displays LVFS in NADPH oxidase−/− and heterozygous control mice at baseline and at the end of the reperfusion period. A significant difference in LVFS was not observed in the NADPH oxidase−/− hearts compared with heterozygous control hearts. A significant decrease (P<0.01) in LVFS at the postreperfusion time point was observed in both the heterozygous control and NADPH oxidase−/− mice compared with baseline values. LVEDD and LVESD diameters for the experimental groups are also shown in Table 2. Both NADPH oxidase−/− hearts and heterozygous control hearts displayed similar-sized areas at risk and infarction after MI/R (Figure 3). The area at risk (AAR/LV) in heterozygous control mice was 55±3% and in NADPH oxidase−/− mice was 61±4%. Infarct/AAR (Inf/ARR) was similar in heterozygous control mice (42±4%) and NADPH oxidase−/− animals (34±5%). The area of infarct/LV (Inf/LV) was also similar between the 2 groups.

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oxidase−/− littersmates and heterozygous littersmates exhibited similar LVEDD and LVESD at baseline and after MI/R. LVESD was significantly increased (P<0.05) at 24 hours of reperfusion in both the heterozygous littermate and the NADPH oxidase−/− groups.

Intravital Microscopy of Perimesenteric Venules

As demonstrated in Table 3, baseline leukocyte rolling and adhesion were similar in the NADPH oxidase−/− group and heterozygous control group. In addition, superfusion of 25 μmol/L NG-nitro-L-arginine methyl ester induced equivalent levels of rolling and adherence in the microcirculation of both groups of mice.

Discussion

Postischemic increases in reactive oxygen species, such as the superoxide anion, hydroxyl radical, and hydrogen peroxide, have been reported in many studies. These toxic oxidants can invoke an array of local inflammatory responses by the cardiac microvasculature. After neutrophil extravasation from the circulation, the role of PMNs as antimicrobial agents becomes detrimental to the surrounding tissue. This inflammatory response can result in myocardial infarct size.

The potential role of neutrophil involvement in MI/R injury has been extensively investigated by several laboratories. PMN depletion has been shown to result in reduced myocardial necrosis after coronary artery occlusion and reperfusion. In sharp contrast, other studies have shown that neutrophil depletion does not alter mechanical function, arrhythmias, and vascular abnormalities. Additionally, other studies have demonstrated that antileukocyte agents fail to reduce myocardial infarct size. Although many differences may be cited among the positive and negative studies, the role of neutrophils in mediating MI/R injury presently remains unresolved.

As bactericidal cells, neutrophils produce large quantities of destructive substances, including proteolytic enzymes and oxygen free radicals. Many studies using oxygen free radical scavengers, such as superoxide dismutase, have demonstrated that these toxic oxidants play a central role in infarct development after reperfusion. With the implication of both neutrophils and reactive oxygen species in MI/R injury, recent emphasis has been placed on PMN-derived oxidants as mediators of cell necrosis. A known major source of superoxide radicals is neutrophilic NADPH oxidase.

In this study, we sought to investigate a correlation between superoxide radicals produced via NADPH oxidase and the development of myocardial necrosis after ischemia and reperfusion. By using a mouse model of chronic granulomatous disease resulting from a defect in the p47phox subunit of NADPH oxidase, we isolated the pathophysiological effects of this enzyme in the postischemic myocardium. Similar to this previous report, we found that NADPH oxidase−/− mice have impaired formation of a respiratory burst (superoxide production) on stimulation. In agreement with our present findings, the same study also found no difference in fertility, weight, and circulating leukocyte counts. It is important to note that Jackson et al found no difference between the wild-type (+/+) and heterozygous (+/−) mice in any of their experiments. As a result of these findings, we used heterozygotes as control mice in the present study.

The present study failed to demonstrate a connection between NADPH oxidase activity and myocardial necro-

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**TABLE 2. LVEDD, LVESD, and LVFS Before and After MI/R in Heterozygous Control Mice (n=10) and NADPH Oxidase−/− Mice (n=8)**

<table>
<thead>
<tr>
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<th>LVEDD, mm</th>
<th>LVESD, mm</th>
<th>LVFS, %</th>
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<tbody>
<tr>
<td>Baseline</td>
<td>Control</td>
<td>2.54±0.12</td>
<td>1.57±0.11</td>
</tr>
<tr>
<td></td>
<td>NADPH oxidase−/−</td>
<td>2.65±0.15</td>
<td>1.66±0.13</td>
</tr>
<tr>
<td>Post-MI/R</td>
<td>Control</td>
<td>2.65±0.09</td>
<td>1.94±0.11*</td>
</tr>
<tr>
<td></td>
<td>NADPH oxidase−/−</td>
<td>2.84±0.15</td>
<td>2.21±0.18*</td>
</tr>
</tbody>
</table>

LVEDD and LVESD were similar between the 2 groups at both baseline and post-MI/R. LVESD was significantly increased (P<0.05) at 24 hours of reperfusion in both heterozygous control mice and the NADPH oxidase−/− mice compared with baseline values. LVFS was significantly reduced (P<0.01) from baseline values after MI/R within each group. However, no significant difference was observed between the 2 groups.

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**TABLE 3. Leukocyte Rolling (Cells/Minute) and Adherence (Cells/100-μm Vessel Length) in Perimesenteric Venules of NADPH Oxidase−/− Mice (n=6) and Heterozygous Control Mice (n=6) Before and After 60 Minutes of 25 μmol/L L-NAME Superfusion**

<table>
<thead>
<tr>
<th></th>
<th>Rolling</th>
<th>Adherence</th>
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<tbody>
<tr>
<td>Basal</td>
<td></td>
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<tr>
<td>L-NAME</td>
<td></td>
<td></td>
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<tr>
<td>Control</td>
<td>34±4</td>
<td>61±10</td>
</tr>
<tr>
<td>NADPH</td>
<td>34±3</td>
<td>68±15</td>
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<tr>
<td>Oxidase−/−</td>
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</table>

No significant differences were noted between the 2 groups before or after L-NAME superfusion.

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**Figure 4.** Myocardial neutrophil (PMN) accumulation after 30 minutes of coronary ischemia and 24 hours of reperfusion in NADPH oxidase−/− littersmates (n=4) and heterozygous control littersmates (n=4). The number of PMNs/mm² was determined in 4 fields for each heart. Myocardial PMN infiltration was significantly higher (P<0.05) in NADPH oxidase−/− mice compared with wild-type littersmates.
sis, contractile dysfunction, or leukocyte-endothelial cell interactions. Both heterozygous control mice and NADPH oxidase–deficient mice suffered similar-sized areas of necrosis after MI/R. Our data concerning leukocyte trafficking demonstrate no difference in the ability of leukocytes to roll, adhere, and transmigrate despite the deficiency of functional NADPH oxidase. This is important for two reasons. First, this clearly demonstrates that NADPH oxidase is not involved in leukocyte-endothelial interactions. Second, the results we present are a function of impaired superoxide production and not related to impaired leukocyte trafficking.

Previous studies have supported the involvement of NADPH oxidase in the development of ischemia and reperfusion injury. Using transgenic mice with an X-linked form of chronic granulomatous disease, Walder et al. reported attenuated microvascular dysfunction after ischemia and reperfusion in the rat mesentery treated with a nonspecific pharmacological inhibitor (PR-39 peptide) of NADPH oxidase. However, our findings do not implicate NADPH oxidase in the development of microvascular dysfunction, MI/R-induced necrosis, or contractile dysfunction after MI/R.

In conclusion, we have not demonstrated an improvement in myocardial necrosis, contractile function, or leukocyte-endothelial interactions in mice lacking functional NADPH oxidase. These results suggest that reactive oxygen species produced via the NADPH oxidase system do not contribute to the development of myocardial infarction and dysfunction after coronary artery occlusion and reperfusion in mice. Furthermore, NADPH oxidase does not seem to be involved in leukocyte rolling, adhesion, or transmigration. The results of the present study provide additional evidence against the role of leukocyte-derived oxidant formation in MI/R injury. In addition, the present study is not in accordance with previous work in our laboratory demonstrating a strong correlation between neutrophil recruitment and infarct size. Additional studies are required to additionally examine the potential role of neutrophils in myocardial reperfusion injury.

Acknowledgments

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References


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