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Review

Nitric Oxide and Peroxynitrite in Postischemic Myocardium

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ABSTRACT

Alterations in the production of nitric oxide ($NO^{\bullet}$) are a critical factor in the injury that occurs in ischemic and reperfused myocardium; however, controversy remains regarding the alterations in $NO^{\bullet}$ that occur and how these alterations cause tissue injury. As superoxide generation occurs during the early period of reperfusion, the cytotoxic oxidant peroxynitrite ($ONOO^-$) could be formed; however, questions remain regarding $ONOO^-$ formation and its role in postischemic injury. Electron paramagnetic resonance spin trapping studies, using the $NO^{\bullet}$ trap Fe^{2+}-*N*-methyl-D-glucamine dithiocarbamate (Fe-MGD), and chemiluminescence studies, using the enhancer luminol, have been performed to measure the magnitude and time course of $NO^{\bullet}$ and $ONOO^-$ formation in the normal and postischemic heart. Isolated rat hearts were subjected to control perfusion, or ischemia followed by reperfusion in the presence of Fe-MGD with electron paramagnetic resonance measurements performed on the effluent from these hearts. Whereas only trace signals were present prior to ischemia, prominent $NO^{\bullet}$ adduct signals were seen during the first 2 min of reflow. The reperfusion associated increase in these $NO^{\bullet}$ signals was abolished by nitric oxide synthase inhibition. In hearts perfused with luminol to detect $ONOO^-$ formation, a similar marked increase was seen during the first 2 min of reperfusion that was blocked by nitric oxide synthase inhibitors and by superoxide dismutase. Either N^G-nitro-L-arginine methyl ester or superoxide dismutase treatment resulted in more than twofold higher recovery of contractile function than in untreated hearts. Immunohistology studies demonstrated that the $ONOO^-$-mediated nitration product nitrotyrosine was formed in postischemic hearts, but not in normally perfused controls. Thus, $NO^{\bullet}$ formation is increased during the early period of reperfusion and reacts with superoxide to form $ONOO^-$, which results in protein nitration and myocardial injury. Antioxid. Redox Signal. 3, 11–22.

INTRODUCTION

NITRIC OXIDE ($NO^{\bullet}$) is a free radical endogenously produced by a variety of mammalian cells and has been shown to be a ubiquitous signal transduction molecule. $NO^{\bullet}$ is known to play an important role in blood pressure regulation, vascular tone, neural signaling, and immunological function (24). It is formed by a class of enzymes, nitric oxide synthases, which synthesize $NO^{\bullet}$ from arginine. $NO^{\bullet}$ binds to and activates guanylate cyclase resulting in the formation of the second messenger molecule cyclic GMP (14), which accounts for many of the physiological effects of $NO^{\bullet}$. Beyond its homeostatic effects, there is evidence that $NO^{\bullet}$ can induce cellular injury due to either direct toxicity (9, 19) or the reaction with superoxide ($^{\bullet}O_2^-$) to form the potent oxidant peroxynitrite ($ONOO^-$) (1, 2).

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Alterations in $NO^{\cdot}$ formation are thought to be of particular importance in the pathogenesis of the injury that occurs in ischemic and reperfused tissues. In postischemic myocardium, alterations in $NO^{\cdot}$ result in endothelial dysfunction and impaired tissue perfusion (6). There has been considerable controversy regarding whether ischemia and reperfusion result in increased or decreased $NO^{\cdot}$ formation in the heart. Studies of endothelial function and vascular reactivity have been interpreted to suggest that $NO^{\cdot}$ production is decreased in postischemic myocardium, and based on this evidence it was hypothesized that a loss of basal $NO^{\cdot}$ production is an important source of postischemic injury (20). Subsequently, other studies have shown that inhibitors of nitric oxide synthase (NOS) can dramatically protect against postischemic injury (27, 29). From these latter studies it was suggested that $NO^{\cdot}$ may be involved in the process of tissue injury and that the production of $NO^{\cdot}$ may actually be increased by the process of ischemia. Subsequent direct electron paramagnetic resonance (EPR) spin tapping measurements were performed that demonstrated that $NO^{\cdot}$ formation is greatly increased in ischemic myocardium (43). As it was previously demonstrated that there is a burst of $^{\cdot}O_2^-$ generation in the postischemic heart, it was hypothesized that $NO^{\cdot}$ might react with this $^{\cdot}O_2^-$ resulting in the formation of the potent cytotoxic oxidant $ONOO^-$ (12, 39).

Although increased $NO^{\cdot}$ and $ONOO^-$ have been hypothesized to be critical biochemical mechanisms of postischemic injury in the heart and in other tissues (7, 27, 40), until recently there was only indirect evidence to support this. As $NO^{\cdot}$ is paramagnetic and binds with high affinity to a variety of metal chelates and metalloproteins, the distinctive EPR spectra of these nitroso complexes can serve as a quantitative measure of $NO^{\cdot}$ generation (25). Recently, the ferrous iron complex of *N*-methyl-D-glucamine dithiocarbamate (MGD), Fe^{2+}-MGD_2 (Fe-MGD), has been shown to be suitable for measurement of $NO^{\cdot}$ in living tissue (18). We have demonstrated using this EPR spin trapping technique that increased levels of $NO^{\cdot}$ are generated in ischemic myocardium (43). It also has been shown that luminol-enhanced luminescence can be used to measure $ONOO^-$ formation specifically (32). Further measurements of $ONOO^-$-mediated protein damage with nitration of the amino acid tyrosine have been reported using specific antibodies directed against nitrotyrosine (4).

To measure and characterize $NO^{\cdot}$ and $ONOO^-$ generation during postischemic reperfusion and to assess the functional importance of this process in the pathogenesis of myocardial reperfusion injury, EPR measurements of the magnitude and time course of $NO^{\cdot}$ formation and superoxide generation were performed along with measurements of $ONOO^-$ formation from luminol-enhanced chemiluminescence and histochemical measurements of the formation of the $ONOO^-$-mediated nitration product nitrotyrosine. The functional importance of this process in the pathogenesis of myocardial injury was determined from hemodynamic studies of the recovery of contractile function in the presence and absence of NOS inhibition or superoxide dismutase (SOD) treatment.

EXPERIMENTAL PROCEDURES

Isolated heart preparation

Female Sprague–Dawley rats (250–300 g) were heparinized with 500 units of heparin and anesthetized with intraperitoneal pentobarbital at a dose of 30–35 mg/kg. The hearts were rapidly excised, and the ascending aorta cannulated and perfused at 37°C with a constant pressure of 80 mm Hg using Krebs bicarbonate perfusate (in m*M*: 16.7 glucose, 120.0 NaCl, 25.0 $NaHCO_3$, 2.5 $CaCl_2$, 5.9 KCl, and 1.2 $MgCl_2$), which was continuously bubbled with 95% O_2/5% CO_2 gas. Two side arms in the perfusion line located just proximal to the heart cannula allowed infusion of treatment agents and spin traps directly into the heart. Coronary flow was continuously measured using a Transonic Flowmeter, model HT107, with a 2N inline flow probe. A fluid-filled latex balloon was inserted into the left ventricle through the mitral valve and secured with a ligature around the left atrium. The balloon was initially in-

flated to an end-diastolic pressure of 8–12 mm Hg and connected to a pressure transducer via a hydraulic line. Left ventricular pressures were recorded with a Gould model RS3400 recorder and stored along with coronary flow values using a MacLab system. Subsequently, the left ventricular developed pressure (LVDP), the difference between peak-systolic and end-diastolic pressures, and the rate pressure product (RPP), the product of heart rate and LVDP, were calculated as indexes of contractile function. Hearts were allowed to stabilize for 10–15 min prior to initiating the experimental protocol.

EPR spin trapping of $NO^{\cdot}$ or oxygen radicals

For measurements of $NO^{\cdot}$, the Fe-MGD spin trap complex was infused through one of the side arms with a final concentration 2 m*M* in Fe^{2+} and 10 m*M* in MGD. Spin trap-containing effluent was collected in 20-s aliquots prior to 30 min of global ischemia and during the first 2 min of reflow, as well as after 5 and 15 min of reperfusion. While samples were collected, heart perfusion was switched from constant pressure to constant flow at 9 ml/min via a syringe infusion pump. Samples were immediately frozen in liquid nitrogen and stored at 77 K until EPR measurement. The protocol for oxygen radical trapping was similar to that utilized for $NO^{\cdot}$ except that the spin trap was 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), used at a 50 m*M* final concentration, with experiments performed in the dark to minimize any light-induced DMPO degradation. DMPO was purchased from Aldrich and further purified by double distillation. All reported spin trapping studies were repeated in a series of at least three hearts for each protocol or treatment. EPR spectra were recorded in flat cells at room temperature with a Bruker ER 300 spectrometer operating at X-band with 100 kHz modulation frequency and a TM_{110} cavity, as described previously (41, 42). The microwave frequency and magnetic field were precisely measured using an EIP 575 microwave frequency counter and Bruker ER 035M NMR gaussmeter. Relative quantitation of the $NO^{\cdot}$ and oxygen free radical signals was performed by double integration.

Chemiluminescence assay of $ONOO^{-}$ generation

Luminol chemiluminescence was used for mesurement of $ONOO^{-}$ in the coronary effluent as previously described (36). An alkaline solution containing 5 m*M* Na_2CO_3 and 40 μM 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol), pH 9.2, was infused during the first minute of reflow, and after 15 min of reflow samples were collected in 10-s intervals and then immediately frozen in liquid nitrogen. The frozen samples were directly transferred and thawed in the measurement chambers of a six-channel Berthold multi-Biolumat LB9505C luminometer.

Immunohistochemistry assay of nitrotyrosine

After either normal perfusion or 45 min of reperfusion, hearts were quickly removed from the cannula, the ventricles sliced into 3–4-mm-thick sections, and immediately immersed in 10% formalin at 4°C. Histological processing was done by conventional methods (8). The sections were incubated for 60 min with the affinity-purified rabbit polyclonal anti-nitrotyrosine antibody, at a 1:25 dilution in a solution of Tris-buffered saline/milk/1% normal goat serum. After the primary incubation, the slides were rinsed in Tris-buffered saline/milk, incubated with the biotinylated secondary for 30 min, then rinsed again, incubated with the tertiary, ExtAvidin alkaline phosphatase, diluted 1:800 in Tris buffer, pH 8.2, for 60 min, and then washed a final time. Fast Red was used as the substrate and produces a red reaction when exposed to the tertiary (8). The stained sections were counterstained with Mayers modified hematoxylin. All incubations were performed at 24°C in a humidity chamber, and all rinses were for 5 min each. Each tissue block was stained with and without the primary antibody to monitor any background staining.

Materials

MGD was synthesized as described previously (43). $ONOO^{-}$ was synthesized in a quenched-flow reactor from the reaction of an aqueous 0.6 *M* HCl, 0.7 *M* H_2O_2 solution with

a solution of 0.6 m*M* nitrite as described previously (2). Perfusate reagents, bovine erythrocyte copper zinc SOD, N^G-nitro-L-arginine methyl ester (L-NAME), N^G-monomethyl-L-arginine (L-NMMA), uric acid, H_2O_2, sodium nitrite, avidin, and ExtrAvidin alkaline phosphatase were purchased from Sigma. *S*-Nitroso-*N*-acetylpenicillamine was purchased from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA), sodium heparin from Elkins Sinn, Inc. (Cherry Hill, NJ), and sodium pentobarbital from Steris Laboratories, Inc. (Phoenix, AZ). Affinity-purified rabbit polyclonal anti-nitrotyrosine antibody was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY).

RESULTS

Measurement of NO$^{\cdot}$ and superoxide generation

Preischemic and postischemic measurements of NO$^{\cdot}$ formation were performed on the coronary effluent of hearts infused with the NO$^{\cdot}$ trap Fe-MGD. Hearts were infused with Fe-MGD for 30 s, and effluent was sampled. The hearts were then allowed to reequilibrate in the absence of Fe-MGD for 5 min and then subjected to 30 min of global ischemia. The hearts were then reperfused in the presence of Fe-MGD with effluent collected in 20-s aliquots. Whereas only a trace signal was observed in the effluent of hearts perfused with Fe-MGD prior to ischemia, following reperfusion after 30 min of global ischemia a prominent triplet EPR spectrum was observed due to formation of the NO–Fe-MGD complex with a characteristic central g value of 2.04 and hyperfine splitting of 12.7 G (Fig. 1). Measurements performed in a series of hearts demonstrated a >10-fold increase in the NO–Fe-MGD signal during the first 20 s of reflow followed by a rapid decline over the first minute, which was followed by a second peak during the second minute of reflow, after which the observed signal intensity gradually declined (Figs. 1 and 2, top). After 5 min of reperfusion, the NO–Fe-MGD signal declined toward, but remained higher than, preischemic values. In additional series of hearts that were pretreated with 1.0

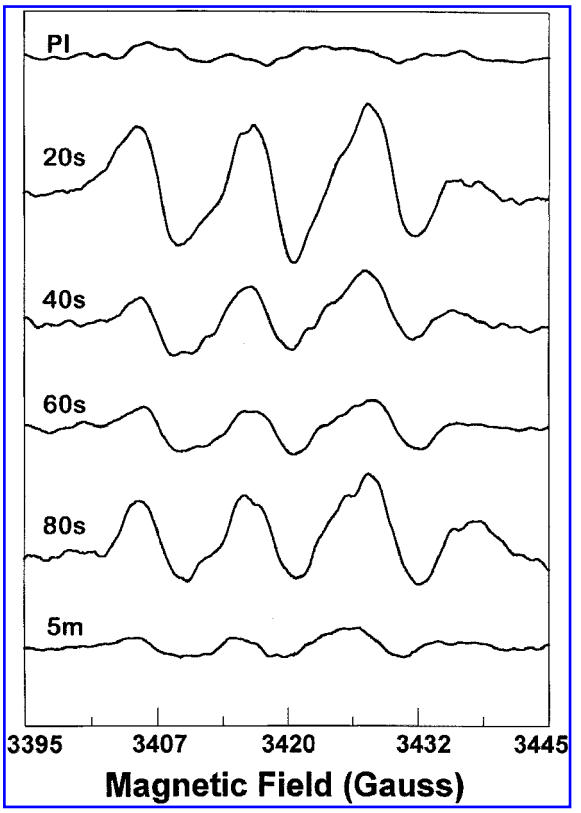

FIG. 1. EPR spectra prior to ischemia (PI) and over the early period of reperfusion. Measurements were performed on the coronary effluent of a heart infused with Fe-MGD (2 m*M* Fe^{2+}, 10 m*M* MGD) prior to and after 30 min of global ischemia. Effluent was collected in 20-s samples with the figure labeled as follows: PI, preischemic; 20 s, 0–20 s of reflow; 40 s, 20–40 s of reflow; 60 s, 40–60 s of reflow; 80 s, 60–80 s of reflow; and 5 m, 300–320 s of reflow. Spectra were recorded at room temperature with microwave frequency of 9.78 GHz using 80-mW microwave power and a modulation amplitude of 4.0 G. Each spectrum was the sum of 20 60-s spectral acquisitions of 50 G sweep width with a time constant of 0.32 s.

m*M* concentrations of either of the NOS inhibitors L-NAME or L-NMMA, no increase was seen after reperfusion with >80% inhibition of the reperfusion associated increase observed (Fig. 2, top).

Spin trapping measurements of oxygen radical generation were performed in hearts in a manner identical to that performed for NO$^{\cdot}$, except that the spin trap DMPO was infused at a final concentration of 40 m*M*. When hearts were subjected to 30 min of 37°C global ischemia followed by reperfusion, prominent radical generation was observed during the early period of reflow, as described previously (41, 42). Prior to ischemia, however, only trace

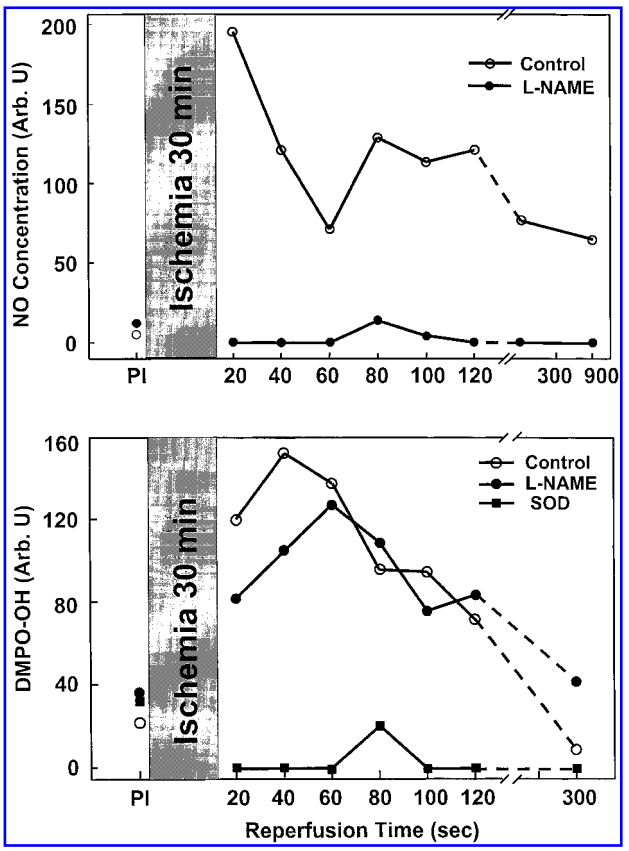

FIG. 2. (**Top**) **Graph of the time course of NO˙ generation in either control untreated or L-NAME-pretreated hearts.** Data were obtained from the intensity of EPR spectra observed in the coronary effluent of hearts perfused with Fe-MGD as described in Fig. 1. In the graph there is a break in the reperfusion time axis between 120 s and 300 s, after which the scale is condensed to show the full measured time course of NO˙ generation. (**Bottom**) **The time course of oxygen radical generation in the postischemic heart.** Untreated control, L-NAME (1 m*M*)-pretreated, or SOD (200 U/ml)-treated hearts were studied. Data were obtained from the intensity of EPR spectra observed in the coronary effluent of hearts perfused with 40 m*M* DMPO. Data are shown with a break in the reperfusion time axis between 120 s and 300 s, after which the scale is condensed to depict the full measured time course of free radical generation after the 30-min period of ischemia. PI, prior to ischemia.

signals were seen. The observed signal was a 1:2:2:1 quartet with $a_N = a_H = 14.9$ G, indicative of DMPO-OH. The time course of this radical generation was measured in a series of four hearts, and it was observed that peak radical generation occurred over the first minute of reperfusion, peaking after 20–60 s of reflow followed by a gradual decline to preischemic levels by 5 min postreflow (Fig. 2, bottom). To determine if the observed DMPO-OH signal was derived from $^{\cdot}O_2^-$, similar experiments were performed in a series of hearts subjected to reperfusion in the presence SOD (200 U/ml). In these SOD-treated hearts, no signal was seen either before or after reperfusion. As SOD totally quenched the observed signal, this suggested that it was derived from $^{\cdot}O_2^-$. It is well known that the DMPO superoxide adduct, DMPO-OOH, can rapidly react or be metabolized to form DMPO-OH in biological systems (11). To determine the effect of NOS inhibition on the process of oxygen radical generation, further experiments were performed in a series of hearts pretreated with 1 m*M* L-NAME, as described above. A similar magnitude and time course of radical generation was seen with L-NAME treatment, indicating that L-NAME did not either quench or enhance radical formation (Fig. 2, bottom).

Measurement of $ONOO^-$ formation

To detect $ONOO^-$ generated *in situ*, hearts were perfused with an alkaline solution of perfusate containing 5 m*M* Na_2CO_3 along with the luminescence enhancer luminol at 40 μM. When the pH is raised above 9, the process of $ONOO^-$ protonation and decomposition is greatly slowed (2), so that it becomes possible to stabilize and detect $ONOO^-$ *ex vivo* by luminol chemiluminescence. Whereas only a very weak luminol-chemiluminescence signal was observed from the effluent of normally perfused hearts prior to ischemia, upon reperfusion following 30 min of global 37°C ischemia the intensity of luminol luminescence markedly increased over the first minute of reperfusion, reaching a maximum after 40 s, with a >10-fold increase seen (Fig. 3). Subsequently, a gradual decline was observed with values returning to baseline by 15 min. This luminescence was quenched when hearts were reperfused in the presence of SOD (200 U/ml) and markedly decreased in hearts pretreated with the NOS inhibitors L-NAME or L-NMMA (1 m*M*). Thus, dismutation of $^{\cdot}O_2^-$ or inhibition of NOS greatly decreased the observed luminol luminescence. When hearts were reperfused in the presence of the $ONOO^-$ scavenger urate (1 m*M*), the observed luminescence was

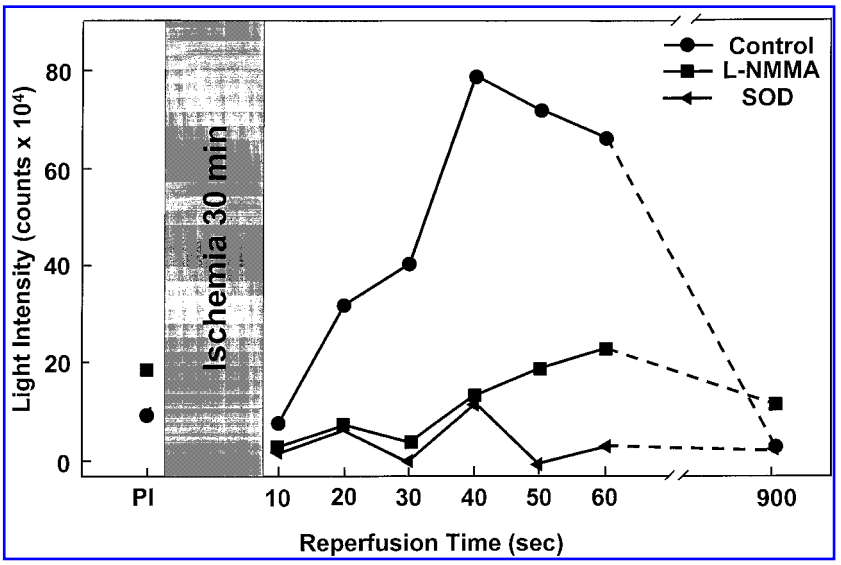

FIG. 3. Graph of the time course of $ONOO^-$ formation in pre- and postischemic hearts. Results are shown from untreated control, L-NMMA (1 m*M*)-pretreated, and SOD (200 U/ml)-treated hearts. Data were obtained from the integral of luminol luminescence measured from the coronary effluent of hearts perfused with probe solution containing 40 μM luminol. The graph is shown with a break in the reperfusion time axis between 60 s and 900 s, after which the scale is condensed to depict the complete time course of $ONOO^-$ generation after 30 min of global ischemia. PI, prior to ischemia.

totally quenched, further demonstrating that this luminescence was derived from $ONOO^-$.

Immunohistology measurements of nitrotyrosine

To determine if $ONOO^-$-mediated cellular injury with amino acid nitration occurred in reperfused myocardium, experiments were performed measuring the formation of the specific $ONOO^-$-mediated nitration product nitrotyrosine. A specific affinity-purified nitrotyrosine antibody was used with measurements performed on normally perfused control heart tissue and on tissue from hearts subjected to 30 min of ischemia followed by reperfusion. To establish a positive control, a series of hearts were perfused with 1 m*M* $ONOO^-$ for 5 min. In these positive controls, strong dense red staining was seen in a vascular distribution pattern indicative of high concentrations of nitrotyrosine formed at the site of endothelial or vascular proteins coming into direct contact with the infused $ONOO^-$ (Fig. 4A). In normally perfused control heart tissue, little if any positive staining was observed (Fig. 4B). In the postichemic myocardium, however, strong positive red staining for nitrotyrosine was observed within myocytes (Fig. 4C and D). This positive nitrotyrosine staining was either diffusely or focally increased within the reperfused myocardium. In control experiments with postischemic myocardium in the absence of the primary nitrotyrosine antibody, no staining was observed. These experiments suggest that $ONOO^-$ is formed in postischemic myocardium and results in nitration of proteins within cardiac myocytes.

Measurements of contractile function

To assess the importance of the reperfusion-associated increase of $NO^{\cdot}$ and $ONOO^-$ formation in the process of postischemic injury, experiments were performed measuring the recovery of contractile function in hearts reperfused after 30 min of ischemia. Three groups of hearts were studied, i.e., untreated control, hearts pretreated with 1 m*M* L-NAME, or hearts treated with 200 U/ml SOD during the first 5 min of reflow, with eight hearts in each group. Baseline contractile function and coronary flow values were identical in each of these groups with left ventricular developed pressures of 164 ± 4 mm Hg, RPP values of 34 ± 2 × 10^3 mm Hg/min, and coronary flow values of 20 ± 1 ml/min. In the control untreated hearts, marked injury was seen upon reperfu-

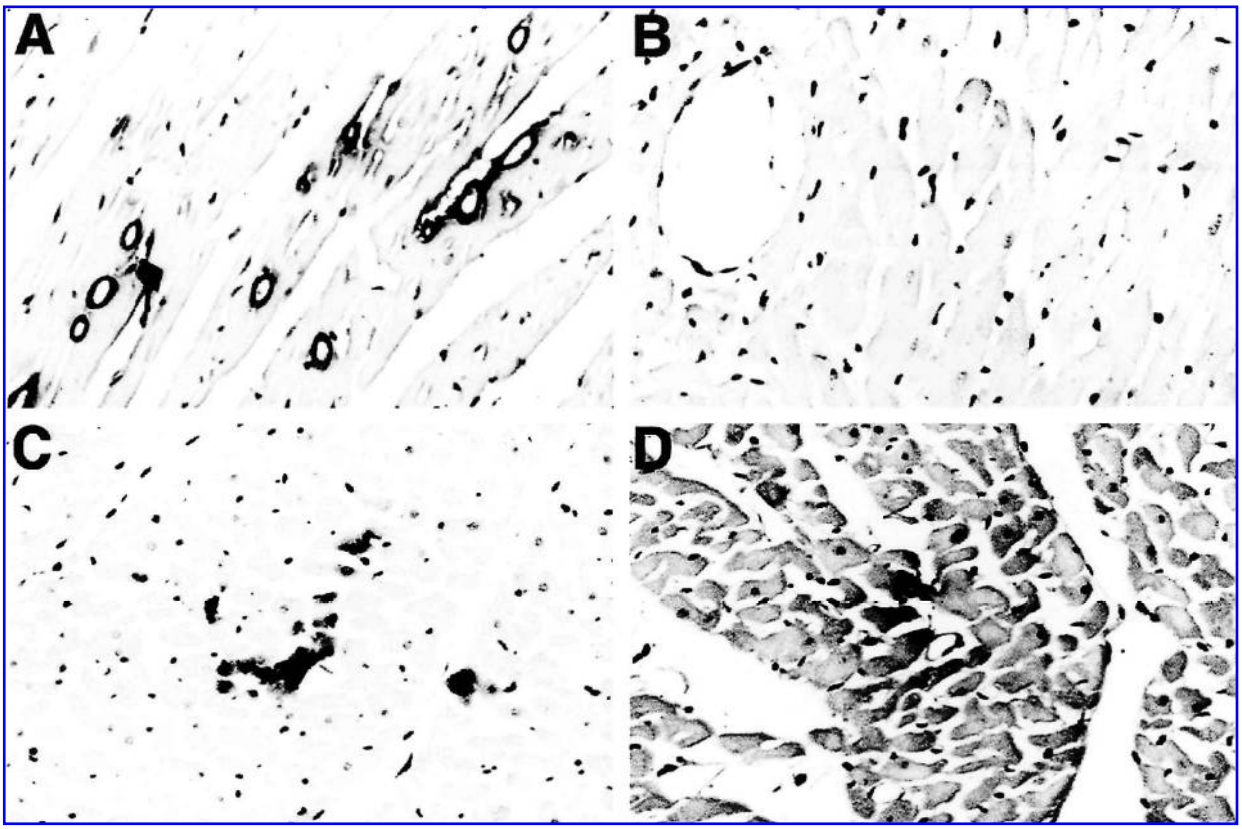

FIG. 4. Immunohistology measurement of the presence and localization of nitrotyrosine formation in normal and postischemic heart tissue. The $ONOO^-$ nitration product nitrotyrosine was visualized using a specific affinity-purified nitrotyrosine antibody as primary, biotinylated secondary, and alkaline phosphatase-conjugated tertiary. (**A**) A positive control heart infused with 1 m*M* $ONOO^-$. (**B**) A normally perfused heart. (**C** and **D**) From hearts subjected to 30 min of global ischemia followed by 45 min of reflow. Strong positive red staining is seen in the vasculature of the heart perfused with $ONOO^-$, (A), whereas no staining is seen in normal heart tissue (B). In postischemic heart tissue, either focal (C) or diffuse (D) positive red staining is observed in myocytes and microvascular endothelium.

sion with a final recovery of only 9 ± 2% of the preischemic RPP; however, with either L-NAME or SOD treatment enhanced recovery of contractile function was seen with final recovered values of 24 ± 2% or 27 ± 3%, respectively (Fig. 5). Thus, with either L-NAME or SOD a similar approximately threefold increase in functional recovery was observed. These results suggest that generation of both $NO^{\cdot}$ and $^{\cdot}O_2^-$ was required for the occurrence of myocardial reperfusion injury with impaired contractile function, and therefore suggest that the observed injury is mediated by $ONOO^-$.

DISCUSSION

Over the last decade, evidence has accumulated demonstrating that superoxide and superoxide-derived oxygen radicals are important mediators of postischemic injury in the heart. Early studies demonstrated that SOD could decrease cell death and enhance the recovery of contractile function (17). Subsequent direct and spin trapping EPR measurements demonstrated that a burst of superoxide and superoxide-derived radical generation occurs during the early period of reperfusion (41, 42). The enzyme xanthine oxidase was shown to be an important source of this radical generation (22, 34). While in these early studies the role of $NO^{\cdot}$ was as yet unrecognized, over the last several years much attention has focused on the potential interaction of these oxygen radicals with $NO^{\cdot}$. A number of studies have shown that $NO^{\cdot}$ rapidly reacts with $^{\cdot}O_2^-$ to form $ONOO^-$ (2, 5, 13), which in turn exerts cytotoxicity via its reaction with a variety of mole-

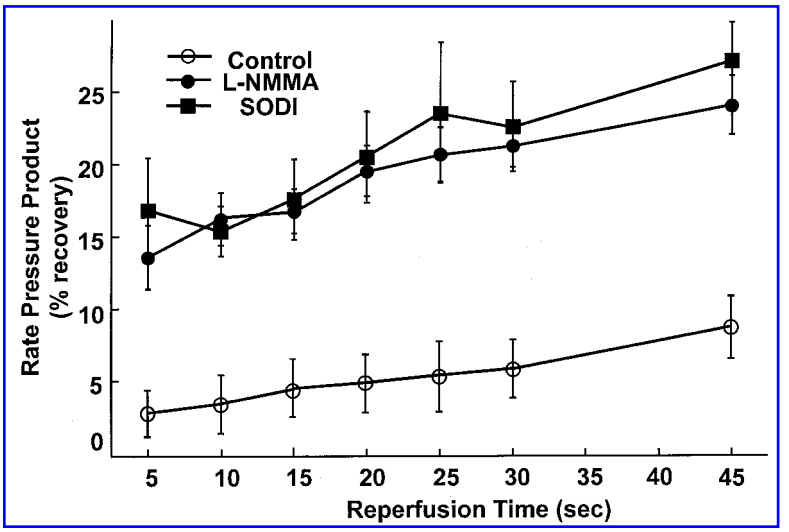

FIG. 5. Measurement of the recovery of RPP, an index of contractile function in untreated control hearts, hearts pretreated with 1 m*M* L-NAME, or hearts reperfused in the presence of 200 U/ml SOD. Hearts were subjected to 30 min of global ischemia followed by 45 min of reflow. RPP was calculated from the measured left ventricular pressure and heart rate. Data are expressed as percent recovery of preischemic values.

cular targets (30, 31, 33). It has also been reported that $ONOO^-$ may cause aggregation of human platelets (28) and impaired vascular relaxation (35), and possibly serve as a mediator of postischemic injury (1, 27).

As early as 1985, it was demonstrated that $NO^\cdot$ could react with $^\cdot O_2^-$ to form $ONOO^-$ and nitrate in alkaline aqueous solution (5). In 1986, it was reported that $^\cdot O_2^-$ contributes to the instability of endothelial derived relaxing factor (EDRF) because it was observed that the effects of EDRF were prolonged by addition of SOD (12). Subsequently, compounds were developed as inhibitors of $NO^\cdot$ that acted by generating $^\cdot O_2^-$ (23). Furthermore, some studies suggested that $NO^\cdot$ can serve as a scavenger of $^\cdot O_2^-$ (10, 37). In 1990, Beckman and co-workers suggested that $ONOO^-$ may be an important mediator of free radical-dependent toxicity because of its strong oxidizing properties (1, 2). Subsequently, some studies demonstrated that $ONOO^-$ could mediate oxidative injury toward a variety of biomolecules, including protein and nonprotein thiols (26), deoxyribose (15), and membrane phospholipids (31). Thus, $ONOO^-$ generation via the reaction of $NO^\cdot$ with $^\cdot O_2^-$ could be an important mechanism of cellular injury in tissues where increased $^\cdot O_2^-$ generation occurs such as during inflammation or postischemic reperfusion.

Based on observations of impaired endothelial reactivity in reperfused myocardium, it was suggested that $NO^\cdot$ generation is altered in the postischemic heart; however, controversy remained regarding the exact nature of these alterations. It was hypothesized that the progressive loss of basal $NO^\cdot$ release after myocardial ischemia and reperfusion might be the cause of this endothelial dysfunction (20). However, it was subsequently reported that the nitrite level in systemic and coronary sinus blood was increased after reoxygenation following 120 min of hypoxia and that this correlated with increased severity of myocardial reoxygenation injury (27). We have measured $NO^\cdot$ formation in the ischemic heart using the $NO^\cdot$ trap Fe-MGD, which binds $NO^\cdot$ giving rise to a unique triplet EPR spectrum, and we observed that $NO^\cdot$ generation markedly increased as a function of the duration of ischemia (43). With short durations of ischemia, this $NO^\cdot$ formation was shown to arise primarily from NOS; however, with prolonged periods of ischemia progressing to necrosis, it was observed that marked $NO^\cdot$ formation occurred from the NOS-independent conversion of

tissue nitrite to $NO^{\cdot}$ that occurs under the acidotic and highly reduced state of the myocardium during ischemia. It was observed that $NO^{\cdot}$ formation from both pathways contributed to reperfusion injury (44). Although these earlier studies demonstrated that $NO^{\cdot}$ was increased during ischemia, until recently there has been a lack of information regarding the alterations in $NO^{\cdot}$ that occur during the critical early period of reperfusion (36). Therefore, experiments were performed measuring $NO^{\cdot}$ during this period of myocardial reperfusion and correlating this with the time course of $^{\cdot}O_2^-$ generation, as well as with the formation of $ONOO^-$.

To trap $NO^{\cdot}$ specifically during reperfusion rather than during ischemia, hearts were subjected to ischemia in the absence of Fe-MGD with the trap infused only at the onset of reflow. Immediately upon reflow, a prominent triplet signal of trapped $NO^{\cdot}$ was seen that was >10-fold higher than preischemic levels. This $NO^{\cdot}$ signal rapidly declined over the first minute of reflow with about a threefold decrease seen after 40–60 s of reflow. A subsequent second peak was observed over the second minute of reflow followed by a gradual decline over the next 15 min. Even after 15 min of reflow, however, the intensity of this $NO^{\cdot}$ signal remained more than fourfold above baseline preischemic levels. These measurements suggest that $NO^{\cdot}$ levels in the heart are greatly increased not only during ischemia, as previously described (43), but also during the early minutes of reperfusion (36). Inhibition of NOS largely blocked the increase in this signal throughout the time course of reflow, indicating that this $NO^{\cdot}$ formation was largely derived from NOS. The initial marked elevation and subsequent rapid decline seen during the first minute of reflow may be secondary, at least in part, to washout of the pool of $NO^{\cdot}$ formed within the heart during ischemia. The subsequent rise seen during the second minute of reflow could be due to increased activation of constitutive NOS, which could occur due to increased intracellular Ca^{2+} concentrations that have been previously shown to occur during reperfusion (21).

To be able to compare precisely the reperfusion time course of $NO^{\cdot}$ with that of $^{\cdot}O_2^-$ and $^{\cdot}O_2^-$-derived oxygen radical generation, parallel studies were performed using the nitrone spin trap DMPO, which is a well characterized trap suitable for measuring the generation of these oxygen radicals (11). As reported previously, a prominent DMPO-OH adduct was observed reaching a maximum during the first 40 s of reflow followed by a gradual decline to preischemic levels over the next 4 min. The $^{\cdot}O_2^-$ dismutating enzyme SOD totally quenched this signal, demonstrating that it was derived from $^{\cdot}O_2^-$. In L-NAME-treated hearts, however, the overall magnitude and time course of this signal was largely unchanged with only a small 20–25% decrease seen over the first 40 s of reflow. Previous studies have shown that xanthine oxidase is the major source of oxygen radical generation in the postischemic rat heart; thus, it is not surprising that this radical generation would be largely unaffected by inhibition of NOS (34, 38). However, recent studies have demonstrated that arginine depletion can cause NOS to also generate $^{\cdot}O_2^-$ with this process inhibited by L-NAME. As global ischemia might be expected to result in some depletion of intracellular arginine, due to a lack of supply, it is possible that the small L-NAME-mediated decrease in $^{\cdot}O_2^-$ generation seen during the early seconds of reflow could be due to inhibition of NOS-mediated $^{\cdot}O_2^-$ generation (39).

Although $ONOO^-$ has been shown to be a potent cytotoxic species (28, 30), questions remain regarding whether $ONOO^-$ formation occurs *in vivo*. Comparison of the time courses measured for $NO^{\cdot}$ with that of $^{\cdot}O_2^-$ demonstrated that both were markedly elevated during the first 1–2 min of reflow. Therefore, assuming that this $NO^{\cdot}$ and $^{\cdot}O_2^-$ occur within the same tissue sites, it would be expected that $ONOO^-$ could be formed. It has been previously reported that $ONOO^-$ can induce luminol chemiluminescence, which is inhibited by SOD or urate directly (32). Chemiluminescence has been used for detecting endothelium-derived $ONOO^-$ (16). However, it is more difficult to detect *in situ* formation of $ONOO^-$ in tissues by chemiluminescence because $ONOO^-$ is rapidly protonated with decomposition at physiological pH. In alkaline solution

(pH > 9), the half-life of $ONOO^-$ is markedly prolonged (2); therefore, we performed measurements in which hearts were perfused with alkaline solutions in the presence of luminol to stabilize and detect $ONOO^-$ formed *in situ*. Although this procedure alkalinized the heart effluent, no persistent hemodynamic alterations were observed when this probe solution was infused for short intervals. A prominent luminol-chemiluminescence signal was observed during the first minute of reflow closely paralleling the time course of $^{\cdot}O_2^-$ generation. A series of experiments were performed that confirmed that the observed luminol luminescence was derived specifically from $ONOO^-$. These included a demonstration that this signal was quenched by SOD or the $ONOO^-$ scavenger urate and markedly decreased by the NOS blockers L-NAME and L-NMMA. Thus, a marked increase in $ONOO^-$ was observed during the early period of reperfusion that was triggered by the increased $^{\cdot}O_2^-$ and $NO^{\cdot}$ formation that occurs.

The functional importance of the increased $NO^{\cdot}$ and $ONOO^-$ formation in the process of postischemic injury was assessed in studies measuring the recovery of contractile function in hearts subjected to 30 min of global ischemia followed by reperfusion. It was observed that either inhibition of $NO^{\cdot}$ formation with the NOS blocker L-NAME or scavenging of $^{\cdot}O_2^-$ with SOD resulted in similar, more than twofold enhancement in the recovery of contractile function. These studies demonstrated that increased formation of both $^{\cdot}O_2^-$ and $NO^{\cdot}$ was required for the induction of myocardial reperfusion injury, indicating that $ONOO^-$ was the oxidant species responsible for this injury. Further immunohistology studies demonstrated markedly increased nitrotyrosine staining in reperfused myocardium, indicating that the $ONOO^-$ generated during reperfusion results in modification of myocardial proteins.

In the early period of postischemic reperfusion, generation of both $NO^{\cdot}$ and $^{\cdot}O_2^-$ is markedly increased leading to the formation of the potent oxidant $ONOO^-$, which in turn causes protein nitration and cellular injury. Blockade or scavenging of either $NO^{\cdot}$ or $^{\cdot}O_2^-$ generation prevents reperfusion injury and greatly enhances the recovery of contractile function. Thus, with increasing severity of ischemia the increased levels of $NO^{\cdot}$ and $^{\cdot}O_2^-$ present upon reperfusion react to form $ONOO^-$ and switch from a homeostatic signaling role to critical mediators of cellular injury.

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ABBREVIATIONS

DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; EDRF, endothelium-derived relaxing factor; EPR, electron paramagnetic resonance; LVDP, left ventricular developed pressure; MGD, *N*-methyl-D-glucamine dithiocarbamate; L-NAME, N^G-nitro-L-arginine methyl ester; L-NMMA, N^G-monomethyl-L-arginine; $NO^{\cdot}$, nitric oxide; NOS, nitric oxide synthase; $ONOO^-$, peroxynitrite; RPP, rate pressure product; SOD, superoxide dismutase.

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