Peroxynitrite, a Two-Edged Sword in Post-Ischemic Myocardial Injury—Dichotomy of Action in Crystalloid- Versus Blood-Perfused Hearts

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ABSTRACT

Peroxynitrite (ONOO⁻) is widely recognized as a mediator of NO⁻ toxicity, but recent studies have indicated that this compound may also have physiologic activity and induces vascular relaxation as well as inhibition of platelet aggregation and neutrophil adhesion. The present experiment was designed to determine whether ONOO⁻ may exert different effects on postischemic myocardial injury in a crystalloid perfusion environment versus a blood perfusion environment and, if it does, to clarify the mechanisms causing any differences. In Krebs-Henseleit buffer-perfused rabbit hearts, administration of ONOO⁻ at the onset of reperfusion enhanced myocardial injury in a concentration-dependent fashion with a significant effective concentration of 30 μM. In contrast, in blood-perfused hearts, administration of ONOO⁻ (1 to 30 μM) significantly attenuated postmyocardial injury as evidenced by improved cardiac function recovery, preserved endothelial function,

Substantial evidence exists indicating that reperfusion of an ischemic myocardium can extend myocardial injury and further jeopardize viable myocardial cells (Flitter, 1993). The cause of reperfusion-induced myocardial injury is likely multifactorial, including reactive oxygen species, calcium overload, and neutrophil-mediated cell damage. Numerous experiments have demonstrated that superoxide (O₂⁻) generation from ischemic/reperfused endothelial cells (ECs) and activated neutrophils (PMNs) is markedly increased in postischemic myocardial tissue (Flitter, 1993). The O₂⁻ further dismutates to H₂O₂ and OH⁻, the latter being highly toxic to biological tissues and causing significant myocardial injury (Flitter, 1993).

Nitric oxide (NO⁻), a free radical gas produced primarily by ECs in the cardiovascular system, has been shown to attenuate myocardial reperfusion injury by a constellation of actions. One of the proposed mechanisms by which NO⁻ attenuates postischemic myocardial injury is through the biradical reaction with O₂⁻ in a nearly diffusion-limited rate (6.7 × 10⁹ M⁻¹ s⁻¹) (Huie and Padmaja, 1993). This reaction scavenges potentially cytotoxic O₂ and prevents the formation of highly toxic OH⁻ (Rubanyi et al., 1991). However, recent studies have shown that the reaction of NO⁻ and O₂⁻ forms the potent cytotoxic anion peroxynitrite (ONOO⁻). In vitro and cell culture studies, ONOO⁻ has been shown to be highly reactive with a wide variety of molecules, including deoxyribose, cellular lipids, and protein sulphydryl moieties and causes direct oxidative tissue damage apparently similar to that caused by OH⁻ in vitro. Moreover, recent chemical and biochemical experimental results have indicated that ONOO⁻ may react with CO₂ and produce even more toxic free radicals such as NO₂⁻ and CO₃²⁻ (Squadrito and Pryor, 1998). However, a recent in vivo study has reported that ONOO⁻ exerts cardioprotective effects on the postischemic...
myocardium (Nossuli et al., 1998). This dichotomy of action may be related to the concentration of ONOO− to which myocardial cells are exposed, to potential detoxification, or to biortransformation to secondary intermediates.

One suspected reaction forming biologically active secondary intermediates is the nitrosylation of thiols (Wu et al., 1994; Ma et al., 1997a; Balazy et al., 1998). Biological thiols include glutathione, albumin, and cysteine groups on proteins. Reaction with these sulfhydryl-containing molecules will both prevent ONOO− from achieving high concentrations in tissues and potentially form nitrosylated or nitrated intermediates that have similar cardioprotective properties as NO. These thiol-containing substances are contained in blood plasma and red blood cells and may be responsible for detoxification of ONOO− in vivo.

The purposes of this study was 1) to determine whether ONOO− is cytotoxic at the organ level under a blood-cell free environment and, if it is, to establish a reliable dose-response relationship; and 2) to examine whether ONOO− can decrease reperfusion injury when blood cells are present and, if it does, to define the mechanisms by which ONOO− may exert cardioprotection against reperfusion injury.

Experimental Procedures

Materials. Chemicals were purchased from Sigma (St. Louis, MO) unless otherwise indicated. ONOO− was prepared by the methods described previously by Beckman et al. (1990), stored at −20°C in 15-ml capped centrifuge tubes, and used within 1 week. Excess H2O2 was removed by passing the ONOO− solution over solid granular manganese dioxide packed into a small column. Before each experiment, an aliquot was taken from the concentrated liquid layer on top of the ice crystals, and the concentration was determined using the extinction coefficient ε = 1670 M−1 cm−1 at 302 nm. Only those ONOO− aliquots that had a calculated concentration ≥180 mM were used. Decomposed ONOO− was obtained by incubating ONOO− in 0.5 M sodium phosphate buffer, pH 7.4, for 5 min at room temperature, and the pH value of this decomposed ONOO− was adjusted back to the same value as that of ONOO− stock solution with NaOH. Both ONOO− and decomposed ONOO− were diluted in Milli-Q water immediately before use, shielded from light, and kept on ice. Under these conditions, the diluted ONOO− stock solution is stable for at least 2 h (Villa et al., 1994). Saturated NO− solution was prepared as previously reported (Weyrich et al., 1994), and the NO− concentration was determined using a chemiluminescence NO− meter (SIEVERS 270B Nitric Oxide Analyzer; Sievers, Boulder, CO).

A total of 143 adult male New Zealand rabbits (3.2 to 3.8 kg) were used in this study. The experiments were performed in adherence to National Institutes of Health “Guidelines for the Use of Laboratory Animals” and approved by the Thomas Jefferson University Committee on Animal Care.

Isolated Heart Preparation. Crystallloid-perfused model. Rabbits were anesthetized (sodium pentobarbital, 35 mg/kg, i.v.) and heparinized (sodium heparin, 1000 U/kg, i.v.). Five minutes after heparin injection, a midsternal thoracotomy was performed, and hearts were rapidly excised and placed into ice-cold Krebs-Henseleit (KH) buffer solution consisting of (in mM): NaCl, 118; KCl, 4.75; KH2PO4, 1.19; MgSO4 (KH) buffer solution consisting of (in mM): NaCl, 118; KCl, 4.75; KH2PO4, 1.19; MgSO4, 0.5; and glucose, 11. Within 30 s, hearts were mounted onto a Langendorff heart perfusion apparatus and perfused at a constant pressure of 60 mm Hg with KH solution oxygenated with NaOH. Both ONOO− aliquots that had a calculated concentration ≥180 mM were used. Decomposed ONOO− was obtained by incubating ONOO− in 0.5 M sodium phosphate buffer, pH 7.4, for 5 min at room temperature, and the pH value of this decomposed ONOO− was adjusted back to the same value as that of ONOO− stock solution with NaOH. Both ONOO− and decomposed ONOO− were diluted in Milli-Q water immediately before use, shielded from light, and kept on ice. Under these conditions, the diluted ONOO− stock solution is stable for at least 2 h (Villa et al., 1994). Saturated NO− solution was prepared as previously reported (Weyrich et al., 1994), and the NO− concentration was determined using a chemiluminescence NO− meter (SIEVERS 270B Nitric Oxide Analyzer; Sievers, Boulder, CO).

Blood-perfused model. The blood-perfused isolated rabbit heart preparation was a modification of the paracorporeal rabbit heart model used previously by Sandhu et al. (1993). Two rabbits (one support rabbit, one heart donor rabbit) were used in each experiment. The support rabbit was anesthetized with sodium pentobarbital through the marginal ear vein. An endotracheal tube was inserted through a midline incision, and the rabbit was ventilated via a Harvard small animal respirator (Harvard Apparatus, Natick, MA). Arterial blood gas determination was measured periodically throughout the experiment, and oxygen flow to the respirator was adjusted to maintain pCO2, pH, and P2O of the animal within the normal physiological range.

After heparinization (1000 IU/kg bolus plus 300 IU/kg/h infusion, i.v.), one catheter was inserted in the left carotid artery to supply arterial blood to the isolated heart, and another catheter was inserted into the left common external jugular vein for venous return. An additional polyethylene catheter was inserted through the left femoral artery and positioned in the abdominal aorta for measurement of mean arterial blood pressure via a COBE CDX III pressure transducer (Lakewood, CO).

The heart donor rabbit was anesthetized and heparinized, and its heart was rapidly excised as described above. Blood was collected and filtered through a blood transfusion filter (40 μm; Baxter, Deerfield, IL) into a polyethylene container. Within 30 s, the heart was mounted onto a Langendorff heart perfusion apparatus and perfused in a retrograde fashion with KH solution. Five minutes after KH perfusion, arterial blood was withdrawn from the support rabbit by a roller pump into an overflow blood reservoir located 82 cm (60 mm Hg) above the isolated heart. The venous return pump was activated at the same time, and the blood collected from the heart donor rabbit was infused into the support rabbit. The arterial pump and venous pump were adjusted to the same speed so that mean arterial blood pressure of the support rabbit remained stable. The overflow blood and coronary venous blood from the isolated heart were collected into the heart chamber, and returned to the support rabbit after passing through a blood transfusion filter. All tubing was made of silicon, and the blood reservoirs and heart chamber were siliconized using Sigmacote.

Experimental protocol. After a 20-min equilibration time, hearts were subjected to complete global ischemia for 30 min by turning off the perfusion system. After the ischemia period, the perfusion system was restarted, and the hearts were reperfused (blood or crystalloid) for an additional 90 min. At the time of reperfusion, hearts were randomized to receive one of the following treatments for 20 min: 1) vehicle (Milli-Q water, pH adjusted to 8.5 with 0.1 N NaOH, continuously infused at rate to achieve 0.1% of the perfusate); 2) ONOO− (concentrations 0.3 to 100 mM in Milli-Q water, pH 8.5, continuously infused at a rate to achieve final concentrations of 0.3 to 100 μM depending on target concentrations); and 3) decomposed ONOO− (equivalent in strength to 100 mM ONOO−, pH 8.5, continuously infused at 0.1% of CF). In order to maximally reduce the exposure time of ONOO− to the perfusate before it entered the coronary circulation, a PE-20 tubing was inserted via a sidearm in the perfusion line and advanced to the tip of cannula. ONOO− was infused through this tubing to a point ~3 to 5 mm above the origin of the coronary arteries. The rate of infusion (0.1% of coronary perfusate flow rate) was continuously adjusted based on the perfusate flow rate so that the target final concentration was achieved. Sham ischemic-reperfusion hearts were perfused with KH solution or blood for 2.0 h without ischemia and reperfusion.

Functional assessment. To assess contractile function, a latex balloon was inserted into the left ventricular cavity through the mitral orifice and connected to a pressure transducer. The balloon was initially inflated with deionized water to produce an end diastolic pressure of 8 to 10 mm Hg. During the 30-min ischemic period, the balloon was deflated using a gas-tight microsyringe to minimize balloon-induced myocardial injury. At 3 min of reperfusion, the same volume of water was injected slowly back to the balloon. Left ven-
tricular pressure (LVP) and coronary venous flow were continuously recorded on a Power Macintosh computer via a MacLab data acquisition system (AD Instruments, Milford, MA). The left ventricular systolic pressure (LVSP), left ventricular end diastolic pressure (LVEDP), left ventricular developed pressure (LVPD; LVPD = LVSP – LVEDP), heart rate (HR), rate pressure product (PRP = HR × LVPD), and mean coronary venous flow rates were derived by computer algorithms.

**Assessment of endothelial function.** Coronary vasorelaxation responses to acetylcholine (ACh), an endothelium-dependent vasodilator, and S-nitroso-N-acetylpenicillamine (SNAP), an endothelium-independent vasodilator, were assessed immediately before ischemia (control) and again at the end of 90 min of reperfusion (or after 2.0 h of normothermic reperfusion in shams). ACh or SNAP was infused via a side port located just above the aortic cannula for 1 min, and the infusion rate was adjusted based on CF rate so that a final concentration of 0.1 mM was achieved (Smith et al., 1992). The ratio of postischemic versus preischemic flow response to ACh was used to evaluate the change in endothelial function after ischemia and reperfusion in comparison to smooth muscle relaxation to SNAP at the end of reperfusion.

**Assessment of necrotic injury.** At the end of each experiment, the heart was removed from the perfusion apparatus and the ventricles were sliced into ~2-mm thick slices. Slices were incubated in 0.1% nitroblue tetrazolium in phosphate buffer at pH 7.4 and 37°C for 15 min. The unstained portion (which is the irreversibly injured, necrotic region) was then separated from the stained (nonnecrotic) portion. Both sections were weighed, and the results were expressed as a percentage of necrotic tissue over total ventricular mass.

**Creatine kinase (CK) measurement.** After assessing necrotic injury, the left ventricular myocardial tissue was separated into two parts. One half of the tissue was weighed and stored in −70°C for later measurement of myeloperoxidase (MPO) activity as an indicator of PMN accumulation. The other half was homogenized in cold 0.25 M sucrose (1:10, w/v) containing 1 mM EDTA and 0.1 mM mercaptoethanol using a PRO 200 homogenizer (PRO Scientific, Monroe, CT) for measurement of tissue CK. Homogenates were centrifuged at 36,000g at 4°C for 30 min. The supernatant were decanted and analyzed spectrophotometrically for CK activity as reported previously (Ma et al., 1997b). Protein concentration was determined by the bicinchoninic acid method (Pierce, Rockford, IL). The CK loss was calculated by subtracting CK activity of postischemic hearts from CK activity in sham hearts; CK activity and loss were expressed in international units per 100 mg of protein.

**Measurement of MPO activity in cardiac tissue.** MPO, an enzyme that is specific for PMNs, was determined in cardiac tissue as described previously (Liu et al., 1998). One unit of MPO was defined as that quantity of enzyme hydrolyzing 1 mmol of peroxide per minute at 25°C, and results were expressed as units of MPO activity per 100 mg wet myocardial tissue.

**PMN Adhesion to Cultured ECs. EC culture.** Microvascular ECs were isolated from rabbit tissue by the methods reported by Renzi and Flynn (1992). Briefly, ~10 g of perirenal fat was taken from each side of pentobarbital-anesthetized rabbits and digested in a 0.3% collagenase solution. The digested tissue was centrifuged at 200g for 5 min. The bovine adipocytes and supernatant were removed, and the remaining cell pellet was washed in PBS plus 1% BSA and re centrifuged. The cell pellet was resuspended in 1 ml of PBS-BSA solution and applied to a preformed, isotonic, 45% continuous gradient of Percoll and centrifuged at 1000g for 10 min. The vascular cell band residing at a density level of ~1.03 g/ml was collected and filtered through a 60-μm nylon mesh. After a final washing and centrifuge step, the cells were resuspended in 2 ml of PBS-BSA solution and placed onto 0.1% gelatin-coated six-well tissue culture plates in a concentration of 250,000 cells/well and incubated with modified RPMI 1640 medium for 1.5 h. The wells were then washed again to remove nonadherent cells, and fresh medium was added. The cells reached confluence within 4 to 5 days and were passed into 24-well plates; experiments were performed within 48 h. Only cells at passage 2 were used.

**Rabbit PMN isolation.** Peripheral blood (20 ml) was collected from the central ear artery and mixed with 3.0 ml of anticoagulating agents, which included 1.6% citric acid and 2.5% sodium citrate at pH 5.4 and 17 ml of 6% Hespan solution. PMNs were isolated by a procedure described by Todd et al. (1996). PMN preparations obtained by this method are typically >95% pure (hematoyxin/eosin staining) and >95% viable (trypan blue exclusion).

**PMN adhesion to hypoxia-reoxygenated ECs.** Glucose-free PBS-BSA solution was first gassed for 5 min with a hypoxic gas mixture (90% N2-5% CO2-5% O2) in a custom-designed hypoxia-reoxygenation incubator. Normal culture medium was quickly replaced with the hypoxia-hypoglycemic PBS-BSA solution (300 μl per well) within the incubator, and ECs were incubated at 37 ± 0.2°C for 60 min. A continuous flow of a hypoxic gas humidified and warmed to 37°C was maintained during hypoxic incubations. After 60 min of hypoxic-hypoglycemic incubation, the culture medium was removed, 1 ml of preoxygenated buffer solution (prepared as described by Estevez et al. to minimize reactions of ONOO− with bicarbonate and other components in the culture medium) was added into each well (Estevez et al., 1995). Two minutes after reoxygenation, an aliquot of 5 × 104 PMNs was added into the each well. Three bolus injections (30-s interval) of vehicle (0.5 μl), ONOO− (3 μM), decomposed ONOO− (5 μM), or authentic NO’ (0.3 μM) were then added along one edge of each well using a repeating dispenser and gas tight microsyringe, and the buffer was rapidly swirled for 5 s after each injection. The plate was agitated (60 rpm) at 37°C in an incubator that is purged with a 20% O2-75% N2-5% CO2 gas mixture. After another 2 h of incubation, the number of PMNs adherent to ECs was quantitated by measuring MPO activity using the method described by Pietersma et al. (1994).

**Statistical Analysis.** All values in the text and figures are presented as means ± S.E. of n independent experiments. All time-related data were analyzed using repeated-measures ANOVA. When group differences were found by ANOVA, the source of differences was located with the Bonferroni correction for post hoc t test comparison. Values of P < .05 were considered to be statistically significant.

**Results**

**Effects of ONOO− on Cardiac Functional Recovery After Reperfusion.** The product of LVDP and HR (PRP) was used as a primary index for cardiac function. Perfusion of sham hearts with KH buffer for 2.0 h without ischemia caused a slow decline in PRP over time. At the end of the 2.0-h perfusion period, PRP decreased to 89 ± 1.8% of control value (P < .05) versus baseline. In the hearts perfused with KH buffer subjected to 30 min of ischemia, postischemic cardiac function was markedly reduced. After reperfusion, PRP gradually recovered over the first 30 min of reperfusion and remained stable thereafter. By the end of 90 min of reperfusion, PRP averaged to 46 ± 1.5% of preischemic value in vehicle-treated hearts (P < .001 versus sham). Infusion of 0.3 or 1 μM ONOO− for as long as 20 min beginning at the onset of reperfusion exerted no significant effects on the recovery of PRP. However, when 3 μM or higher concentrations of ONOO− were infused, PRP recovery decreased significantly in a dose-dependent fashion (Fig. 1A). The detrimental effects of increasing concentrations of ONOO− could not be explained by an alkalinic pH or by an excess amount of nitrite because administration of decomposed ONOO− at the same pH value had no effect (data not shown).

Sham hearts perfused with blood without ischemia main-
tained more stable cardiac function than KH-perfused hearts. By the end of the 120-min perfusion period, PRP remained at 96 ± 1.5% of control. After 30 min of ischemia, the hearts perfused with blood exhibited a faster recovery of cardiac function than KH-perfused hearts in the first 10 min. However, PRP showed a secondary decline starting at 60 min of reperfusion (Fig. 1B). This may be a result of additional injury induced by activated PMNs, which becomes significant when reperfusion is prolonged. In contrast to the observations in KH buffer-perfused hearts, administration of ONOO⁻ to blood-perfused hearts significantly improved postischemic cardiac function. Although there was no appreciable effect of 1 μM or 3 μM ONOO⁻ in KH-perfused hearts, there was a significantly greater PRP with these concentrations at 90-min reperfusion compared with the vehicle group (Fig. 1B). When the ONOO⁻ concentration was further increased, the cardioprotective effects of ONOO⁻ began to diminish (30 μM) or were cardiodepressive (100 μM) relative to vehicle.

Effects of ONOO⁻ on Endothelial Dysfunction. The changes in ACh-induced CF increase after myocardial ischemia and reperfusion, and the effects of administration of ONOO⁻ to reperfusion-induced endothelial dysfunction are presented in Figs. 2 and 3. Perfusion of the hearts with either KH solution or blood for 2.0 h without ischemia did not result in significant endothelial dysfunction. In contrast, after 30 min of ischemia and 90 min of reperfusion without ONOO⁻, ACh-induced CF increase was markedly attenuated in both KH-perfused (Fig. 2A) and blood-perfused hearts (Fig. 3A). This endothelial dysfunction was more severe in blood-perfused hearts than KH-perfused hearts (44 ± 3.8% of baseline increase in blood flow-perfused hearts versus 64 ± 3.9% of baseline in KH-perfused hearts, P < .05). In KH-perfused hearts, administration of 0.3 to 3 μM ONOO⁻ had no significant effect on postischemic endothelial dysfunction. However, administration of 100 μM ONOO⁻ further exaggerated endothelial dysfunction (Fig. 2A). The vasorelaxation response to SNAP, an endothelium-independent vasodilator, was not changed at the end of reperfusion (98 ± 1.9% in KH-perfused hearts and 97 ± 2.2% in blood-perfused hearts), indicating that the abundant responses were due to endothelial dysfunction and not to other causes of CF deficits (edema, no reflow). In contrast, in blood-perfused hearts, administration of as low as 3 μM ONOO⁻ significantly attenuated endothelial dysfunction in a concentration-dependent manner between 1 and 30 μM (Fig. 3A). Administration of ONOO⁻ at any concentration had no significant effect on the endothelium-independent effect to SNAP (Figs. 2B and 3B).

Effects of ONOO⁻ on Myocardial Cellular Injury. To further clarify the effect of ONOO⁻ on myocardial ischemia-reperfusion injury, we measured the myocardial CK loss and myocardial necrotic injury at the end of 90 min of reperfu-
tion period was more severe in untreated (vehicle) blood-perfused hearts than in KH-perfused hearts. The additional damage is likely caused by activated PMNs, which are absent from crystalloid perfusate. However, in contrast to KH-perfused hearts, administration of ONOO\textsuperscript{−} to blood-perfused hearts significantly attenuated both CK loss and necrosis between 0.3 μM and 30 μM. Furthermore, this protective effect of ONOO\textsuperscript{−} occurred at much lower concentrations than that required to exert significant detrimental effects in KH-perfused hearts. Thus, when 1 μM ONOO\textsuperscript{−} was given, myocardial CK loss and necrotic size were significantly decreased. The greatest reduction in CK loss and necrosis occurred at 3 μM ONOO\textsuperscript{−}. Further increasing the concentration of ONOO\textsuperscript{−} to 30 μM did not exert more protection against myocardial CK loss and necrotic injury, and increasing ONOO\textsuperscript{−} concentration to 100 μM significantly increased myocardial CK loss and increased necrotic size to levels comparable with the vehicle group (Table 1).

**Effects of ONOO\textsuperscript{−} on Myocardial MPO Activity.** To determine the role of PMNs on the effects of ONOO\textsuperscript{−} in blood-perfused hearts, myocardial tissue MPO activity, a reliable index of PMN accumulation in ischemia-reperfusion myocardial tissue, was measured. In KH-perfused hearts, there was no detectable MPO activity in any group. In contrast, a 7-fold increase in MPO activity was measured in blood-perfused hearts subjected to ischemia and reperfusion (Fig. 4). Administration of ONOO\textsuperscript{−} attenuated MPO increase in a dose-dependent manner (Fig. 4). Administration of decomposed ONOO\textsuperscript{−} had no effect on MPO activity after ischemia and reperfusion (data not shown).

**Effects of ONOO\textsuperscript{−} on PMN Adhesion to Cultured ECs.** To further elucidate the effect of ONOO\textsuperscript{−} on PMN-EC interactions, the effects of ONOO\textsuperscript{−} on isolated PMN adhesion to cultured ECs was studied in vitro. Exposing cultured microvascular ECs to hypoxia (pO\textsubscript{2} in the culture medium decreased from 149 ± 5 mm Hg to 47 ± 5 mm Hg) followed by reoxygenation significantly increased PMN adhesion to ECs (Fig. 5). Addition of three multiple doses of 3 μM ONOO\textsuperscript{−}, a concentration that exerted the maximal protection in blood-perfused hearts, at the time of reoxygenation markedly inhibited PMN adherence to the microvascular ECs (Fig. 5). Administration of decomposed ONOO\textsuperscript{−} had no effect. However, the inhibitory effects of ONOO\textsuperscript{−} on PMN-ECs interactions was much weaker than that exerted by NO\textsuperscript{•−}. When three multiple doses of 0.3 μM authentic NO\textsuperscript{•−}, a concentration that is 10 times lower than ONOO\textsuperscript{−}, was added, PMN adhesion induced by hypoxia-reoxygenation was almost completely blocked (Fig. 5).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Dose-response relationship of ONOO\textsuperscript{−} on myocardial CK loss and necrotic size after 30 min of ischemia and 90 min of reperfusion in KH buffer-perfused hearts versus blood-perfused hearts</th>
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<tr>
<td></td>
<td><strong>Myocardial CK Loss (LU/100 mg Protein)</strong></td>
</tr>
<tr>
<td></td>
<td>KH buffer</td>
</tr>
<tr>
<td>Vehicle</td>
<td>337 ± 32 (n = 11)</td>
</tr>
<tr>
<td>ONOO\textsuperscript{−} (0.3 μM)</td>
<td>334 ± 36 (n = 10)</td>
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<tr>
<td>ONOO\textsuperscript{−} (3 μM)</td>
<td>415 ± 42 (n = 10)</td>
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<tr>
<td>ONOO\textsuperscript{−} (30 μM)</td>
<td>488 ± 38 (n = 10)</td>
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<tr>
<td>ONOO\textsuperscript{−} (100 μM)</td>
<td>612 ± 34 (n = 10)</td>
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\( ^*P < .05; ^{**}P < .01 \) versus vehicle group.
In 1995, we first reported that SIN-1, a molecule that releases NO\(^{\cdot}\) and O\(_{2}\)\(^{\cdot}\) simultaneously and produces ONOO\(^{\cdot}\), exerts opposite effects on postischemic myocardial injury in crystalloid buffer-perfused versus blood-perfused hearts (Lopez et al., 1995). The results of the present study demonstrate that 1) ONOO\(^{\cdot}\) is a “double-edged sword”; it has the ability to directly enhance myocardial damage and is also capable of indirectly protecting myocardial cells from reperfusion injury by inhibiting PMN-endothelial interactions; 2) the ONOO\(^{\cdot}\) concentration that is needed to exert indirect protective effects is much lower than that required to exert its direct toxic effect; and 3) the effects of ONOO\(^{\cdot}\) on PMN accumulation is linear in the concentration range of 1 to 100 μM; however, the maximal protective effects of ONOO\(^{\cdot}\) are observed at a ONOO\(^{\cdot}\) concentration of 3 μM. Exposing hearts to a higher concentration of ONOO\(^{\cdot}\) on PMN accumulation is linear in the concentration range of 1 to 100 μM; however, the maximal protective effects of ONOO\(^{\cdot}\) are observed at a ONOO\(^{\cdot}\) concentration of 3 μM. Exposing hearts to a higher concentration of ONOO\(^{\cdot}\) (i.e., 100 μM) enhanced reperfusion injury despite even less PMN accumulation at this concentration. The results suggest that ONOO\(^{\cdot}\) could increase postischemic myocardial injury when formed at a high concentration.

In vitro toxicity of ONOO\(^{\cdot}\) is well documented. However, the effects of ONOO\(^{\cdot}\) on postischemic myocardial function and morphological changes associated with ischemia and reperfusion are less clear, and there are only a few studies in which the effects of ONOO\(^{\cdot}\) on postischemic injury are examined. Wang and Zweier (1996) reported that in crystalloid buffer-perfused hearts, ONOO\(^{\cdot}\) concentration is markedly increased during ischemia and early reperfusion. Administration of L-N\(^{\cdot}\)monomethyl-L-arginine not only decreased ONOO\(^{\cdot}\) formation, but also attenuated posts ischemic cardiac dysfunction. These results suggest that endogenous ONOO\(^{\cdot}\) may contribute to postischemic myocardial injury. Subsequent studies by Schulz et al. (1997; Yasmin et al., 1997) confirmed these findings using a similar model (i.e., crystalloid buffer perfusion) but with a different method of measuring ONOO\(^{2}\) generation. These investigations further demonstrated that ONOO\(^{\cdot}\) impairs cardiac contractile function by decreasing cardiac efficiency. However, in these studies, only one or two concentrations of ONOO\(^{\cdot}\) were studied. Moreover, because the half-life of ONOO\(^{\cdot}\) in biologic solution is very short (<1 s), any time delay before ONOO\(^{\cdot}\) enters the coronary circulation can decrease the ONOO\(^{\cdot}\) activity to which cardiac tissue is actually exposed. Consequently, the ONOO\(^{\cdot}\) effects on cardiac tissue may therefore be under- or overestimated. To establish a reliable dose-response relationship of ONOO\(^{\cdot}\) in postischemic myocardial injury, we have used a specially designed perfusion apparatus. Our results demonstrated that in crystalloid buffer-perfused hearts, ONOO\(^{\cdot}\) enhances postischemic myocardial injury in a concentration-dependent manner in the range of 3 to 100 μM. These results suggest that under a blood cell-free environment, such as might be found in organ transplantation and open-heart surgery using crystalloid cardioplegia solutions, ONOO\(^{\cdot}\) may greatly enhance tissue injury.

**Fig. 4.** Dose-response relationships of ONOO\(^{\cdot}\) on PMN accumulation as measured by MPO activity in blood-perfused hearts. Bar heights are means and brackets indicate ±S.E.M. Numbers at the bottom of the bars represent number of hearts studied. *P < .05; **P < .01 versus vehicle.

**Discussion**

In 1995, we first reported that SIN-1, a molecule that releases NO\(^{\cdot}\) and O\(_{2}\)\(^{\cdot}\) simultaneously and produces ONOO\(^{\cdot}\), exerts opposite effects on postischemic myocardial injury in crystalloid buffer-perfused versus blood-perfused hearts (Lopez et al., 1995). The results of the present study demonstrate that 1) ONOO\(^{\cdot}\) is a “double-edged sword”; it has the ability to directly enhance myocardial damage and is also capable of indirectly protecting myocardial cells from reperfusion injury by inhibiting PMN-endothelial interactions; 2) the ONOO\(^{\cdot}\) concentration that is needed to exert indirect protective effects is much lower than that required to exert its direct toxic effect; and 3) the effects of ONOO\(^{\cdot}\) on PMN accumulation is linear in the concentration range of 1 to 100 μM; however, the maximal protective effects of ONOO\(^{\cdot}\) are observed at a ONOO\(^{\cdot}\) concentration of 3 μM. Exposing hearts to a higher concentration of ONOO\(^{\cdot}\) on PMN accumulation is linear in the concentration range of 1 to 100 μM; however, the maximal protective effects of ONOO\(^{\cdot}\) are observed at a ONOO\(^{\cdot}\) concentration of 3 μM. Exposing hearts to a higher concentration of ONOO\(^{\cdot}\) (i.e., 100 μM) enhanced reperfusion injury despite even less PMN accumulation at this concentration. The results suggest that ONOO\(^{\cdot}\) could increase postischemic myocardial injury when formed at a high concentration.

In vitro toxicity of ONOO\(^{\cdot}\) is well documented. However, the effects of ONOO\(^{\cdot}\) on postischemic myocardial function and morphological changes associated with ischemia and reperfusion are less clear, and there are only a few studies in which the effects of ONOO\(^{\cdot}\) on postischemic injury are examined. Wang and Zweier (1996) reported that in crystalloid buffer-perfused hearts, ONOO\(^{\cdot}\) concentration is markedly
In contrast to the linear dose-response relationship observed in the crystalloid-perfused hearts, a bell-shaped dose-response relationship to ONOO\(^-\) was observed in blood-perfused hearts. The minimal protective concentration of ONOO\(^-\) in this model was found to be 1 \(\mu\)M, a concentration that is 1/30th the concentration that results in significant myocardial damage in crystalloid-perfused hearts. The maximal protection was obtained at 3 \(\mu\)M ONOO\(^-\). The protective effects decreased when ONOO\(^-\) concentration was increased to 30 \(\mu\)M and even became detrimental when 100 \(\mu\)M ONOO\(^-\) was administered in blood-perfused hearts. In the present study, the specially designed ONOO\(^-\) delivery system enabled us to administer precise concentrations of ONOO\(^-\) to the local coronary circulation. This method eliminated the variability of ONOO\(^-\) concentration that may occur in the coronary circulation if ONOO\(^-\) was administered systemically because the time required for ONOO\(^-\) travel into the coronary circulation is affected significantly by many hemodynamic parameters such as cardiac output, aortic blood pressure, and coronary vasculature resistance.

The different dose-response relationships observed in crystalloid buffer-perfused versus blood-perfused hearts can be explained by several mechanisms. First, in crystalloid-perfused hearts, myocardial cells are the only cellular oxidant targets. In contrast, in blood-perfused hearts, multiple reaction targets exist and some other biological molecules may be preferentially oxidized other than cardiac tissue. Second, in the blood-perfused hearts, ONOO\(^-\) can be detoxified by glutathione (Wu et al., 1994; Balazy et al., 1998) and uric acid (particularly in those species that maintain a high level of urate, such as the human) (Skinner et al., 1998) present in erythrocytes, platelets, and plasma. The reaction of glutathione and uric acid with ONOO\(^-\) not only reduces ONOO\(^-\) concentration and thus decreasing myocardial oxidative injury induced by ONOO\(^-\) but also regenerates NO\(^-\), thereby converting the toxic species, ONOO\(^-\), to the protective species, NO\(^-\). Third, it is well known that PMNs play a critical role in postischemic myocardial injury. The results from the present study as well as those reported by other investigators (Lefer et al., 1997) demonstrate that ONOO\(^-\) possesses significant anti-PMN effect (although not as strong as that exerted by NO\(^-\), as illustrated in Fig. 5). These results indicate that ONOO\(^-\) may reduce postischemic myocardial injury indirectly through its anti-PMN activity. In this connection, we have recently demonstrated that administration of low dose ONOO\(^-\) significantly reduced infarct size in a rabbit in vivo myocardial ischemia-reperfusion model. However, when PMN adhesion is first blocked with a monoclonal antibody against CD 18 adhesion molecules on PMNs (R15.7), administration of the same concentration of ONOO\(^-\) exerted no cardioprotection (Huffman et al., 1999). These results provide strong evidence that ONOO\(^-\) attenuates myocardial injury via its anti-PMN activity. Fourth, it is well known that platelet and platelet-derived mediators play a significant role in acute myocardial ischemic injury in the absence of reperfusion (Stamler and Loscalzo, 1991). However, recent experiments have revealed that platelet may also contribute significantly to reperfusion injury via a platelet-PMN interaction (Nash, 1994). It has been reported that platelet activation significantly facilitates PMN adhesion to ECs and thus increases PMN accumulation in inflammatory tissue (Diacovo et al., 1996). Moreover, the platelet-PMN interaction markedly increases superoxide anion generation by PMNs (Colli et al., 1996). In the present experiment, we have observed that administration of ONOO\(^-\) significantly inhibited platelet aggregation ex vivo (maximal amplitude from 46.2 ± 2.3 to 36.7 ± 3.5\%, slope from 49.2 ± 2.5 to 39.8 ± 3\%; \(P < .05\)). Therefore, ONOO\(^-\) might protect myocardial tissue by decreasing the platelet-PMN interaction.

There are several potential sources of ONOO\(^-\) formation in the in vivo ischemic-reperfused heart. The vascular ECs generate NO\(^-\) constitutively and produce a burst of O\(_2^+\) production on reperfusion, suggesting that ECs may generate ONOO\(^-\) following ischemia and reperfusion. However, it is
unlikely that ONOO\textsuperscript{−} production from EC constitutive NO synthase would be able to reach a high enough concentration to cause significant cellular injury. In contrast, ONOO\textsuperscript{−} produced in the early phase of reperfusion may reach levels that could inhibit PMNs from adhering to the ECs, thus protecting myocardial cells from PMN-induced damage. Another potential source of ONOO\textsuperscript{−} is inducible NO synthase expressed in ischemic/reperfused cardiac tissue. Several recent studies have demonstrated that both inducible and constitutive NO synthase can generate NO\textsuperscript{·} and O\textsubscript{2}\textsuperscript{−} simultaneously and produce ONOO\textsuperscript{−} when the L-arginine concentration is either decreased or depleted for a period of time (Xia et al., 1996; Huk et al., 1997; Xia and Zweier, 1997). Liu et al. have recently reported that in ischemic-reperfused myocardial tissue in which inducible NO synthase is expressed, O\textsubscript{2}\textsuperscript{−} and NO\textsuperscript{·} levels are increased and nitrotyrosine formation is detected (Liu et al., 1997), suggesting that a significant amount of ONOO\textsuperscript{−} can be generated. However, the most significant source of ONOO\textsuperscript{−} is likely to be infiltrated leukocytes. It is well documented that there is a substantial leukocyte accumulation in ischemia-reperfusion myocardium in vivo. Recent studies have demonstrated that activated leukocytes generate a large amount of NO\textsuperscript{·} and O\textsubscript{2}\textsuperscript{−} simultaneously and yield ONOO\textsuperscript{−} (Ischiropoulos et al., 1992; Carreras et al., 1994; Rodenas et al., 1995; Evans et al., 1996; Xia and Zweier, 1997). Because this ONOO\textsuperscript{−} is generated by those leukocytes that have adhered to the endothelium and myocytes, a high local concentration of ONOO\textsuperscript{−} may be formed on the intercellular compartment between PMN-EC and PMN-myocyte cells and thus may cause significant damage directly to the endothelium and myocardium.

**Limitation of the Study.** In the present experiment, the dose-response relationships were established based on the effects of exogenously infused ONOO\textsuperscript{−} on postischemic injury. Caution must be taken when extrapolating these dose-response relationships to the effects of endogenous ONOO\textsuperscript{−} on myocardial reperfusion injury for the following reasons. First, when ONOO\textsuperscript{−} is infused exogenously, the concentration to which the myocytes are actually exposed is likely to be lower than that calculated in the perfusate due to its fast decay in biological solution. Therefore, the toxic concentration of endogenously formed ONOO\textsuperscript{−} is likely to be lower than that determined using exogenous ONOO\textsuperscript{−}. Second, when ONOO\textsuperscript{−} is generated from intracellular sources, or from adhered leukocytes, a high ONOO\textsuperscript{−} concentration compartment may be formed. Therefore, the local concentration of ONOO\textsuperscript{−} is likely to be much higher than that determined from extracellular fluid, such as coronary effluent. Thus, it is possible that intracellular ONOO\textsuperscript{−} concentrations may vary extensively and that ONOO\textsuperscript{−}, depending on its concentrations and environments, may act as a “double-edged sword” as demonstrated by the present study.

**References**


Beckman JS, Beckman TW, Chen J, Marshall PA and Freeman BA (1990) Apparent hydroxyl radical production by peroxynitrite: Implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci USA 87:1620–1624.


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