

Inhaled Nitric Oxide Attenuates Reperfusion Inflammatory Responses in Humans

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Background: Reduced bioavailability of endothelium-derived nitric oxide associated with reperfusion could potentially exacerbate the inflammatory response during reperfusion. Evidence suggests the pharmacologic effects of inhaled nitric oxide may extend beyond the pulmonary vasculature, and this is attributed to nitric oxide–derived complexes in blood that ultimately orchestrate antiinflammatory effects. In this study, the authors evaluated the potential for inhaled nitric oxide (80 ppm) to attenuate inflammation instigated by ischemia–reperfusion in a human model using patients undergoing knee surgery where a tourniquet was used to produce a bloodless surgical field.

Methods: Inhaled nitric oxide (80 ppm) was administered before tourniquet application and continued throughout reperfusion until the completion of surgery. Venous blood samples were collected before and after reperfusion, for the measurements of nitrate and nitrite, CD11b/CD18, soluble P-selectin, and lipid hydroperoxide. Muscle biopsies were obtained from the quadriceps muscle before skin closure and analyzed for myeloperoxidase, conjugated dienes, and nuclear factor- κ B translocation.

Results: Administration of inhaled nitric oxide (80 ppm) significantly attenuated the inflammatory response characterized by reduced expression of CD11b/CD18, P-selectin, and nuclear factor κ B compared with the control group. This was accompanied by increased plasma levels of nitrate and nitrite and reduced oxidative stress.

Conclusions: Administration of inhaled nitric oxide at 80 ppm significantly reduces inflammation in lower extremity ischemia–reperfusion in humans. This observation supports the concept that during diseases characterized by dysfunction in nitric oxide metabolism, inhaled nitric oxide may be an effective therapy to replenish systemic nitric oxide, thus retarding inflammatory-mediated injury.

AN ischemia–reperfusion event occurring in skeletal muscles initiates a cascade of cellular processes and molecular events reminiscent of an inflammatory response, frequently accompanied by local and remote organ injury.¹ Reactive oxygen intermediates and cytokines have been implicated in the pathogenesis of ischemia–reperfusion injury.² The inflammatory response of ischemia–reperfusion up-regulates the expression of ad-

hesion molecules and promotes endothelial–leukocyte interactions.^{3,4}

A growing body of evidence suggests that bioavailability of endothelium-derived nitric oxide is reduced after an ischemia–reperfusion event. Excessive generation of superoxide anion in the vasculature is linked to the reduced bioavailability of endothelium-derived nitric oxide.⁵ Nitric oxide otherwise plays a critical role in maintaining vascular homeostasis *via* multiple mechanisms, which includes inducing vasodilation and scavenging low levels of reactive oxygen species generated by metabolism.⁶ Nitric oxide supplementation either as authentic gas or as a nitric oxide donor can attenuate ischemia–reperfusion injury. This cytoprotective effect is attributed to inhibition of neutrophil adherence activation and scavenging of superoxide radicals.^{5,7} Furthermore, nitric oxide inhibits the activity of the proinflammatory transcription factor nuclear factor (NF)- κ B *via* S-nitrosylation.⁸ Inhalation of nitric oxide in concentrations ranging from 10 to 40 ppm has been reported to cause selective pulmonary vasodilation and improves gas exchange in patients with adult respiratory distress syndrome. This selective pulmonary vasodilation is attributed to nitric oxide inactivation by its reaction with oxyhemoglobin forming methemoglobin and nitrite (NO₂⁻).⁹ However, more recent evidence suggests that pharmacologic effects of inhaled nitric oxide may extend beyond the pulmonary vasculature to affect microvascular beds, specifically during conditions associated with loss of endogenous nitric oxide.^{10–12} This is attributed to nitric oxide reacting with elements in blood forming relatively more stable complexes that can be transported, including S-nitrosothiols (*e.g.*, S-nitrosohemoglobin), nitrite (NO₂⁻), and S-nitrosoalbumin. These compounds can simulate effects similar to endothelium-derived nitric oxide, including vasodilatation, although the precise mechanisms remain unclear.^{13,14}

Reduced bioavailability of nitric oxide has been demonstrated in humans during reperfusion in orthotopic liver transplantation.¹⁵ Interestingly, inhaled nitric oxide has been shown to increase peripheral blood flow and reduce leukocyte adhesion in the setting of ischemia–reperfusion injury, suggesting that inhaled nitric oxide has both pulmonary and extrapulmonary antiinflammatory properties.^{10,12,16,17} This is consistent with the concept that replenishing nitric oxide may provide an efficacious therapy in ischemia–reperfusion injury.¹⁰ In this study, we evaluated the potential for inhaled nitric oxide (administered at 80 ppm) to attenuate inflammation instigated by ischemia–reperfusion in a clinically relevant

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Table 1. Clinical Characteristics, Tourniquet Times, and Outcomes in Patients Undergoing Knee Surgery

	Control	INO	
Patients, n	9	9	NS
Men/women	6/3	5/4	NS
Age, yr	34 ± 4	36 ± 5	NS
Weight, kg	76.8 ± 8.6	77.5 ± 6.2	NS
Smoking history	0	0	NS
FiO ₂	0.4	0.4	NS
Systolic blood pressure, mmHg	135 ± 6	138 ± 7	NS
Diastolic blood pressure, mmHg	85 ± 6	87 ± 7	NS
Duration of surgery	180 ± 35	198 ± 45	NS
Tourniquet Time	120 ± 15	120 ± 27	NS
Urine output, ml/h	40 ± 10	45 ± 9	NS
PaO ₂ /FiO ₂ (FiO ₂ = 0.5)	282 ± 13	295 ± 13	NS
Methemoglobin, %	0.2 ± 0.6	1.6 ± 0.6	
Outcome			
Death	0	0	NS
Complications	0	0	NS

FiO₂ = fraction of inspired oxygen; INO = inhaled nitric oxide; NS = not significant; PaO₂ = partial pressure of oxygen in arterial blood.

human model using patients undergoing knee surgery where a tourniquet was used to produce a bloodless surgical field.¹⁸

Materials and Methods

The Institutional Review Board of the University of Texas Medical Branch, Galveston, Texas, approved this study. Written informed consent was obtained from each patient. Patients (n = 18) undergoing elective knee surgery were included in this study (table 1). They were divided into two groups: group A (control; n = 9) and group B (inhaled nitric oxide; n = 9). Anesthesia was induced with a combination of thiopental (3–5 mg/kg) and fentanyl (1–1.5 µg/kg). Tracheal intubation was accomplished with succinylcholine after preoxygenation with 100% oxygen. Anesthesia was maintained with fentanyl, isoflurane, nitrous oxide:oxygen gas mixture (50:50), and vecuronium for muscle relaxation. Patients were mechanically ventilated, and minute ventilation was adjusted to maintain normocapnia. Total tourniquet time was approximately 2 h. Venous samples were collected before tourniquet application and 2 h after tourniquet release. Muscle biopsies were collected from the

quadriceps femoris before ischemia (pretourniquet) and after reperfusion (release of tourniquet) just before skin closure.

Administration of Inhaled Nitric Oxide

Nitric oxide was supplied as a 400-ppm mixture diluted in nitrogen (BOC Gases, Port Allen, LA). The nitric oxide/nitrogen mixture was delivered by a nitric oxide delivery device (INOvent; Ohmeda, Inc., Madison, WI). This delivery device is capable of delivering a consistent concentration of inspired inhaled nitric oxide regardless of ventilator volume and flow settings. The final blended gas was introduced into the inspiratory limb of the respiratory circuit at a concentration of 80 ppm, immediately after intubation and then continued through the reperfusion period until the termination of the surgical procedure. The inhaled concentration of nitric oxide was measured by the INOvent delivery device using electrochemical cell analysis with a sampling of the inspired gases at the distal inspiratory limb of the breathing circuit occurring in a continuous fashion. A scavenging system was used to reduce atmospheric pollution in the operating room.

Study Protocol

Study protocol for the administration of inhaled nitric oxide, blood sampling for adhesion molecules, nitrite and nitrate, and muscle biopsies (for NF-κB, conjugated dienes, and myeloperoxidase activity) is diagrammed in figure 1 and was performed in each patient.

Isolation of Blood Cells

Granulocytes and mononuclear cells were isolated from venous and arterial blood using a density gradient centrifugation on Hypopaque 1077 and 1119 (Sigma, St. Louis, MO).

Assessment of Neutrophil Activation

Neutrophils from patients were isolated by sequential sedimentation in 6% dextran (molecular weight 520,000 Da; Sigma, Manchester, United Kingdom) in 0.9% sodium chloride for 45 min at 22°C, centrifugation in Ficoll-Paque (Pharmacia LKB Biotechnology, Piscataway, NJ) at 300g for 30 min to pellet granulocytes and remain-

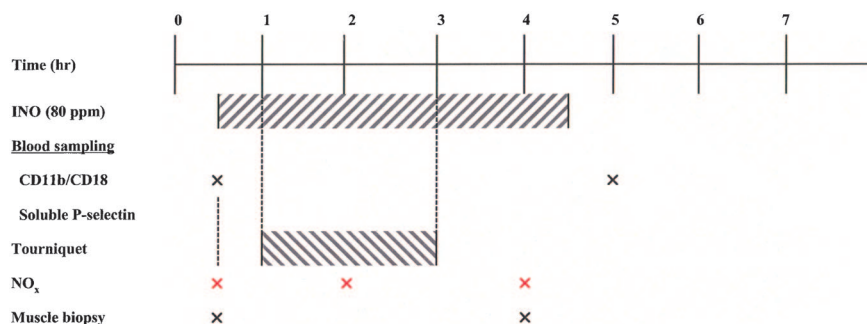


Fig. 1. Study protocol flow diagram. INO = inhaled nitric oxide; NO_x = nitrite and nitrates.

ing erythrocytes, and centrifugation of the resuspended pellet over an 81% isotonic Percoll (Sigma, Manchester, United Kingdom) gradient at 350g for 15 min to pellet erythrocytes. The diffuse layer at the interface containing neutrophils was then harvested, washed, resuspended in medium, and counted. The cells were kept at 0°C for at least 30 min before use. Viability of cells by trypan blue exclusion was greater than 98%.

Neutrophils were incubated with 10 μ l PE-conjugated antileu 15 (anti-CR3/CD11b) mouse anti-human antibody (Becton Dickinson, San Jose, CA) for 20 min, and 10 μ l fluorescein-isothiocyanate-conjugated anti-CR3/CD18 mouse anti-human antibody (Becton Dickinson) for 20 min. After centrifugation at 250g for 5 min, the cell pellets were washed.

Because of considerable variation of CD11b/CD18 expression depending on the neutrophil donor, the data were normalized to the respective basal value (before incubation with conditioned medium) and expressed as percent increase. CD11b and CD18 expression were analyzed on a Fluorescence-Activated Cell-sorter Scanner (FACScan Cytofluorometer; Becton Dickinson). The mean channel fluorescence intensity of stained cells was detected on the basis of a minimum number of 5,000 cells collected analyzed using the FACScan Research Software version B (Becton Dickinson).

Circulating Soluble P-selectin Concentration

Serum concentrations of soluble P-selectin were measured with a sandwich enzyme-linked immunosorbent assay kit (Bender MED-Systems, Vienna, Austria).

Lipid Hydroperoxide Assay

Plasma lipid hydroperoxide were measured colorimetrically with a commercial kit (Lipid Hydroperoxide Assay Kit; Cayman Chemicals, Ann Arbor, MI). This assay measures the hydroperoxide-driven production of the thiocyanate ion, which serves as a chromogen. Lipid hydroperoxides were extracted from the samples (500 μ l) with extract R (methanol) and chloroform following the protocol of the assay kit, and hydroperoxide-driven production of the thiocyanate ion served as the chromogen measured. Standard curve and samples were read at 500 nm. The sensitivity of the method is 0.25–5 nmol hydroperoxide.

Skeletal Muscle Biopsy

Muscle biopsies were obtained from the midzone of the quadriceps femoris during general anesthesia. The biopsies were immediately frozen in liquid nitrogen and stored at -80°C for subsequent analysis.

Isolation of Nuclear Proteins

Briefly, skeletal muscle biopsies were homogenized in 800 μ l ice-cold buffer (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1.0 mM dithiothreitol, 1.0 mM phe-

nylmethanesulfonyl fluoride, 10 $\mu\text{g}/\text{ml}$ aprotinin, 10 $\mu\text{g}/\text{ml}$ pepstatin, 10 $\mu\text{g}/\text{ml}$ leupeptin). The samples were incubated on ice for 30 min; vortexed for 30 s after addition of 50 μ l Nonidet-P40, 10% (Roche Diagnostics GmbH, Penzberg, Germany); and then centrifuged for 10 min at 4°C in an Eppendorf centrifuge (Fisher Scientific, Inc., Pittsburgh, PA). The pellets were suspended in an ice-cold buffer (20 mM HEPES, 400 mM NaCl, 1.0 mM EDTA, 1.0 mM EGTA, 1.0 mM dithiothreitol, 1.0 mM phenylmethanesulfonyl fluoride, 10 $\mu\text{g}/\text{ml}$ aprotinin, 10 $\mu\text{g}/\text{ml}$ pepstatin, 10 $\mu\text{g}/\text{ml}$ leupeptin), incubated on ice for 2 h, mixed frequently, and centrifuged for 10 min at 4°C . The supernatants were collected as nuclear extract and stored at -70°C . The total protein concentration of the samples was determined by the pierce protein assay reagent (Pierce, Rockford, IL).

NF- κ B DNA Binding Assay

Nuclear factor- κ B DNA binding activity was assessed with the Trans-AM NF- κ B family transcription factor assay kits (Active Motif Europe, Rixensart, Belgium) developed by Renard *et al.*¹⁹ as a new sensitive assay to estimate the amount of activated NF- κ B in nuclear protein extracts. The enzyme-linked immunosorbent assay-like test measures the level of the active form of NF- κ B contained in nuclear extracts specifically able to bind to an oligonucleotide containing the NF- κ B consensus site (5'-GGGACTTCC-3') attached to a 96-well plate.^{20,21} Ten-microgram extracts were added to the 96-well plates. The binding of NF- κ B to the DNA was visualized by incubation with anti-p50, anti-p52, anti-p65/Rel-A, and anti-Rel-B antibodies that specifically target activated NF- κ B, followed by a secondary antibody conjugated with horseradish peroxidase. Antibody binding was determined as absorbance values at 450 nm.

Evaluation of Conjugated Dienes Content

Estimation of the tissue content of conjugated dienes was performed to evaluate the extent of lipid peroxidation in tissue as previously shown.^{22,23} Muscle samples were collected in polyethylene tubes and then washed with 1 ml butylated hydroxytoluene, 1 mg/ml, in phosphate buffer. After the samples were dried in absorbent paper, they were frozen at 4°C until analysis. The biochemical assay of conjugated dienes required previous lipid extraction from the tissue samples by chloroform:methanol (2:1). The lipid layer was dried under nitrogen atmosphere and then dissolved in cyclohexane. Muscle content of conjugated dienes was measured at 232 nm by using a spectrophotometric technique. The amount of muscle conjugated dienes was expressed as Δ acrylonitrile butadiene styrene/mg protein.

Myeloperoxidase Assay

Myeloperoxidase was completely extracted from the neutrophils in the muscle samples using hexadecyltri-

methyl-ammonium bromide (Sigma, St. Louis, MO) to solubilize the enzyme. As described previously in the literature, with this detergent treatment, $97.8 \pm 0.02\%$ of total recoverable myeloperoxidase is extracted from neutrophils.²⁴ Myeloperoxidase was assayed spectrophotometrically; 0.01 ml of the homogenized tissue was mixed with 29.0 ml phosphate buffer (50 mM; pH 6.0) containing 0.167 mg/ml dianisidine dihydrochloride (Sigma, St. Louis, MO) and 0.0005% hydrogen peroxide (Mallinckrodt, Paris, KY). The change in absorbance at 460 nm was measured with a Beckman DU spectrophotometer (Beckman Instruments, Fullerton, CA) with a recording attachment (Gilford Instrument Labs, Oberlin, OH). One unit of myeloperoxidase activity was defined as that degrading $1 \mu\text{mol}$ peroxide per minute at 25°C .²⁵ Values were calculated as milliunits of myeloperoxidase per milligram of protein using bicinchonic acid protein assay (Pierca, Rockford, IL) as the reagent.

Nitrite and Nitrate (NO_2^- and NO_3^-)

Concentration of nitrite/nitrate (NO_2^- and NO_3^-) was measured using the vanadium-based assay used for simultaneous nitrite and nitrate measurement in biologic specimens as described by Miranda *et al.*²⁶ Briefly, in this method, vanadium is used to reduce nitrate to nitrite, which reacts with Greiss reagents. This reaction product is measured calorimetrically at 540 nm.

Statistics

Immunologic data are expressed graphically as the mean \pm SD and as maximal percentage changes. To examine differences between the control and nitric oxide groups and the effects of time on immune parameters, a two-way repeated analysis of variance was performed. When a significant F ratio was obtained, pairwise *post hoc* comparisons were performed to isolate differences among treatment means using a Huynh-Feldt correction for multiple comparisons. For all comparisons, a probability of 0.05% or less was considered to be statistically significant.

Results

The control group ($n = 9$) and the inhaled nitric oxide group ($n = 9$) were comparable in terms of demographics and preoperative risk factors (blood pressure and glucose concentrations measured on the day of surgery). Likewise, duration of anesthesia and mean time of muscle ischemia (tourniquet time) were similar in both groups (table 1).

Adhesion Molecule Expression

The proportion of circulating granulocytes was not different between the control and inhaled nitric oxide group. Reperfusion of the lower extremity provoked an up-regu-

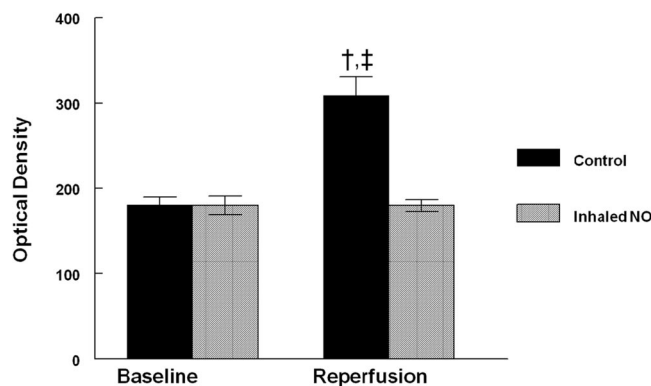


Fig. 2. The granulocyte cellular adhesion molecule receptor CD11b/CD18 was measured as a marker of inflammation. Its up-regulation was blunted during ischemia-reperfusion in patients who received inhaled nitric oxide (NO) during general anesthesia. $P < 0.05$, † compared with baseline values and ‡ compared with inhaled NO reperfusion group.

lation of CD11b/CD18 (minimum alveolar concentration [MAC] 1) from a mean value of 181 ± 22 at baseline to 309 ± 8 during reperfusion ($P < 0.05$). However, in the group receiving inhaled nitric oxide (182 ± 7), this response was diminished compared with the control group during reperfusion ($P < 0.05$; figs. 2 and 3).

Soluble P-selectin significantly increased from a mean value of 178 ± 42 at baseline to 334 ± 43 ($P < 0.05$) during reperfusion in the control group. Again, this response was significantly reduced with nitric oxide treatment to a mean value of $174 (\pm 19)$ compared with the control group during reperfusion (fig. 4).

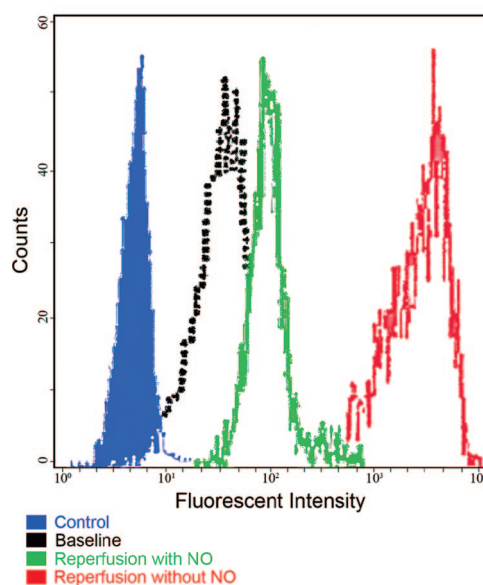


Fig. 3. Effect of inhaled nitric oxide (NO) on the expression of adhesion molecules (CD11b/CD18) on neutrophils. The dark area represents the histogram obtained with the control antibody. The results are representative of four separate experiments using neutrophils isolated from four different patients. Blue represents control group, black represents baseline, green represents reperfusion with NO, and red represents reperfusion without NO.

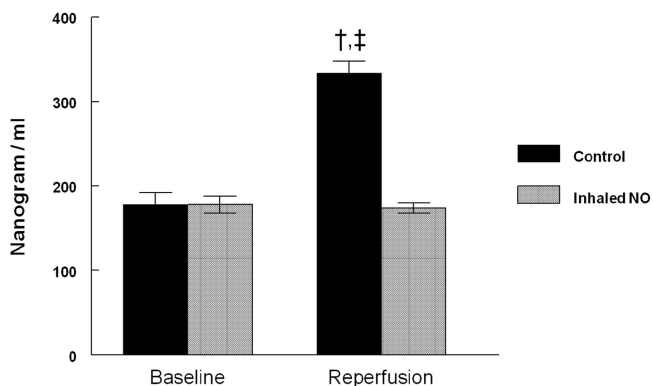


Fig. 4. Soluble P-selectin, an adhesion molecule of granulocytes, was measured as a marker of inflammation during ischemia-reperfusion. Its up-regulation was blunted in patients receiving inhaled nitric oxide (NO). $P < 0.05$, [†] compared with baseline values and [‡] compared with inhaled NO reperfusion group.

Plasma Lipid Hydroperoxide

Plasma lipid hydroperoxide levels were similar in both groups at baseline during reperfusion. The control group showed a significant increase during reperfusion signifying increases in oxidative stress. Plasma lipid hydroperoxide levels were markedly decreased in patients receiving inhaled nitric oxide compared with the control group ($P < 0.05$; fig. 5).

NF-κB Translocation in Post-Ischemia-Reperfusion Muscle

Inhaled nitric oxide significantly decreased NF-κB translocation in muscle during reperfusion (1.5 ± 0.2 vs. 3.2 ± 0.2 ; $P < 0.05$; fig. 6). Baseline values between both control and inhaled nitric oxide were not different (1.50 ± 0.1 vs. 1.60 ± 0.1).

Conjugated Dienes

Conjugated dienes contents were similar in both groups at baseline. During reperfusion, the control group showed oxidative stress damage characterized by a significant in-

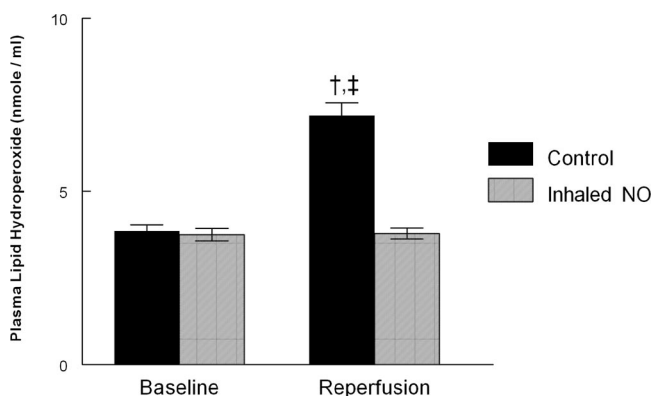


Fig. 5. Lipid hydroperoxide was measured in plasma as a reflection of oxidative stress during ischemia-reperfusion. Plasma lipid hydroperoxide levels were markedly decreased in patients receiving nitric oxide (NO) compared with the control group. $P < 0.05$, [†] compared with baseline values and [‡] compared with inhaled NO reperfusion group.

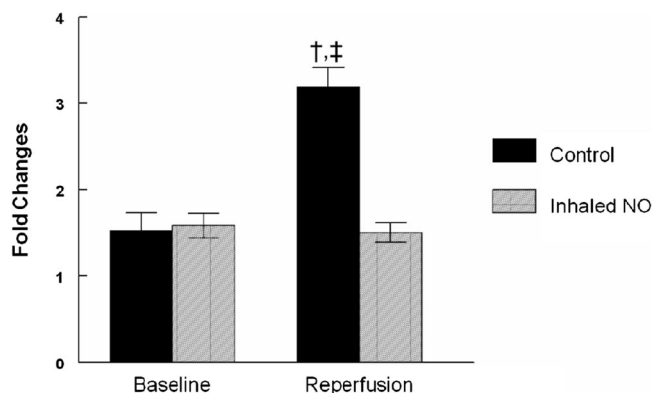


Fig. 6. Nuclear factor-κB translocation in the quadriceps femoris muscle was measured as a marker of inflammation due to ischemia-reperfusion. A threefold higher increase in translocation occurred in the control group. Inhaled nitric oxide (NO) significantly suppressed any increase in nuclear factor-κB translocation. $P < 0.05$, [†] compared with baseline values and [‡] compared with inhaled NO reperfusion group.

crease in muscle content conjugated dienes. Administration of inhaled nitric oxide resulted in a reduction in conjugated dienes during reperfusion (fig. 7).

Myeloperoxidase Activity in Muscle

Before ischemia, both the control group and the inhaled nitric oxide group had minimal measurable myeloperoxidase activity. However, reperfused muscle in the control group showed a significant increase in myeloperoxidase activity ($P < 0.05$) compared with the inhaled nitric oxide group in the same period. This activity was decreased to values approaching baseline values (fig. 8).

Nitrite and Nitrate (NO₂⁻ and NO₃⁻)

There was no significant difference in baseline values of nitrate and nitrite between the control group and the group receiving inhaled nitric oxide (77 ± 8 vs. 73 ± 7 ; nonsignificant; fig. 9). Patients receiving inhaled nitric

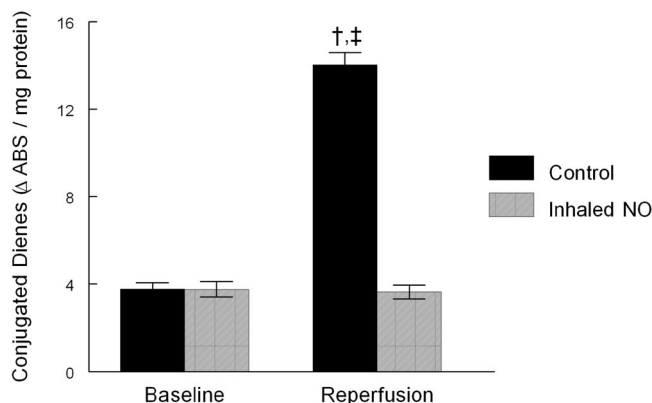


Fig. 7. Conjugated diene activity in muscle was measured as a marker of oxidative stress during ischemia-reperfusion. Low conjugated diene content was observed in patients receiving inhaled nitric oxide (NO) compared with the control group. ABS = acrylonitrile butadiene styrene. $P < 0.05$, [†] compared with baseline values and [‡] compared with inhaled NO reperfusion group.

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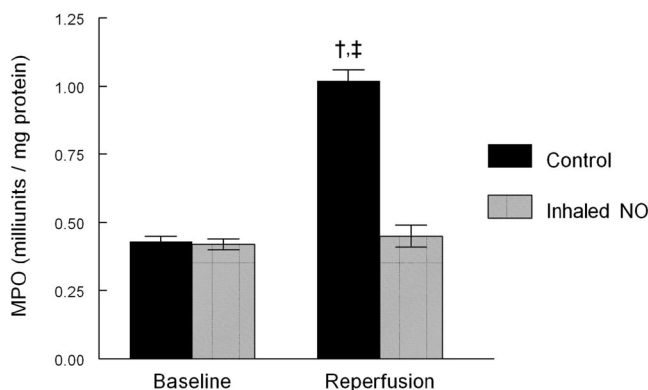


Fig. 8. Myeloperoxidase (MPO) activity in muscle was measured as a marker of inflammation and oxidative stress during ischemia-reperfusion. MPO activity was decreased in patients receiving inhaled nitric oxide (NO). During reperfusion compared with the control group. $P < 0.05$, † compared with baseline values and ‡ compared with inhaled NO reperfusion group.

oxide had significantly greater nitrate and nitrite concentrations compared with the control group 45 min after inhaled nitric oxide administration (119 ± 9 vs. 73 ± 7 ; $P < 0.01$) and at the completion of reperfusion (the time of surgical closure) (61 ± 7 ; $P < 0.01$).

Discussion

The current study shows that following reperfusion of a lower extremity after ischemia in humans, an inflammatory response results; this is characterized by activation of NF- κ B in muscles with parallel increases in oxidase stress in the affected skeletal muscle. The enhanced expression of NF- κ B in muscle was accompanied by augmented expression of adhesion molecules CD11b/CD18 and soluble P-selectin in peripheral blood. These

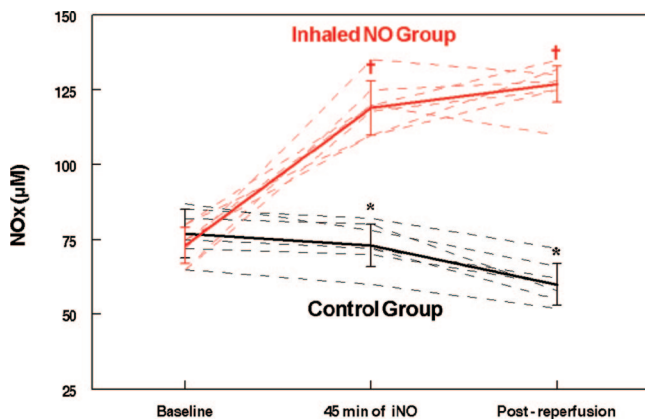


Fig. 9. Plasma nitrate and nitrite (NOx) were measured in control patients and patients receiving inhaled nitric oxide (INO). Baseline values were not different between groups. A significant increase occurred in patients receiving INO compared with control patients and compared with the baseline values of patients randomized to INO. $P < 0.01$, * compared with INO groups at 45 min after INO administration and at the completion of the surgical procedure (postreperfusion), and † compared with baseline values. NO = nitric oxide.

alterations were significantly attenuated in patients who received inhaled nitric oxide.

Activation of NF- κ B, a rapid response transcription factor, has been demonstrated in experimental models of skeletal muscle ischemia-reperfusion, and inhibition of NF- κ B activity decreases inflammation and increases survival rate.²⁷⁻²⁹ Several lines of experimental evidence suggest that ischemia-reperfusion-induced leukocyte-endothelial cell adhesion (hours after reperfusion) is associated with transcription-dependent expression of adhesion molecules such as P-selectin, E-selectin, and intracellular adhesion molecule 1.¹⁻⁵ Recent evidence suggests that the activation of human neutrophils by β_2 integrin (CD11b/CD18) aggregation is mediated *via* activation of the inhibitory unit of NF- κ B/NF- κ B pathway.³⁰ Therefore, the directionally similar responses seen between NF- κ B, CD11b/CD18, and oxidative stress (increased conjugated dienes and myeloperoxidase in our patient) goes along with this paradigm. Reactive oxygen intermediates seem to mediate the transcription-dependent expression of endothelial cell and neutrophil adhesion molecules that are elicited by ischemia-reperfusion, a response that is most likely mediated by the transcription factor NF- κ B.³¹ Consistent with this interpretation, we demonstrated increased oxidative stress both in blood (increased lipid peroxidation) and in muscle (increased conjugated dienes and myeloperoxidase) with parallel increases in NF- κ B activity in muscle during reperfusion. Furthermore, nitric oxide, which blocks the degradation of inhibitory unit of NF- κ B by scavenging superoxide (O_2^-) and/or by S-nitrosylation was able to mitigate the reperfusion-induced expression of P-selectin and MAC 1 expression.^{6,12,32,33} Consistent with this contention that inhaled nitric oxide reduced oxidative stress (decreased lipid peroxidation and myeloperoxidase activity) and directionally similar changes in NF- κ B in muscle supports this paradigm. A reduction in bioavailability of nitric oxide also seems to contribute to the leukocyte-endothelial cell adhesion response to ischemia-reperfusion.^{5,34} This is supported by the observation that inhibition of nitric oxide synthase exacerbates and administration of nitric oxide donor attenuates many of the same oxidant-dependent responses that are observed during ischemia-reperfusion.^{34,35} The reduced bioavailability of nitric oxide during reperfusion is attributed to the inactivation of nitric oxide by superoxide.⁵

Changes in nitrate and nitrite levels during inhaled nitric oxide in our patients suggest parallel changes in bioavailability of nitric oxide. However, caution should be exercised in making such an interpretation. Recent evidence suggests that nitrite and nitrate levels are only a rough estimate of the total body nitrogen/nitric oxide turnover, whereas plasma nitrite represents a useful marker of acute changes in bioavailability of nitric oxide.³⁶ Furthermore, we cannot exclude nitrite/nitrate redistribution away from the circulation into tissues after

ischemia-reperfusion. Our primary objective for these measurements was to show increased flux of nitric oxide and presumably associated metabolites upon inhaled nitric oxide administration, which is supported by data presented in figure 9. Whether the observed antiinflammatory effects of inhaled nitric oxide in the current study reflects nitrite (O_2^-) scavenging or another action, *e.g.*, *S*-nitrosylation, remains unclear.

Our findings have worthy clinical implications. Reperfusion injury remains a significant limiting factor in the successful outcome of major microsurgical operations involving limb replantation, free tissue transfers, and organ transplantation. Inhaled nitric oxide with its ability to affect neutrophil endothelial interaction *via* down-regulation of NF- κ B activity may be administered when the specific disorder is characterized by reduced bioavailability of nitric oxide, *e.g.*, an ischemia-reperfusion event.³⁷ Inhaled nitric oxide (unlike nitric oxide donor compounds), with its lack of effect on systemic blood pressure, is particularly useful in patients undergoing ischemia-reperfusion with unstable hemodynamics, *e.g.*, patients undergoing coronary intervention and emerging from cardiopulmonary bypass.^{38,39} However, in those clinical scenarios in which functional deficiencies of nitric oxide are accompanied by severe lung injury requiring high concentrations of inspired oxygen, it would be preferable to administer a "nitric oxide donor" to avoid the reaction between inhaled nitric oxide and high inspired oxygen concentration, which could potentially generate injurious products such as peroxynitrite.⁴⁰

The absolute levels of nitric oxide in reperfused tissues and its regulation by the subtle interplay with superoxide and the subsequent generation of highly toxic peroxynitrite anion are important factors that determine the optimal timing for the delivery of inhaled nitric oxide in the context of ischemia reperfusion injury.⁴¹ Experimental evidence suggests that in skeletal muscle there is decreased nitric oxide production during early reperfusion (the first 2–3 h), which is followed by a second higher peak commencing in the later stages of reperfusion.^{42,23} Therefore, administering inhaled nitric oxide during early stages of reperfusion as a surrogate for endothelial-derived nitric oxide would enable the cytoprotective effects of nitric oxide to be maintained.⁴³ However, some studies suggest that a nitric oxide donor administered before reperfusion increases both plasma nitric oxide and endothelial nitric oxide synthase expression, and suppresses inducible nitric oxide synthase expression, with the net effect of increased muscle survival.^{44,45} Until the exact nitric oxide spatiotemporal dynamics during ischemia-reperfusion is delineated in humans, the optimal time to administer inhaled nitric oxide during ischemia-reperfusion will remain unidentified. Finally, "off-label" use of inhaled nitric oxide to attenuate ischemia-reperfusion injury is an expensive therapeutic option. A recent estimate for in-hospital,

off-label use is approximately \$125.00 per hour irrespective of the concentration being administered.⁴⁶

The current study has important strengths and limitations. Among the strengths is the ability to demonstrate the extrapulmonary, antiinflammatory effects of inhaled nitric oxide in a clinically relevant human model of ischemia-reperfusion.¹⁸ Because of the small sample size of the study population, no conclusions can be drawn regarding the clinical effectiveness of inhaled nitric oxide. We also acknowledge that a lack of measurement of specific nitric oxide metabolites (*e.g.*, nitrite, *S*-nitrosothiols) preclude assessment of the potential mediators produced by inhaled nitric oxide that may mediate the antiinflammatory effects observed. One may question our wisdom of administering 80 ppm nitric oxide to our patients in view of the nitric oxide reactivity with superoxide and with hemoglobin yielding peroxynitrite and methemoglobin.⁴⁷ In a pilot study ($n = 3$), we were unable to demonstrate antiinflammatory effects with lower concentrations (20 or 40 ppm) of nitric oxide. Accordingly, we chose to administer 80 ppm nitric oxide in this study. Similar findings have been reported for other systemic effects of inhaled nitric oxide.^{10–13} It is conceivable that inhalation of high concentrations of nitric oxide gas is required to down-regulate the adhesion receptors in leukocytes, load nitric oxide transporters, or increase plasma nitrite. Furthermore, despite breathing 80 ppm, nitric oxide methemoglobin levels remained less than 3% in patients receiving nitric oxide. The safety of inhaled nitric oxide in normal individuals breathing a high concentration (128 volumes/million) greater than any dose used clinically has already been established.⁴⁸ Our findings could be conceived, in part, as a reflection of the effects of lower extremity ischemia-reperfusion on remote organ inflammatory response, *e.g.*, the lung.⁴⁹ However, this seems unlikely because gas exchange remained normal (partial pressure of oxygen in arterial blood/fraction of inspired oxygen) during reperfusion.

In conclusion, we have demonstrated that inhaled nitric oxide administered at 80 ppm blunts the inflammatory response of lower extremity ischemia-reperfusion in humans. This is supported by reduced expression of adhesion molecules, *e.g.*, CD11b/CD18 (MAC 1), soluble P-selectin, and accompanied by reduced expression of NF- κ B in reperfused skeletal muscles. The conclusions drawn from the current study must be tempered by the appreciation that in the context of compartmentalized models of organ injury, inhaled nitric oxide has also been demonstrated to exacerbate injury.⁵⁰ As more nitric oxide-related therapies become clinical realities, we will have a better understanding of the role of nitric oxide in reperfusion injury, and efforts to manipulate nitric oxide to attenuate reperfusion-induced inflammatory response in a clinical setting will grow.

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