Cardioprotective actions of endogenous IL-10 are independent of iNOS

STEVEN P. JONES, STEVEN D. TROCHA, AND DAVID J. LEFER
Department of Molecular and Cellular Physiology, Louisiana State University Health Sciences Center, Shreveport, Louisiana 71130

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Jones, Steven P., Steven D. Trocha, and David J. Lefer. Cardioprotective actions of endogenous IL-10 are independent of iNOS. Am J Physiol Heart Circ Physiol 281: H48–H52, 2001.—Myocardial ischemia-reperfusion (I/R) is a well-known stimulus for acute inflammatory responses that promote cell death and impair pump function. Interleukin-10 (IL-10) is an endogenous, potent anti-inflammatory cytokine. Recently, it has been proposed that IL-10 inhibits inducible nitric oxide synthase (iNOS) activity after myocardial I/R and consequently exerts cardioprotective effects. However, whether this actually occurs remains unclear. To test this hypothesis, we utilized iNOS-deficient (−/−), IL-10−/−, and IL-10/iNOS−/− mice to examine the potential mechanism of IL-10-mediated cardioprotection after myocardial I/R. Wild-type, iNOS−/−, IL-10−/−, and IL-10/iNOS−/− mice were subjected to in vivo myocardial ischemia (30 min) and reperfusion (24 h). Deficiency of iNOS alone did not significantly alter the extent of myocardial necrosis compared with wild-type mice. We found that deficiency of IL-10 resulted in a significantly (P < 0.05) larger infarct size than that in wild-type hearts. Interestingly, deficiency of both IL-10 and iNOS yielded significantly (P < 0.01) larger myocardial infarct sizes compared with wild-type animals. Histological examination of myocardial tissue samples revealed augmented neutrophil infiltration into the I/R myocardium of IL-10−/− and IL-10/iNOS−/− mice compared with hearts of wild-type mice. These results demonstrate that 1) deficiency of endogenous IL-10 exacerbates myocardial injury after I/R; 2) the cardioprotective effects of IL-10 are not dependent on the presence or absence of iNOS; and 3) deficiency of IL-10 enhances the infiltration of neutrophils into the myocardium after I/R.

INTERLEUKIN-10 (IL-10) is an important anti-inflammatory cytokine that attenuates the severity of various disease states. Consequently, many investigators have addressed the possible protective role of IL-10 in multiple pathological models. Lentsch et al. (19) demonstrated that IL-10 administration diminished pulmonary inflammatory cell infiltration in rats. In addition, recent studies (22, 24) suggest that IL-10 may impede the development of atherosclerosis. IL-10 therapy may also inhibit intimal hyperplasia after vascular injury in hypercholesterolemic subjects (2). Much of the interest in IL-10 arose from the generation of mice deficient (−/−) in the IL-10 gene and their development of colitis (14). Subsequent work in IL-10−/− mice demonstrated a vital role for IL-10 in attenuating cardiac allograft rejection (26) and lipopolysaccharide (LPS)-induced vascular dysfunction (5). These findings and others fuel further investigation into the potential mechanisms of IL-10-mediated cytoprotection.

Unlike IL-10, the role of inducible nitric oxide (NO) synthase (iNOS) in inflammation is highly controversial. Many studies have implicated iNOS as a pathogenic mediator in renal ischemia-reperfusion (I/R) injury (20) and shock states (30). Others have found iNOS to be protective in the setting of allograft arteriosclerosis (28) and ischemic preconditioning (31). Although the role of iNOS in myocardial I/R injury is unclear, recent studies have investigated its role. Using isolated, perfused iNOS−/− mouse hearts, Xi et al. (36) did not demonstrate a role for iNOS in myocardial I/R injury. Conversely, another study (35) demonstrated an injurious role for iNOS using an iNOS inhibitor.

Recently, interest in the role of IL-10 in the development of injury after myocardial I/R has grown. It has previously been demonstrated that exogenous administration of IL-10 attenuates the extent of polymorphonuclear neutrophil (PMN) infiltration and I/R injury in rats (6). Recently, Yang et al. (37) demonstrated that endogenously produced IL-10 is critical in diminishing myocardial injury after myocardial I/R. The authors of this recent study (37) suggest that endogenous IL-10 inhibits the activity of iNOS after myocardial I/R. Accordingly, IL-10 deficiency might augment the production of iNOS-derived NO and possibly form potent oxidants such as peroxynitrite (ONOO−). In fact, other studies of the protective effects of IL-10 directly or indirectly implicated iNOS as a pathogenic mediator of the injury/dysfunction in IL-10−/− mice (5, 23, 26).

This study was designed to answer the following questions: 1) Does deficiency of IL-10 promote myocardial injury after myocardial I/R? 2) Is the cardioprotective mechanism of IL-10 mediated by inhibition of...
iNOS? We attempted to definitively answer these questions through the use of IL-10−/− and iNOS/IL-10−/− mice in an in vivo model of left anterior descending (LAD) coronary artery occlusion and reperfusion.

MATERIALS AND METHODS

Transgenic mice. IL-10−/− (14), iNOS−/− (15), and IL-10/iNOS−/− mice were generated using homologous recombination. Male, age-matched, mutant, and littermate wild-type mice were used in this study. All experimental procedures complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals approved by the Council of the American Physiological Society and with federal and state regulations. All experimental procedures were approved by the Louisiana State University Health Sciences Center Animal Care and Use Committee.

Surgical procedures. Wild-type (n = 15), iNOS−/− (n = 9), IL-10−/− (n = 9), and IL-10/iNOS−/− (n = 7) mice were allowed free access to standard rodent chow, exposed to 12:12-h light-dark cycles, and housed in a climate-controlled room. The surgical protocol and infarct size determination were performed similar to methods described previously (8, 11). Briefly, the mice were anesthetized with pentobarbital sodium (50 mg/kg ip) and ketamine (50 mg/kg ip). Through direct visualization, the mice were orally intubated with polyethylene-90 tubing. Body temperature was maintained between 36 and 37°C by using a rectal thermometer and infrared heating lamp. The animals were then connected to a rodent ventilator (model 683, Harvard Apparatus). After a median sternotomy was performed, the LAD coronary artery was visualized and ligated with a 7-0 silk suture. Ischemia was confirmed by pallor and hypokinesis distal to the occlusion. After 30-min LAD coronary artery occlusion, the ligation was removed and reperfusion was confirmed visually. The chest wall was closed with three interrupted sutures (4-0 silk), and the skin was approximated with 10-0 silk. The animals were given butorphanol tartrate (0.1 mg/kg sc) for analgesia. The mice were given supplemental oxygen via a nasal cone and allowed to recover in a temperature-controlled area.

The next day (at the end of 24-h reperfusion), a tracheostomy was performed, and the mouse was connected to the respirator. The right common carotid artery was cannulated for Evans blue infusion. The LAD was religated and Evans blue (1.5 ml of 1.0% solution) was retrogradely infused into the carotid artery catheter to delineate the ischemic from the nonischemic zones. Ex vivo incubation in 2,3,5-triphenyltetrazolium chloride for 5 min at 37°C allowed differentiation of ischemic from the nonischemic zones. Myocardial infarct size determination was performed using computer-assisted planimetry (National Institutes of Health Image 1.57).

Assessment of myocardial PMN infiltration. Routine histological staining was performed on multiple sections of midventricular cardiac sections to determine the extent of PMN infiltration. Wild-type (n = 8), iNOS−/− (n = 4), IL-10−/− (n = 4), and IL-10/iNOS−/− (n = 4) mice were subjected to 30 min of LAD coronary artery occlusion and 24-h reperfusion as described above. Midventricular tissue slices (1 mm thick) were prepared from hearts subjected to the myocardial I/R protocol after the completion of all experimental procedures. The tissue sections were immediately fixed and stored in a 10% neutral buffered formalin solution (Sigma). The tissue slices were then embedded in paraffin, cut into 10-μm sections, and placed on slides. The tissue specimens were then stained with Gill no. 3 hematoxylin and eosin. The slides were then viewed microscopically, and the number of PMNs per millimeter squared was determined for each of the hearts examined, the number of PMNs was counted in six fields of three independent tissue sections by an observer blinded to the experiment.

Statistical analyses. All data were subjected to ANOVA with Scheffe’s post hoc test. All values are reported as means ± SE. Statistical significance was set at P < 0.05.

RESULTS

Myocardial infarct size determination. Wild-type (n = 15), iNOS−/− (n = 9), IL-10−/− (n = 9), and IL-10/iNOS−/− (n = 7) mice were subjected to 30 min of in vivo, regional coronary ischemia and 24 h reperfusion. The area-at-risk (AAR) per left ventricle (AAR/LV) for infarction was identified using in vivo Evans blue injection. All groups of mice were subjected to similar AAR (P value not significant). The percentage of infarction per AAR (Inf/AAR) in wild-type hearts was 37 ± 4% (Fig. 1). Deficiency of iNOS did not significantly affect the degree of myocardial necrosis (48 ± 4%). However, IL-10−/− hearts (60 ± 6%) suffered ~90% more necrosis than wild-type hearts (P < 0.01). In addition, deficiency of both IL-10 and iNOS (65 ± 6%) resulted in ~120% more necrosis than wild-type hearts (P < 0.01). When expressed as area of Inf/LV, a similar pattern was also observed.

Myocardial histology. PMN infiltration was assessed by routine histological examination of midventricular slices after in vivo I/R (Fig. 2). Wild-type hearts exhibited 58 ± 2 PMNs/mm². Deficiency of iNOS (62 ± 4 PMNs/mm²) did not significantly alter PMN infiltration compared with wild-type hearts. However, IL-10 deficiency (92 ± 2 PMNs/mm²) resulted in ~60% more PMNs compared with wild-type hearts (P < 0.01). Furthermore, deficiency of both IL-10 and iNOS (100 ± 4 PMNs/mm²) resulted in 72% more PMNs than wild-type hearts (P < 0.01).

Fig. 1. Myocardial area-at-risk (AAR) per left ventricle (AAR/LV) and infarct per area-at-risk (Inf/AAR) were assessed in wild-type (n = 15), inducible nitric oxide synthase (iNOS)-deficient (−/−) (n = 9), interleukin (IL)-10−/− (n = 9), and IL-10/iNOS−/− hearts (n = 7). AAR/LV was similar among all groups. **Inf/AAR was significantly greater (P < 0.01) in IL-10−/− and IL-10/iNOS−/− hearts compared with wild-type hearts after ischemia and reperfusion.
longer than that in a previous study (37) of IL-10 fusion period in the present study was significantly I/R myocardium. It is important to note that the reper-
significant augmentation of PMN infiltration into the vivo myocardial I/R. Furthermore, we demonstrated confirmed the cardioprotective actions of IL-10 after in
hepatic I/R (38), atherosclerosis (22, 24), allograft re-
infarct size was not due to increased myocardial oxy-
demons
ted during in vivo myocardial I/R (17).

The present study demonstrates elevated PMN infiltration in IL-10/−/− and IL-10/iNOS−/− hearts after regional myocardial I/R. The role of PMNs in myocardial I/R injury has received much attention (1, 10, 18, 29, 34). The mechanism of neutrophil recruitment into an area of inflammation occurs via the interaction between leukocyte adhesion molecules and endothelial adhesion molecules. Transmigration of activated PMNs into the I/R myocardium can further damage cardiac tissue. Consequently, demonstration of enhanced PMN infiltration in IL-10−/− and IL-10/iNOS−/− mice may partially address the mechanism

DISCUSSION
This study answers two important questions: 1) Is endogenous IL-10 cardioprotective in the setting of myocardial I/R? 2) Are the cardioprotective effects of endogenous IL-10 mediated by inhibition of iNOS? In this study, we have shown that deficiency of endogenous IL-10 augments PMN infiltration and exacer-
ated myocardial I/R injury. To answer the pressing question of the mechanism of IL-10-mediated cardioprotection we examined the possible role of iNOS, as suggested previously (37). The present study clearly demonstrates that inability to upregulate iNOS (in the absence or presence of IL-10) does not protect the I/R myocardium from necrosis. Furthermore, the elevated infarct size was not due to increased myocardial oxygen demand during ischemia (Table 1).

IL-10 is a potent anti-inflammatory cytokine. Prior studies of IL-10 have demonstrated a protective role in a variety of pathological states including colitis (14), hepatic I/R (38), atherosclerosis (22, 24), allograft rejection (26), leukocyte-endothelial cell interactions (7), and myocardial I/R (6, 37). In the present study, we confirmed the cardioprotective actions of IL-10 after in vivo myocardial I/R. Furthermore, we demonstrated significant augmentation of PMN infiltration into the I/R myocardium. It is important to note that the reper-
fusion period in the present study was significantly longer than that in a previous study (37) of IL-10−/− mice. Another difference between these studies is the need for donor blood in the previous study (37), whereas we did not utilize blood transfusions in the present study.

Endothelial cell-derived NO exhibits a multitude of physiological functions. NO promotes vasodilation, inhibits smooth muscle cell proliferation (3), impairs platelet aggregation (25), attenuates leukocyte-endothelial cell interactions (13, 16), decreases microvascular permeability (12), and diminishes myocardial I/R injury (9). Although the role of endothelial-derived NO appears to be cardioprotective, the role of iNOS-de-
ized NO is unclear. In the present study, deficiency of iNOS did not significantly affect the extent of myocardial necrosis. Similarly, a previous ex vivo study of myocardial I/R injury in iNOS−/− mice (36) demonstrated no role for iNOS in the development of myocardial I/R injury. Contrary to this report, other studies (32, 35) using an iNOS inhibitor after myocardial I/R found iNOS to be injurious. The role of iNOS in myocardial I/R injury will continue to be highly controvers-
ial.

One of the primary arguments for an injurious role of iNOS after myocardial I/R is the hypothetical forma-
tion of ONOO−. ONOO− is formed from approximately equimolar concentrations of NO and superoxide (4). It has previously been reported to be formed and promote injury after myocardial ischemia in isolated rat hearts (21, 33). However, evidence for the formation of ONOO− in vivo is often indirect at best. Although tyrosine can be nitrosylated after the formation of ONOO−, it is not a specific marker for ONOO− formation (4, 27). Even if ONOO− were formed in vivo, it is not likely to be injurious in light of a previous report that demonstrated protective effects of ONOO− administered during in vivo myocardial I/R (17).

The present study demonstrates elevated PMN infiltration in IL-10−/− and IL-10/iNOS−/− hearts after regional myocardial I/R. The role of PMNs in myocardial I/R injury has received much attention (1, 10, 18, 29, 34). The mechanism of neutrophil recruitment into an area of inflammation occurs via the interaction between leukocyte adhesion molecules and endothelial adhesion molecules. Transmigration of activated PMNs into the I/R myocardium can further damage cardiac tissue. Consequently, demonstration of enhanced PMN infiltration in IL-10−/− and IL-10/iNOS−/− mice may partially address the mechanism

Table 1. Baseline, ischemia, and reperfusion values of all groups of mice tested

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Ischemia</th>
<th>Reperfusion</th>
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<tbody>
<tr>
<td>Wild type</td>
<td></td>
<td></td>
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<tr>
<td>MABP</td>
<td>93 ± 3</td>
<td>72 ± 5</td>
<td>67 ± 10</td>
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<tr>
<td>Heart rate</td>
<td>456 ± 41</td>
<td>510 ± 31</td>
<td>504 ± 53</td>
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<tr>
<td>RPP</td>
<td>42.9 ± 5.2</td>
<td>36.0 ± 2.2</td>
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<tr>
<td>iNOS−/−</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>MABP</td>
<td>86 ± 3</td>
<td>59 ± 10</td>
<td>54 ± 6</td>
</tr>
<tr>
<td>Heart rate</td>
<td>423 ± 11</td>
<td>485 ± 30</td>
<td>474 ± 17</td>
</tr>
<tr>
<td>RPP</td>
<td>36.2 ± 0.9</td>
<td>29.6 ± 6.3</td>
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<tr>
<td>IL-10−/−</td>
<td></td>
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<tr>
<td>MABP</td>
<td>88 ± 6</td>
<td>70 ± 3</td>
<td>60 ± 3</td>
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<tr>
<td>Heart rate</td>
<td>483 ± 40</td>
<td>494 ± 22</td>
<td>557 ± 46</td>
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<tr>
<td>RPP</td>
<td>43.0 ± 5.7</td>
<td>34.9 ± 2.9</td>
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<tr>
<td>IL-10/iNOS−/−</td>
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<td></td>
<td></td>
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<tr>
<td>MABP</td>
<td>67 ± 8</td>
<td>58 ± 10</td>
<td>59 ± 12</td>
</tr>
<tr>
<td>Heart rate</td>
<td>410 ± 23</td>
<td>482 ± 18</td>
<td>514 ± 24</td>
</tr>
<tr>
<td>RPP</td>
<td>27.3 ± 2.7</td>
<td>28.4 ± 5.5</td>
<td>30.3 ± 5.4</td>
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Values are means ± SE; n = 5 mice/group. Mean arterial blood pressure (MABP, mmHg), heart rate (beats/min), and rate-pressure product (RPP, MABP × heart rate/1,000) in wild-type, inducible nitric oxide synthase-deficient (iNOS−/−), interleukin (IL)-10−/−, and IL-10/iNOS−/− mice. Mice were instrumented with carotid artery catheters and subjected to ischemia and reperfusion. *P < 0.05 vs. wild-type baseline.
of augmented myocardial injury in these hearts. However, the proximal signal responsible for this injurious cascade is not known.

Future studies could address the role of IL-10 in possible alterations in contractile performance after myocardial I/R. Our present findings indicate that endogenous IL-10 is important in attenuating the extent of PMN infiltration and myocardial injury after I/R. Furthermore, we clearly demonstrate that deficiency of IL-10 does not exacerbate myocardial injury via iNOS.

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REFERENCES


