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Attenuated Nitric Oxide Synthase Activity and Protein Expression Accompany Intestinal Ischemia/Reperfusion Injury in Rats

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Intestinal ischemia/reperfusion (I/R) leads to bowel impairment via the release of reactive oxygen species (ROS) and neutrophil infiltration. In addition to modulating intestinal integrity, nitric oxide ($NO^{\bullet}$) inhibits neutrophil activation and scavenges ROS. Attenuated endogenous $NO^{\bullet}$ formation may result in the accrual of these deleterious stimuli. Therefore, we determined nitric oxide synthase (NOS) activity in anesthetized rats subjected to 1 h of superior mesenteric ischemia or ischemia followed by reflow. NOS activity was measured in intestinal tissue homogenates as the conversion rate of ^{3}H-L-arginine to ^{3}H-L-citrulline. Our results demonstrate that intestinal ischemia leads to a decrease in NOS activity indicating lower $NO^{\bullet}$ formation in the animal model. The attenuation in NOS activity was not reversed following 4 h of reperfusion. Western blot analysis revealed that the decline in enzyme activity was accompanied by reduced intestinal NOS III (endothelial constitutive NOS) expression. These findings provide biochemical evidence for impaired $NO^{\bullet}$ formation machinery in intestinal I/R injury.

Key Words: **nitric oxide; nitric oxide synthase; intestinal ischemia/reperfusion.**

Intestinal ischemia/reperfusion (I/R) injury is a serious medical problem often necessitating surgical intervention (1, 2). Reperfusion of ischemic tissue, although necessary for reparative mechanisms, has been shown to worsen acute ischemic injury via release of reactive oxygen species (ROS) and accumulation of activated neutrophils (3). Leukocyte activation and subsequent mucosal and microvascular infiltration, result in loss of "selective" barrier function of the bowel culminating in increased intestinal permeability (4). This eventually leads to the translocation of enteric bacterial products, multiple organ failure, and death (1).

The role of nitric oxide ($NO^{\bullet}$) in modulating intestinal inflammation has been recently investigated (5). In addition to its function as a vasodilator, $NO^{\bullet}$ has been shown to inhibit neutrophil activation, decrease cell-cell interactions, and scavenge ROS (6, 7). Local release of $NO^{\bullet}$ has been shown to regulate bowel homeostasis via modulating mesenteric perfusion and neutrophil-endothelium interactions (8). Inhibition of gut $NO^{\bullet}$ release results in deleterious effects similar to intestinal I/R injury such as diminished blood flow, increased neutrophil infiltration and augmented mucosal permeability (9). In animal models of I/R injury, administration of NOS-inhibitors has been shown to exacerbate intestinal impairment (9). Furthermore, $NO^{\bullet}$ supplementation ameliorates structural and functional damage accompanying experimental I/R (5, 10). These studies raise the possibility that intestinal I/R injury may be associated with decreased levels of endogenous $NO^{\bullet}$. Presently three nitric oxide synthase (NOS) isoforms, namely NOS-I (neuronal), NOS-II (inducible), and NOS-III (endothelial), have been identified as being responsible for $NO^{\bullet}$ formation (11, 12). NOS III (also termed ecNOS) is the predominant isoform in the gastrointestinal tract (8, 13, 14). Hence, the goal of this study was to determine if intestinal NOS activity and NOS III expression were altered in a rat model of intestinal I/R injury.

MATERIALS AND METHODS

Materials. ^{3}H-arginine, secondary antibodies and the ECL kit were purchased from Amersham Inc., Arlington Heights, IL. DOWEX resin was obtained from BioRad Laboratories, Hercules, CA. Primary antibodies were acquired from Transduction Laborato-

Abbreviations used: $NO^{\bullet}$, nitric oxide; NOS, nitric oxide synthase; I/R, intestinal ischemia/reperfusion; NEC, necrotizing enterocolitis; ROS, reactive oxygen species; ecNOS, endothelial constitutive (Type III) NOS; NADPH, nicotinamide adenine dinucleotide (reduced form); HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethane sulfonic acid.

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ries, Lexington, KY (monoclonal anti-ecNOS and human endothelial cell lysate), Affinity Bioreagents Inc., Golden, CO (polyclonal anti-ecNOS) and Zymed Laboratories Inc., San Francisco, CA (anti-actin). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO). ^{3}H-arginine was purified prior to each use in accordance with published reports (15).

Intestinal ischemia/reperfusion model. All procedures used in the present study have been approved by The State University of New York at Buffalo Animal Care Committee. Adult male Sprague-Dawley rats (225–275 g) were fasted but had free access to water the night before the experiment. Animals were anesthetized by the administration of intramuscular ketamine (90 mg/kg) and xylazine (9 mg/kg). Following a midline laparatomy, rats were subjected to 60 min of ischemia by occluding the superior mesenteric artery (SMA), either with or without subsequent reperfusion (4 h). Between surgical interventions, the incision was sutured and covered with plastic wrap to minimize fluid losses. To maintain an adequate anesthetic plane, ketamine/xylazine was administered as necessary and the rats were placed on heating pads at 37°C throughout the experiment. Control animals were subjected to midline laparatomy and dissection without SMA occlusion. At the end of the experiment, the entire small bowel was harvested and used for determining intestinal NOS activity or protein expression.

Assessment of NOS activity. NOS activity was determined by measuring the conversion rate of arginine (substrate) to citrulline (15). Following induction of injury, the small bowel was isolated, weighed, and homogenized in ice cold buffer (1 gm tissue/1 ml buffer; HEPES 10 mM, sucrose 320 mM, dithiothreitol 1 mM, aprotonin 2 μg/ml and leupeptin 10 μg/ml; pH 7.4). The homogenate was centrifuged for 20 min at 10,000*g* and 4°C. The resulting supernatant (S9-fraction) was incubated at 37°C for 20 min in the presence of purified ^{3}H-L-arginine (60 μM) and cofactors essential for NOS activity, viz., calmodulin (10 μg/ml), tetrahydrobiopterin (3 μM), flavin adenine dinucleotide (1 μM), flavin mononucleotide (1 μM), $CaCl_2$ (1 mM), $MgCl_2$ (2 mM), valine (1 mM), and NADPH (2 mM), in HEPES (20 mM, adjusted to pH 7.4). The enzymatic reaction was terminated by adding "STOP" buffer (in mM, HEPES 25, EDTA 4 and nitro-L-arginine 5; pH 5.5). ^{3}H-L-citrulline was separated by passing the reaction mixture through a column of DOWEX cation exchange resin (Na^+-form, AG 50W-8, pre-equilibrated to pH 5.5). Radioactivity in the effluent was measured using a Tri-Carb Liquid Scintillation Analyzer (Model 1900, Packard Instrument Company, Meriden, CT). Background i.e. non-enzymatic conversion of ^{3}H-arginine to ^{3}H-citrulline was determined by incubating substrate in the absence of intestinal tissue. The percent of added radioactivity converted to ^{3}H-citrulline was determined and normalized to background conversion, incubation time, and tissue weight. Hence, NOS activity was expressed as picomoles of citrulline formed per min per gm tissue.

Western blot analysis of NOS III. Expression of NOS III protein was determined following 1 h of ischemia. S9 fractions were prepared as described above and protein content determined by the Bradford assay with bovine serum albumin as standard (16). Aliquots of 500 μg protein were diluted (1:1) in gel-loading buffer (Tris 100 mM pH 6.8, containing 1% β-mercaptoethanol, 4% sodium dodecyl sulfate and 20% glycerol), denatured by heating at 96°C for 3 min, and loaded onto 8% SDS–PAGE gels. Electrophoresis was carried out at 50 V followed by transfer of protein to nitrocellulose membrane. To block non-specific binding membranes were treated with 5% nonfat dry milk in Tris-buffered saline (TBS) with 0.1% Tween 20. Rabbit polyclonal or mouse monoclonal anti-NOS III antibodies (1:1000 dilution in 5% nonfat dry milk with TBS) were incubated with the membranes for 1 h at room temperature. Incubation with secondary antibody (horseradish peroxidase conjugated sheep anti-rabbit or anti-mouse IgG) was carried out for 1 h at a dilution of 1:2000 in 5% nonfat dry milk with TBS. The membrane was washed and detected using the enhanced chemiluminescence (ECL) method. The resulting signal was detected by exposure to radiographic film.

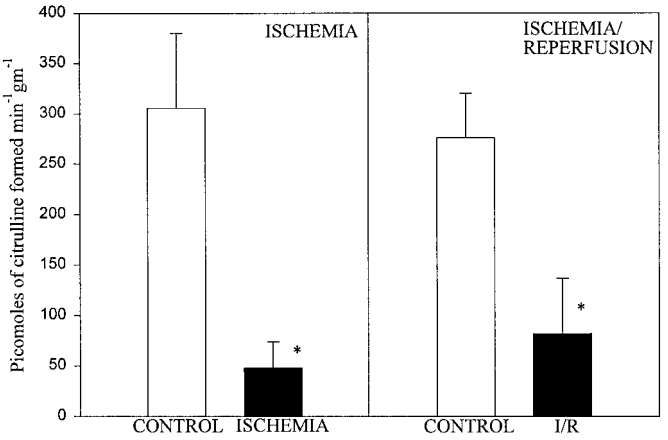

FIG. 1. Effect of superior mesenteric ischemia (left panel) or ischemia followed by reperfusion (right panel) on intestinal NOS activity. The disease group was associated with attenuated NOS activity (*$p < 0.001$ versus time-matched control, n = 6 rats/group). Data expressed as mean ± S.D.

Western blot analysis of actin. Actin was used as a housekeeping protein. The protocol followed was similar to NOS III except that the primary antibody used was mouse anti-actin (monoclonal, 1:500) and the secondary antibody was sheep anti-mouse IgG (1:2000).

Optical densitometry. For quantification of Western blot analysis, the developed radiographic film was scanned, and optical density measured using a BIORAD imaging densitometer (Model GS-700).

Statistical analyses. All data are reported as mean ± S.D. Comparisons between groups were made using Student's t-test at the $p < 0.05$ level.

RESULTS

Alterations in NOS activity following intestinal ischemia/reperfusion. One h of mesenteric ischemia resulted in a drastic decrease in intestinal NOS activity (n = 6 rats/group; $p < 0.001$, Fig. 1, left panel). The attenuation in NOS activity following ischemia was not reversed by re-establishing blood flow. Hence, animals subjected to mesenteric occlusion followed by reperfusion were also associated with significantly lower intestinal NOS activity in comparison to their time-matched controls (n = 6 rats/group; $p < 0.001$, Fig. 1, right panel).

Effect of intestinal ischemia on NOS III expression. Western blot analysis of intestinal tissue from control rats demonstrated a band of approximately 140 kDa (Fig. 2), consistent with NOS III (14). This was confirmed by co-electrophoresing human endothelial cell lysate as a positive control (denoted by "+" in Fig. 2). Intestinal ischemia was associated with a substantial reduction in size of the 140 kDa band suggesting that the decrease in NOS activity observed may be partly due to loss of NOS III protein (Fig. 2). Similar results were obtained irrespective of the type of primary antibody used. The reduction in NOS III protein was confirmed by optical densitometry where the data showed

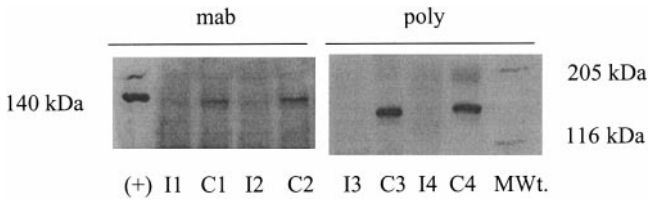

FIG. 2. Immunoblot of NOS III in intestines from control and ischemic rats (n = 3 animals/group). Membrane was probed using both monoclonal (mab) and polyclonal (poly ab) antibodies. Intestinal ischemia results in decreased NOS III protein (band detected at approximately 140 (kDa) irrespective of antibody used. The band in the lane labeled (+) is endothelial cell lysate (positive control). Lanes labeled I(x) denote NOS III expression in bowels from individual animals subjected to ischemia whereas C(y) denote expression in intestinal tissue from time-matched controls. Lane marked MWt. represents molecular weight markers.

significant reductions in band density using both monoclonal (0.31 ± 0.02 arbitrary units/cm^2 in ischemic animals versus 1.15 ± 0.42 arbitrary units/cm^2 in controls, n = 3 rats/group; $p < 0.001$) and polyclonal (1.29 ± 0.74 arbitrary units/cm^2 in ischemic animals versus 6.89 ± 0.57 arbitrary units/cm^2 in control animals, n = 3 rats/group; ($p < 0.001$) antibodies. This decrease in protein expression was similar to the reduction in NOS activity (86%) that was observed following 1 h of ischemia (Fig. 1, left panel).

Actin expression following intestinal ischemia. To demonstrate that alterations in NOS III protein expression were not due to universal changes in expression of all proteins, we also assessed for changes in actin expression. Actin (45 kDa) is commonly used as a housekeeping protein in the gastrointestinal tract (17). Our results demonstrate significant actin expression in intestinal tissue from all rats. Actin expression was unchanged in rats subjected to 1 h of intestinal ischemia (Fig. 3). These results were confirmed by quantification of the immunoblots using optical densitometry, with similar actin expression density in intestinal tissue from control and diseased animals. (43.9 ± 10.3 arbitrary units/cm^2 in controls versus 45.0 ± 1.57 arbitrary units/cm^2 in ischemic bowels, $p > 0.05$, n = 3 rats/group).

DISCUSSION

Intestinal ischemia/reperfusion (I/R) is thought to play an important role in numerous clinical disorders including neonatal necrotizing enterocolitis (NEC), tissue dysfunction following small bowel transplantation and mesenteric insufficiency in the elderly (1). I/R injury, although initiated by vascular occlusion, has been shown to be mediated by superoxide release and neutrophil activation (3). The end result of I/R injury is a loss of "selective" barrier function of the intestine subsequent to increased mucosal and microvascular permeability (4). Current therapy is limited and hence these diseases are associated with significant mortality rates (18). Identification of key mediators of I/R injury may permit the development of suitable treatment modalities.

Recent evidence has suggested that nitric oxide ($NO^{\bullet}$) may play a significant role in maintenance of mucosal integrity (5, 8, 19). In the mesenteric endothelium, low level continuous release of $NO^{\bullet}$ by NOS III is thought to be a major determinant of vascular tone and regulation of blood flow to the mucosa (8). In addition to suppressing neutrophil activation and scavenging reactive oxygen species (ROS) such as superoxide, $NO^{\bullet}$ has also been shown to inhibit enzymes responsible for the release of superoxide (20, 21). Attenuated $NO^{\bullet}$ levels have been demonstrated in pathological conditions associated with increased formation of ROS and activation of neutrophils (8, 9, 22). Hence, we have investigated the link between I/R injury and $NO^{\bullet}$ formation in a well-documented rat I/R model (23, 24). Due to its ephemeral nature, direct *in vivo* measurements of $NO^{\bullet}$ are difficult. Therefore, we have used the conversion of ^{3}H-arginine to ^{3}H-citrulline via NOS as an indicator of $NO^{\bullet}$ formation (15).

Following 1 h of ischemia we have observed a significant decrease in intestinal NOS activity. This attenuation in activity was maintained even after reestablishing blood flow. The decline in NOS activity suggests that the animal model may be associated with lower production of intestinal $NO^{\bullet}$. Consistent with this argument, Kurose *et al.* (22) have demonstrated that intestinal I/R in the rat was accompanied by decreased plasma levels of nitrite/nitrate (metabolic products of $NO^{\bullet}$). Although we observed decreased NOS activity following intestinal ischemia, Kanwar *et al.* have reported that reperfusion, but not mesenteric ischemia, induced a significant decrease in intestinal NOS activity in a feline I/R model (9). Differences in species, especially with regard to collateral flow during ischemia (25–27), may provide an explanation for these

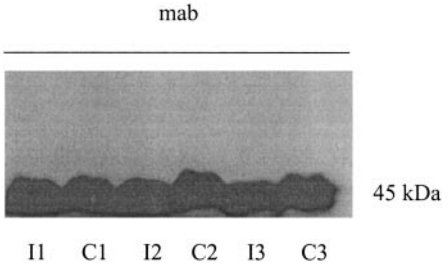

FIG. 3. Immunoblot of actin in intestines from control and ischemic rats (n = 3 animals/group). Membrane was probed by anti-actin monoclonal (mab) antibody. Actin expression (band detected at approximately 45 kDa) is preserved after 1 h of intestinal ischemia. Lanes labeled I(x) denote actin expression in bowels from individual rats subjected to ischemia whereas C(y) denote expression in intestinal tissue from controls.

dissimilar results. In agreement with our data, Giraldez *et al.* have demonstrated decreased NOS activity in cardiac tissue following *in vitro* myocardial ischemia in rats (28).

To assess whether alterations in protein expression accounted for the ischemia-induced decrease in NOS activity, we carried out Western blot analysis of NOS III in intestinal tissue homogenates. NOS III is the predominant NOS isoform found in the gastrointestinal tract and accounts for the majority of $NO^{\bullet}$ production (8, 13, 14). Due to the length of time required for up-regulation of NOS II (inducible NOS) it was not expected to play a significant role in this acute injury model (9). Our results demonstrate significant NOS III expression in intestinal tissue from control animals. However, following 1 h of ischemia there was a marked decrease in expression of NOS III protein suggesting that the attenuation in NOS activity may be due to reduced protein levels. Similar results have been obtained in an *in vitro* model of myocardial I/R injury where the authors demonstrated that NOS III expression was decreased following ischemic periods of 60 min or greater (28). Intracellular pH drops to approximately 5.5 in ischemic tissues (29). Due to the relative instability of ecNOS at lower pH values (30), it has been suggested that decreased NOS III expression following ischemia may be due to acidosis-dependent denaturation and proteolysis (28).

Actin has been commonly used as a housekeeping protein in the gastrointestinal tract (17). We have observed substantial actin expression in intestinal tissue homogenates from all animals. Following 1 h of mesenteric occlusion, expression of actin was preserved indicating that the effect of intestinal ischemia on NOS III may not be due to widespread ischemia-induced tissue damage. Similarly, expression of enzymes such as alkaline phosphatase have been shown to be insensitive to ischemia alone (28) indicating the selective susceptibility of NOS III to ischemia.

In summary, we have demonstrated that mesenteric ischemia leads to a sustained decrease in intestinal nitric oxide synthase activity and expression, resulting in lower nitric oxide production in the animal model. The end result of attenuated $NO^{\bullet}$ formation may be enhanced superoxide levels and neutrophil infiltration that may contribute to impaired intestinal function in I/R injury. Clinical evidence supports this line of reasoning. Plasma levels of arginine (substrate for NOS), have been found to be lower in a cohort of premature infants that subsequently developed NEC, a condition caused by mesenteric I/R (31). This suggests that substrate limitation, and hence $NO^{\bullet}$ availability, may have contributed to the loss of mucosal integrity, resulting in NEC. A critical role for $NO^{\bullet}$ in I/R is also supported by observations that administration of NOS-inhibitors exacerbate, whereas $NO^{\bullet}$ donors ameliorate, experimental I/R injury (5). These results demonstrate a link between I/R injury and impaired endogenous $NO^{\bullet}$ formation and support the rationale for supplementation of the nitric oxide pathway, via exogenous $NO^{\bullet}$ delivery or stabilization of NOS III protein, in treating this condition.

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