Measurement of Nitric Oxide and Peroxynitrite Generation in the Postischemic Heart

EVIDENCE FOR PEROXYNITRITE-MEDIATED REPERFUSION INJURY*

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Altered nitric oxide (NO) production is a critical factor in tissue reperfusion injury; however, controversy remains regarding these alterations and how they cause injury. Since superoxide (O2−) generation is triggered during the early period of reperfusion the cytotoxic oxidant peroxynitrite (ONOO−) could be formed, but it is not known if this occurs. Therefore electron paramagnetic resonance and chemiluminescence studies were performed of the magnitude and time course of NO−, O2−, and ONOO− formation in the postischemic heart. Isolated rat hearts were subjected either to normal perfusion or to reperfusion after 30 min of ischemia in the presence of the NO− trap Fe2+-N-methyl-D-glucaminedithiocarbamate with electron paramagnetic resonance measurements performed on the effluent. Although only trace signals were present prior to ischemia, prominent NO− adduct signals were seen during the first 2 min of reflow which were abolished by nitric oxide synthase (NOS) inhibition. Similar studies with the O2− trap 5,5-dimethyl-1-pyrroline N-oxide demonstrated a burst of O2− generation over the first 2 min of reflow. Chemiluminescence measurements using 5-aminomethyl-2,3-dihydro-1,4-phthalazinedione (luminol) demonstrated a similar marked increase in ONOO− which was blocked by NOS inhibitors or superoxide dismutase. NOS inhibition or superoxide dismutase greatly enhanced the recovery of contractile function in postischemic hearts. Immunohistochemistry demonstrated that the ONOO−-mediated nitration product nitrotyrosine was formed in postischemic hearts but not in normally perfused controls. Thus, NO− formation is increased during the early period of reflow and reacts with O2− to form ONOO−, which results in amino acid nitration and cellular injury.

Nitric oxide (NO) is a free radical endogenously produced by a variety of mammalian cells and has been shown to be a ubiquitous signal transduction molecule. NO− is known to play an important role in blood pressure regulation, vascular tone, neural signaling, and immunological function (1). It is formed by a class of enzymes, nitric oxide synthases, which synthesize NO− from arginine. NO− binds to and activates guanylate cyclase resulting in the formation of the second messenger molecule cyclic GMP (2), which accounts for many of the physiological effects of NO−. Beyond its homeostatic effects, it has been proposed that NO− can induce cellular injury either due to direct toxicity (3, 4) or to the reaction with superoxide (O2−) to form the potent oxidant peroxynitrite (ONOO−) (5, 6).

It has been suggested that alterations in NO− formation are particularly important in the pathogenesis of the injury that occurs in ischemic and reperfused tissues. In the postischemic heart it has been reported that alterations in NO− result in altered endothelial function with impaired tissue perfusion. In the postischemic heart it has been reported that alterations in NO− result in altered endothelial function with impaired tissue perfusion (7).

There has been considerable controversy regarding whether reperfusion results in increased or decreased NO− formation in the heart. Studies of endothelial function and vascular reactivity have been interpreted to suggest that NO− production is decreased in postischemic myocardium, and based on this evidence it was hypothesized that a loss of basal NO− production is an important source of postischemic injury (8). Subsequently, other studies have shown that inhibitors of nitric oxide synthase (NOS) can dramatically protect against postischemic injury (9, 10). From these latter studies it was suggested that NO− may be involved in the process of tissue injury and that the production of NO− may actually be increased during reperfusion. Subsequent direct electron paramagnetic resonance (EPR) spin trapping measurements were performed which demonstrated that NO− formation is greatly increased in ischemic myocardium (11). Since it was demonstrated previously that there is a burst of O2− generation in the postischemic heart, it was hypothesized that if NO− is also increased upon reperfusion it might react with this O2− resulting in the formation of the potent cytotoxic oxidant ONOO− (6).

Although increased NO− and ONOO− has been hypothesized to be a critical biochemical mechanism of postischemic injury in the heart and in other tissues (9, 12, 13), there is only indirect evidence to support this since no direct measurements of NO− and ONOO− formation during reperfusion have been performed. Since NO− 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− generation (14). Recently, the ferrous iron complex of N-methyl-D-glucaminedithiocarbamate (MGD), Fe2+-MGD2 (Fe-MGD), has been shown to be suitable for measurement of NO− in living tissues (15). We have demonstrated previously using this EPR spin trapping technique that increased levels of NO− are generated in ischemic myocardium (11). It also has been shown that 5-aminomethyl-2,3-dihydro-
1,4-phthalazinidene (luminol)-enhanced luminescence can be used to specifically measure ONOO– formation (16). Further measurements of ONOO–-mediated protein damage with nitration of the amino acid tyrosine have been reported using specific antibodies directed against nitrotyrosine (17).

The current study was designed to measure and characterize the process of NO– and ONOO– generation during postischemic reperfusion and to assess the functional importance of this process in the pathogenesis of myocardial reperfusion injury. EPR spin trapping measurements of the relative magnitude and time course of NO– formation in the postischemic heart were performed using the NO– trap Fe-MGD. These measurements were correlated with EPR measurements of oxygen radical generation using the spin trap spin, 5,5-dimethyl-1-pyrroline N-oxide (DMPO) and measurements of ONOO– formation performed using luminol-enhanced chemiluminescence. To assess the functional importance of this process in the pathogenesis of myocardial injury, hemodynamic studies of the recovery of contractile function were also performed, in the presence and absence of NOS inhibition or superoxide dismutase (SOD), along with histochemical measurements of the formation of the ONOO–-mediated nitration product nitrotyrosine.

**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 excised rapidly, and the ascending aorta was cannulated and perfused at 37 °C with a constant pressure of 80 mm Hg using Krebs bicarbonate perfusate (in mM: 16.7 glucose, 120.0 NaCl, 25.0 NaHCO3, 2.5 CaCl2, 5.9 KCl, and 1.2 MgCl2), which was bubbled continuously with 95% O2, 5% CO2 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 measured continuously using a Transonic flowmeter, model HT107, with a 2N in-line 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 inflated 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 RS400 recorder and stored along with coronary flow values using a MacLab system. Subsequently, the left ventricular developed pressure (the difference between peak-systolic and end-diastolic pressures) and the rate pressure product (product of heart rate and left ventricular developed pressure) 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– or Oxygen Radicals**—For measurements of NO–, the Fe-MGD spin trap complex was infused through one of the side arms with a final concentration of 2 mM in Fe(II) and 10 mM 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 identical to that utilized for NO– except that the spin trap DMPO was used, with experiments performed in the dark to minimize any light-induced DMPO degradation. DMPO was purchased from Aldrich and purified further 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 TM110 cavity, as described previously (18, 19). The microwave frequency and magnetic field were measured precisely with an EIP 575 microwave frequency counter and Bruker ER 035M NMR gaussmeter. Relative quantification of the NO– and oxygen free radical signals was performed by double integration.

**Chemiluminescence Assay of ONOO– Generation**—Luminol chemiluminescence was used for measurement of ONOO– in the coronary effluent. An alkaline solution containing 5 mM Na2CO3 and 40 µM luminol, pH 9.2, was infused during the 1st or 2nd min of reflow and after 5 and 15 min of reflow with samples 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-Biodum LB9505C luminescence reader.

**Immunohistochemistry Assay of Nitrotyrosine**—After either normal perfusion or 45 min of reperfusion hearts were quickly removed from the cannula, and the ventricles were sliced into 3–4-mm-thick sections and immersed immediately in 10% formalin at 4 °C. Histological processing was done by conventional methods (20). The formalin-fixed paraffin sections were cleared in xylene for 5 min and then rehydrated and washed in Tris-buffered saline, pH 7.6 (TBS). To block any naturally occurring biotin within the tissue, the slides were preincubated with 0.1 mg/ml avidin for 20 min, then rinsed in TBS and incubated in 0.05 mg/ml biotin for 20 min. The sections were then rinsed in a TBS/milk solution for 20 min. The slides were rinsed in a TBS/milk solution (0.5% Carnation milk) and blocked with TBS, milk, 1% normal goat serum for 10 min. 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 TBS, milk, 1% normal goat serum. After the primary incubation, the slides were rinsed in TBS/milk and incubated with the biotinylated secondary for 30 min and then rinsed again and incubated with the tertiary, ExtrAvidin alkaline phosphatase, diluted 1,900 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 (20). The stained sections were counterstained with Mayer’s modified hematoxylin, washed in ethanol solutions, cleared with xylene, and coverslipped. 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 (11, 21). Peroxynitrite was synthesized in a quenched flow reactor from the reaction of an aqueous 0.6 mM 1,1′-sulfonyl-2,2′-dihydroxybenzene and 0.5 mM FeCl2 in a solution with a solution of 0.6 mM nitrite as described previously (5). Perfusion reagents, bovine erythrocyte copper zinc SOD, l-NAME, l-NMMA, uric acid, H2O2, sodium nitrite, avidin, and ExtrAvidin alkaline phosphatase were purchased from Sigma. S-Nitrosoacetylpenicillamine was purchased from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA); sodium heparin was from Elkins Sinn, Inc. (Cherry Hill, NJ); and sodium pentobarbital was from Steris Laboratories, Inc. (Phoenix, AZ). Affinity-purified rabbit polyclonal anti-nitrotyrosine antibody was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY).

**Statistical Analysis**—Data are expressed as mean ± S.E. The statistical significance of differences between the groups were calculated using two-way analysis of variance. p values of < 0.05 were considered significant.

**RESULTS**

**Measurement of NO– Generation**—Preischemic and postischemic measurements of NO– formation were performed from the coronary effluent of hearts infused with the NO– trap Fe-MGD. Hearts were infused with Fe-MGD for 30 s, and the effluent was sampled. The hearts were then allowed to reequilibrate in the absence of Fe-MGD for 5 min and were 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. No EPR spectrum was observed from the Fe-MGD complex in Krebs bicarbonate buffer; however, after addition of the NO– donor compound S-nitrosoacetylpenicillamine (50 nM) 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, A and B). Although only a trace signal was observed in the effluent of hearts perfused with Fe-MGD prior to ischemia, a prominent triplet spectrum, similar to that seen with the NO– donor, was observed from the effluent of rat hearts reperfused after 30-min global ischemia (Fig. 1, C and D). In measurements performed in a series of four hearts, more than a 30-fold increase in the NO–Fe-MGD signal was observed during the first 20 s of reflow followed by a decline over the 1st min, but it still remained more than 10-fold elevated relative to controls. Subsequently, a second peak occurred during the 2nd min of reflow after which the observed signal intensity gradually declined (Figs. 2 and 3). 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 mM concentrations of either of the NOS inhibitors L-NAME or L-NMMA, no increase was seen after reperfusion with more than 80% inhibition of the reperfusion-associated increase observed (Fig. 3).

Measurement of Oxygen Radical Generation—Spin trapping measurements of oxygen radical generation were performed in hearts in a manner identical to that performed for NO\textsuperscript{\textcircled{z}}, except that the spin trap DMPO was infused at a final concentration of 40 mM. 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 (18, 19). Prior to ischemia, however, only trace signals were seen. The observed signal was a 1:2:2:1 quartet with \( \alpha_N = \alpha_H = 14.9 \) G, indicative of DMPO-OH (Fig. 4). 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 1st min of reperfusion, peaking after 20–60 s of reflow followed by a gradual decline to preischemic levels by 5 min post-reflow (Fig. 5). To determine if the observed DMPO-OH signal was derived from O\textsubscript{2}\textsuperscript{\textcircled{z}} or NO\textsuperscript{\textcircled{z}}, similar experiments were performed in which a series of hearts was subjected to reperfusion in the presence of 200 units/ml SOD. In these SOD-treated hearts no signal was seen either before or after reperfusion. Since SOD totally quenched the observed DMPO-OH signal, this suggested that this radical adduct was derived from O\textsubscript{2}\textsuperscript{\textcircled{z}}. It is well known that the DMPO superoxide adduct, DMPO-OOH, can rapidly react or be metabolized to form DMPO-OH in biological systems (22). To determine the effect of NOS inhibition on the process of oxygen radical generation further experiments were performed in a series of hearts that were pretreated with 1 mM L-NAME, as described above. As shown in Fig. 5, a similar magnitude and time course of radical generation were seen in these L-NAME-treated hearts, indicating that L-NAME neither quenched nor enhanced radical formation. A small 20–25% decrease in the...
radical signals was seen over the first 40 s of reflow; however, this was not statistically significant.

Measurement of Peroxynitrite Formation—To detect ONOO\(^{-}\) generated in situ, hearts were perfused with an alkaline solution of perfusate containing 5 mM Na\(_2\)CO\(_3\) along with the luminescence enhancer luminol, 40 \(\mu\)M. When the pH is raised above 9 the process of ONOO\(^{-}\) protonation and decomposition is slowed greatly (5), so that it becomes possible to stabilize and detect ONOO\(^{-}\) ex vivo by luminol chemiluminescence. Although 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 1st min of reperfusion reaching a maximum after 40 s, with more than a 10-fold increase seen (Fig. 6). 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 200 units/ml SOD and markedly decreased in hearts pretreated with L-NAME or L-NMMA (Figs. 6 and 7). With L-NAME-treated hearts, increased background luminescence was observed both in preischemic and postischemic samples; however, the reperfusion-associated increase in luminescence was quenched. In L-NMMA-treated hearts, less increase in background occurred, and again the reperfusion-associated increase in luminol luminescence was quenched. Thus, inhibition of NOS greatly decreased the observed luminol luminescence, suggesting that it was derived from NO\(^{+}\) synthesized by NOS. When hearts were reperfused in the presence of the ONOO\(^{-}\) scavenger urate, the observed luminescence was quenched totally, further demonstrating that this luminescence was derived from ONOO\(^{-}\). In repeat experiments performed with SOD, L-NAME, L-NMMA, or urate, with four hearts/group, similar results were obtained.
with highly significant quenching of luminescence with each of these treatments, \( p < 0.001 \) versus untreated control for each group (Fig. 7).

To further confirm that the chemiluminescence observed in these studies was derived specifically from \( \text{ONOO}^- \), experiments were performed in which the pH of effluent solutions was lowered to pH 7.4 just prior to measurement, and it was observed that the luminescence was quenched. In further experiments with the addition of 50 mM DMPO to these samples no radical signals were observed, further confirming that no measurable radical generation continued in these effluent samples after exiting the coronary circulation. These experiments suggested that the observed luminal chemiluminescence was specifically due to \( \text{ONOO}^- \).

**Immunohistology Measurements of Nitrotyrosine—**To determine if \( \text{ONOO}^- \)-mediated cellular injury with amino acid nitration occurred in reperfused myocardium, experiments were performed measuring the formation of the specific \( \text{ONOO}^- \)-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 mM \( \text{ONOO}^- \) 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 \( \text{ONOO}^- \) (Fig. 8A).

In normally perfused control heart tissue, little if any positive staining was observed (Fig. 8B). In the postischemic myocardium, however, strong positive red staining for nitrotyrosine was observed within myocytes (Fig. 8). 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 \( \text{ONOO}^- \) 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 in NO\(^\cdot\) and \( \text{ONOO}^- \) formation in the process of postischemic injury, experiments were performed measuring the recovery of contractile function in a series of hearts reperfused after 30 min of ischemia. Three groups of hearts were studied: untreated control, pretreated with 1 mM \( \text{l-NAME} \), or treated with 200 units/ml SOD during the first 5 min of reflow, with eight hearts in each group. Base-line contractile function and coronary flow values were identical in each of these groups with left ventricular developed pressures of \( 164 \pm 4 \text{ mmHg} \), rate pressure product values of \( 34.3 \pm 2.2 \times 10^3 \text{ mmHg/min} \), and coronary flow values of \( 19.8 \pm 0.8 \text{ ml/min} \). In the control untreated hearts, marked injury was seen upon reperfusion with a final recovery of only \( 8.7 \pm 1.8\% \) of the preischemic rate-pressure product. As shown in Fig. 9, both \( \text{l-NAME} \) and SOD resulted in greatly enhanced recovery of contractile function with final recovered rate pressure product of \( 24 \pm 2\% \) or \( 27 \pm 2.6\% \), respectively. Thus, with either \( \text{l-NAME} \) or SOD, a similar almost 3-fold increase in functional recovery was observed throughout the time course of reflow. In either untreated control hearts or hearts treated with SOD, a similar recovery of coronary flow was observed with final recoveries of \( 60 \pm 6\% \) and \( 56.3 \pm 6\% \), respectively. In hearts pretreated with \( \text{l-NAME} \), however, lower values of coronary flow of \( 27.5 \pm 4\% \) were observed throughout the time course of reflow as expected due to the inhibition of endothelial NOS (Fig. 10). These results suggest that both NO\(^\cdot\) and \( \text{O}_2\)\(^-\) generation were required for the occurrence of myocardial reperfusion injury with impaired contractile function and therefore suggest that much of the observed injury is mediated by \( \text{ONOO}^- \).

**DISCUSSION**

Over the last decade much evidence has accumulated demonstrating that \( \text{O}_2\)\(^-\) and \( \text{O}_2\)\(^-\)-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 (23). Subsequent direct and spin trapping EPR measurements demonstrated that a burst of \( \text{O}_2\)\(^-\) and \( \text{O}_2\)\(^-\)-derived radical generation occurs during the early period of reperfusion (18, 19). The enzyme xanthine oxidase was shown to be an important source of this radical generation (24). Although 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\(^\cdot\) reacts rapidly with \( \text{O}_2\)\(^-\) to form \( \text{ONOO}^- \) (5, 25–27), which in turn exerts cytotoxicity via its reaction with a variety of molecular targets (28–31). It has also been reported that \( \text{ONOO}^- \) may cause aggregation of human platelets (32) and impaired vascular relaxation (33). Although proposed as a possible mediator of tissue injury, it was not known previously.
if ONOO\(^-\) is actually formed upon reperfusion of ischemic tissues, such as the heart, and its role in the pathogenesis of reperfusion injury was also unknown (34).

As early as 1985, it was demonstrated that NO\(^-\) could react with O\(_2\)\(^2\) to form ONOO\(^-\) and nitrate in alkaline aqueous solution (25). In 1986, it was reported that O\(_2\)\(^2\) contributes to the instability of endothelial-dependent relaxing factor since it was observed that the effects of endothelial-dependent relaxing factor were prolonged by the addition of SOD (35). Subsequently, compounds were developed as inhibitors of NO\(^-\) which acted by generating O\(_2\)\(^2\) (36). Furthermore, some studies suggested that NO\(^-\) can serve as a scavenger of O\(_2\)\(^2\) (37, 38).

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 (5, 6). Subsequently, some studies demonstrated that ONOO\(^-\) could mediate oxidative injury toward a variety of biomolecules including protein and nonprotein thiols (39), deoxyribose (40), and membrane phospholipids (29, 41). Thus, ONOO\(^-\) generation via the reaction of NO\(^-\) with O\(_2\)\(^2\) could be an important mechanism of cellular injury in tissues where increased O\(_2\)\(^2\) generation occurs such as during inflammation or posts ischemic reperfusion.

Based on observations of impaired endothelial reactivity in reperfused myocardium it was suggested that NO\(^-\) generation is altered in the posts ischemic heart; however, controversy remained regarding the exact nature of these alterations. It was hypothesized that the progressive loss of basal NO\(^-\) release after myocardial ischemia and reperfusion might be the cause of this endothelial dysfunction (8). However, it was reported subsequently that the nitrite level in systemic and coronary sinus blood was increased after reoxygenation following 120-min hypoxia and that this correlated with increased severity of myocardial reoxygenation injury (9). We have recently reported the direct measurement of NO\(^-\) formation in the ischemic heart using the NO\(^-\) trap Fe-MGD, which binds NO\(^-\), giving rise to a unique triplet EPR spectrum. It was observed that NO\(^-\) generation increased markedly as a function of the duration of ischemia (11). With short durations of ischemia this NO\(^-\) formation was shown to arise primary from NOS; however, with prolonged periods of ischemia progressing to necrosis it was observed that marked NO\(^-\) formation occurred from the NOS-independent conversion of tissue nitrite to NO\(^-\) which occurs under the acidic and highly reduced state of the myocardium during ischemia (42). Although these earlier studies demonstrated that NO\(^-\) was increased during ischemia, there has been a lack of information regarding the alterations in NO\(^-\) which occur during the critical early period of reperfusion. Therefore in the present study, experiments were performed to measure NO\(^-\) during this period of myocardial reperfusion and to correlate this with the time course of O\(_2\)\(^2\) generation as well as with the formation of ONOO\(^-\).

To trap NO\(^-\) 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\(^-\) was seen which was more than 30-fold higher than preischemic levels. This NO\(^-\) signal remained more than 10-fold above preischemic values throughout the first 5 min of reflow. Over the 1st min of reflow, a partial decrease was observed which was followed by a second peak at 80 s and then a gradual decline over the next 15 min. Even after 15 min of reflow, however, the intensity of this NO\(^-\) signal remained above baseline preischemic levels. These measurements suggest that NO\(^-\) levels in the heart are not only greatly increased during ischemia, as described previously (11), but also during the early minutes of reperfusion. Inhibition of NOS largely blocked the increase in this signal throughout the time course of reflow, indicating that this NO\(^-\) 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\(^-\) formed within the heart during ischemia. The subsequent rise seen during the 2nd min of reflow could be due to increased activation of constitutive NOS which could occur due to increased intracellular Ca\(^{2+}\) concentrations, which have been previously shown to occur during reperfusion (43).

To compare the reperfusion time course of NO\(^-\) with that of O\(_2\)\(^2\) and O\(_2\)\(^2\) derived oxygen radical generation, parallel studies were performed using DMPO, which is a well characterized trap suitable for measuring the generation of these oxygen radicals (22). 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 O\(_2\)\(^2\) dismutating enzyme SOD totally quenched this signal, demonstrating that it was derived from O\(_2\)\(^2\). Although the DMPO-OH signal was derived from O\(_2\)\(^2\), it could be formed by three different chemical pathways. First, DMPO-OH could be derived from the direct trapping of O\(_2\)\(^2\) with formation of DMPO-OOH which is degraded rapidly to DMPO-OH. Alternatively, O\(_2\)\(^2\) along with H\(_2\)O\(_2\) can react via the Fenton reaction (29, 30).
reaction to form 'OH which reacts with DMPO to form DMPO-OH (19). Lastly, it has been also reported that ONOO⁻, which is derived from the reaction of O₂ and NO, can react with DMPO to form DMPO-OH (44, 45). In l-NAME treated hearts the overall magnitude and time course of the DMPO-OH signal were largely unchanged. Although elimination of NO generation would be expected to increase the formation of DMPO-OH, which arises due to direct trapping of O₂, it would abolish that from ONOO⁻. This may explain why no increase in the DMPO-OH signal was seen in l-NAME treated hearts. 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 (46, 47). However, recent studies have demonstrated that arginine depletion can also cause NOS to generate O₂⁻ with this process inhibited by l-NAME (48, 49). Since global ischemia might be expected to result in some depletion of intracellular arginine, due to a lack of supply, it is possible that l-NAME could partially decrease the primary process of O₂ generation, and this may explain the small 20–25% decrease in the DMPO-OH signal seen over the early seconds of reflow.

Although ONOO⁻ has been shown to be a potent cytotoxic species (28, 32), questions remain regarding whether ONOO⁻ formation occurs in vivo (50, 51). Comparison of the time courses measured for NO' with that of O₂ was demonstrated that both were markedly elevated during the first 1–2 min of reflow. Therefore, assuming that this NO' and O₂ occur within the same tissue sites, it would be expected that ONOO⁻ could be formed. It has been reported previously that ONOO⁻ can induce luminol chemiluminescence, which is inhibited by SOD or urate directly (16). Chemiluminescence has been used for detecting endothelium-derived ONOO⁻ (52). However, it is more difficult to detect in situ formation of ONOO⁻ in tissues by chemiluminescence since ONOO⁻ is protonated rapidly with decomposition at physiological pH. In alkaline solution (pH > 9), the half-life of ONOO⁻ is prolonged markedly (5); therefore, we performed measurements in which hearts were perfused with alkaline solutions in the presence of luminol to stabilize ONOO⁻ and detect ONOO⁻ formation (53). However, it is more difficult to detect in situ formation of ONOO⁻ in tissues by chemiluminescence since ONOO⁻ is protonated rapidly with decomposition at physiological pH. In alkaline solution (pH > 9), the half-life of ONOO⁻ is prolonged markedly (5); 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 alkalized 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 1st min of reflow closely paralleling the time course of O₂ generation. A series of experiments were performed which 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 decreased markedly by the NOS blockers l-NAME and N-NMMA. Although both NOS inhibitors were effective in decreasing the observed luminescence, it was observed that l-NAME was somewhat less effective than l-NMMA and directly increased the background, as reported previously (52). Thus, a marked increase in ONOO⁻ was observed during the early period of reperfusion which was triggered by the increased O₂ and NO' formation that occurs. The functional importance of the increased NO' and ONOO⁻ formation in the process of postischemic injury was assessed in hemodynamic 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' formation with the NOS blocker l-NAME or scavenging of O₂⁻ with SOD resulted in similar but more 2-fold enhancement in the recovery of contractile function. These studies demonstrated that both increased O₂⁻ and NO' formation were 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.

Thus, we have demonstrated by direct trapping measurements that during the early period of posts ischemic reperfusion NO' is increased greatly above normal control values. Markedly increased NO' levels occurred concurrently with the reperfusion associated burst of O₂ generating, leading to the formation of the potent oxidant ONOO⁻ with a magnitude and time course paralleling that of O₂. This peroxynitrite generation caused protein nitration and cellular injury. Blockade or scavenging of either NO' or O₂ was sufficient to prevent reperfusion injury and greatly enhance the recovery of contractile function. This process of peroxynitrite-mediated cellular damage may be an important common mechanism of reperfusion injury in a variety of organs and tissues. Recognition of the reperfusion-associated increases in NO' and peroxynitrite generation may be of great importance in the development of therapeutic approaches to prevent reperfusion injury.

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