Hindlimb Ischemia/Reperfusion-Induced Remote Injury to the Small Intestine: Role of Inducible Nitric-Oxide Synthase-Derived Nitric Oxide

Kazuhiro Katada, Aurelia Bihari, Amit Badhwar, Norimasa Yoshida, Toshikazu Yoshikawa, Richard F. Potter, and Gediminas Cepinskas

Centre for Critical Illness Research, Lawson Health Research Institute, London, Ontario, Canada (K.K., A.Bi., A.Ba., R.F.P., G.C.); Department of Surgery, University of Western Ontario, London, Ontario, Canada (R.F.P.); and Department of Molecular Gastroenterology and Hepatology, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kyoto, Japan (N.Y., T.Y.)

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ABSTRACT

Systemic inflammatory response syndrome, as a consequence of ischemia/reperfusion (I/R), negatively influences the function of the affected organs. The objective of this study was to assess the role of nitric oxide (NO) in remote intestinal inflammatory response elicited by hindlimb I/R. To this end, C57BL/6 (wild type; WT) and inducible nitric-oxide synthase (iNOS)-deficient mice were subjected to bilateral hindlimb ischemia (1 h) followed by 6 h of reperfusion. Some WT mice were injected with iNOS inhibitor N-[3-(aminomethyl)benzyl] acetamidine (1400W) (5 mg/kg s.c.) immediately before reperfusion, and proinflammatory response was assessed 6 h later. Hindlimb I/R resulted in dysfunction of the small intestine as assessed by the increase in permeability [blood-to-lumen clearance of Texas Red-dextran (molecular mass 3 kDa)] and an increase in the luminal levels of tumor necrosis factor (TNF)-α protein and nitrate/nitrite (NO$_2^-$/NO$_3^-$). The above-mentioned changes were accompanied by up-regulation of the proinflammatory phenotype in the mucosa of small intestine with respect to 1) an increase in TNF-α and iNOS protein expression, 2) leukocyte accumulation, 3) formation of edema, 4) an increase in leukocyte rolling/adhesion in the submucosal microvasculature, and 5) activation of transcription factor nuclear factor-κB and up-regulation of adhesion molecule expression. Interestingly, the most profound changes with respect to intestinal dysfunction were found in jejunum and ileum, whereas duodenum was affected the least. Interfering with iNOS activity (1400W and iNOS-deficient mice) significantly attenuated hindlimb I/R-induced inflammatory response and dysfunction of the small intestine with respect to the above-mentioned markers of inflammation. The obtained results indicate that hindlimb I/R induces remote inflammatory response in the small intestine through an iNOS-derived NO-dependent mechanism.

The systemic inflammatory response syndrome (SIRS) is a consequence of many conditions, such as surgery, trauma, burn, pancreatitis, hemorrhagic shock, or bacterial infection (Carden and Granger, 2000), that, if not treated in a timely manner, results in the development of potentially fatal complication known as multiple organ dysfunction syndrome (MODS). The small intestine has been implicated in the development of SIRS and MODS (Deitch, 1990; Fink, 2003; Balzan et al., 2007); however, the mechanism(s) remain controversial and poorly investigated.

Bilateral hindlimb I/R has been extensively used in our laboratory as a model of SIRS, which closely resembles the acute traumatic and ischemic insult seen in SIRS patients (Wunder et al., 2002). It has been demonstrated that hindlimb I/R causes cellular injury in remote organs (e.g., liver, lung, and intestine) and contributes to the development of MODS (Wunder et al., 2002; Yassin et al., 2002).

Induction of SIRS is a complex response to the initiating factor(s) involving proinflammatory cytokines, prostaglandins, thromboxanes, reactive oxygen species, and nitric oxide.
(NO) by both organ-specific cells (e.g., pneumocytes, hepatocytes) and organ-nonspecific cells (e.g., vascular endothelial cells and circulating leukocytes) (Jean-Baptiste, 2007; Cepinskas and Wilson, 2008).

NO is a gaseous molecule produced from the amino acid L-arginine by nitric-oxide synthase (NOS), which consists of neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS) (Grisham et al., 1999). It has been reported that NO produced via constitutive NOS (i.e., NOS or eNOS) may be an important endogenous inhibitor of leukocyte-endothelial cell interactions, whereas high levels of NO associated with the induction of iNOS may be detrimental to targeted tissues/organisms (Levy et al., 2005). Bacterial endotoxins and SIRS-relevant inflammatory cytokines, in contrast, have been shown to induce iNOS expression in various organs/tissues, including intestine (Kleinert et al., 2003; Lozano et al., 2005). With respect to the latter, it has been shown that sustained up-regulation of NO production in the intestine leads to intestinal epithelial injury through the formation of peroxynitrite, a highly cytotoxic molecule capable of initiating lipid peroxidation and formation of nitrotyrosine (Potoka et al., 2002; Lush et al., 2003). Selective inhibition of iNOS offers protection against ischemia/reperfusion-induced injury to the gut and kidney (Naito et al., 2004; Mark et al., 2005), suggesting that iNOS-derived NO can potentially be implicated in the pathophysiology of SIRS.

Therefore, in the present study we assessed the role and potential mechanisms of iNOS-derived NO in modulation of hindlimb I/R-induced remote injury to the small intestine (a clinically relevant model of SIRS-induced remote organ injury) by using pharmacological (iNOS inhibitor 1400W; Garvey et al., 1997) and genetic (iNOS-deficient mice) experimental approaches. The results indicate that interfering with iNOS activity attenuates hindlimb I/R-induced remote injury in the small intestine.

**Materials and Methods**

**Experimental Protocol.** This study was approved by the Animal Research Ethics Board of the University of Western Ontario and met the guidelines of the Canadian Council on Animal Care. Male C57BL/6 mice (20–25 g) were randomized into the following three groups to determine the regional distribution of iNOS within the small intestine: sham (no treatment; n = 6); bilateral hindlimb ischemia (1 h) followed by 6 h of reperfusion (I/R; n = 6); and I/R-challenged group treated with a specific iNOS inhibitor, 1400W (Calbiochem, San Diego, CA; Garvey et al., 1997) (5 mg/kg, injected subcutaneously immediately before reperfusion; n = 6). In addition, C57BL/6 male iNOS-deficient (iNOS−/−) mice (The Jackson Laboratory, Bar Harbor, ME) were randomized into the following groups: sham (n = 6) or I/R (n = 6). All mice were initially anesthetized with 5% isoflurane (Abbott Laboratories, Mississauga, ON, Canada) and maintained at 2% in a 50%-50% oxygen/nitrogen mixture throughout the procedure. Ischemia was induced by applying tourniquets directly above the greater trochanter of each leg. Analgesia (0.1 mg/kg buprenorphine, Buprenex; Reckitt and Colman Pharmaceuticals, Richmond, VA) was administered subcutaneously immediately after the tourniquets (#4 silk; Johnson & Johnson, Markham, ON, Canada) were applied or after induction of anesthesia in sham mice. After a period of 60 min, the tourniquets were removed, and 1.0 ml of 0.9% NaCl was injected subcutaneously just before recovery for fluid resuscitation. Recovered mice had free access to food (standard laboratory diet) and water ad libitum. Six hours later, mice were used for various experimental endpoints.

**Intestinal Permeability.** Intestinal permeability was determined by measuring the blood-to-lumen clearance of Texas Red-conjugated dextran using a previously published assay method, with some modification (Nagahama et al., 2002; Balzan et al., 2007). In brief, 6 h after hindlimb ischemia, mice were reanesthetized by isoflurane inhalation, and the small intestine was cannulated at proximal duodenum and terminal ileum. To this end, the duodenum was first ligated 1 cm distal from the bile duct opening to omit the bile juice effect. Next, a polyethylene-50 tube (VWR, West Chester, PA) was inserted (approximately 1 cm) into duodenum immediately distal from the ligation site and tied. Subsequently, the terminal ileum was cut from the cecum, and the intestine was perfused through the cannulated duodenum with 2 ml of PBS at a rate of 0.3 ml/min using syringe pump (Harvard Apparatus Inc., Holliston, MA) to remove the luminal content. Subsequently, the intestine was cannulated at the terminal ileum using polyethylene-200 tubing (VWR). The above-mentioned procedure did not result in overdistending or overextending the cannulated intestine, nor did it cause any perforation or internal bleeding.

After cannulation of the small intestine, Texas Red-conjugated dextran (2 mg/ml) (molecular mass, 3 kDa; Invitrogen, Carlsbad, CA) was administered intravenously (tail vein) in a total volume of 0.1 ml of PBS, and the cannulated intestinal segment was perfused with 1.5 ml of PBS every 30 min for a total of 90 min. The collected luminal samples (perfusate; similar flush volumes between 1.3 and 1.5 ml were obtained from each sampling) were centrifuged (3000 rpm at 4°C) for 5 min, and supernatants (100 μl) were mixed with 3.9 ml of distilled H2O. The presence of Texas Red in the samples was determined spectrofluorometrically (RF-1501 spectrofluorometer; Shimazu, Kyoto, Japan) at the excitation/emission wavelengths of 595 nm/615 nm, respectively.

**Assessment of Intestinal Remote Inflammation/Injury Induced by Hindlimb I/R.** After reperfusion, mice were reanesthetized and immediately exsanguinated via cardiac puncture. The entire small intestine was removed, and the lumen was washed with PBS (1.5 ml). The samples were centrifuged and assessed for luminal contents of nitrate/nitrite (NO2−/NO3−; Griess reaction) and TNF-α (ELISA kit; BioLegend, San Diego, CA; the detection limit of the assay was 5 pg/ml). In parallel, the individual small intestine segments including duodenum (pyloric sphincter to ligament of Treitz, −4 cm), jejunum (−12 cm between duodenum and ileal segments), and ileum (−12 cm proximal to ileocecal junction) were dissected and homogenized in PBS buffer, pH 7.4, containing protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Subsequently, the samples were sonicated (5 s), centrifuged, and analyzed for tissue levels of TNF-α.

For histological evaluation, segments of the small intestine were fixed in 10% neutral-buffered formalin. Then, they were embedded in paraffin, cut into 7-μm sections, and stained with hematoxylin and eosin.

**Expression of iNOS, ICAM-1, and E-Selectin Proteins (Western Blot).** Frozen small intestine tissue samples were homogenized in radioimmunoprecipitation assay buffer (150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 1% sodium deoxycholate, and 50 mM Tris-HCl containing protease inhibitor cocktail [Sigma-Aldrich]) and subjected to 7% SDS-polyacrylamide gel electrophoresis (10 μg of protein) and Western blot (polyvinylidene difluoride membrane; GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) as described previously by us (Scott et al., 2005). The membranes were probed for detection of iNOS (rabbit anti-mouse iNOS antibody), ICAM-1 (hamster anti-mouse ICAM-1 antibody), and E-selectin (rat anti-mouse E-selectin antibody) (all antibodies were obtained from BD Biosciences, San Jose, CA) using enhanced chemiluminescence reagents (GE Healthcare). As a loading control, the levels of actin were detected by using rabbit anti-mouse actin antibody (Sigma-Aldrich). The band intensity was visualized on radiograph films (Eastman Kodak, Rochester, NY), quantified using Multi-Analyst software (Bio-Rad, Hercules, CA), and expressed as relative change in band density.
Preparations of Nuclear Extracts and Electrophoretic Mobility Shift Assay. Activation of NF-κB (nuclear appearance of p65 NF-xB protein) at the whole tissue level in the small intestine (jejunum) was assessed by EMSA as described previously by us (Lush et al., 2003). In brief, jejunum was homogenized in 4 volumes (w/v) of PBS containing the following protease inhibitors: 2 mM 4-(2-aminophenyl)benzenesulfonyl fluoride hydrochloride; 1 mM E-64; and 10 μg/ml each of pepstatin A, bestatin, leupeptin, and aprotinin. The homogenate was centrifuged at 3000g for 10 min, and the pellet was resuspended in buffer A (0.3 M sucrose, 5 mM dithiothreitol, 5 mM MgCl2, 10 mM Tris-Cl, and 0.1% Triton X-405) and further homogenized using a Dounce homogenizer. After filtration through a 100-μm nylon mesh, the obtained suspension was centrifuged at 1000g for 5 min at 4°C. The pellet (nuclei) was washed in buffer A without 0.1% Triton X-405 and centrifuged (1000g for 5 min at 4°C). Subsequently, the nuclei were extracted on ice for 30 min in a buffer containing 20 mM HEPES, 0.75 mM spermidine, 0.15 mM spermine, 0.2 mM EDTA, 2 mM dithiothreitol, 20% glycerol, and 1 mM phenylmethylsulfonyl fluoride (4°C) in the presence of 0.4 M NaCl. Finally, the samples were centrifuged for 10 min at 21,000g at 4°C, and the supernatants (nuclear extracts) were stored at −80°C until further analysis.

For EMSA, 5 μg of total nuclear protein was incubated with 1.0 pmol of double-stranded [γ-32P]ATP end-labeled oligonucleotides containing consensus binding sequences for NF-xB and electrophoresed on 4% polyacrylamide gel under nondenaturing conditions. Subsequently, the gels were dried and exposed to X-ray film (Eastman Kodak) for 2 to 4 h at −80°C. The specific bands were quantified by densitometry (GS-690 densitometer; Bio-Rad, Hercules, CA).

Intravital Microscopy and Video Analysis. C57BL/6 mice were randomly assigned to the following groups for intravital microscopy: sham (1-h anesthesia, followed by a 6-h recovery period; n = 6), I/R (6 h of reperfusion; n = 6), and I/R + 1400W (n = 6). Moreover, iNOS(-/-) mice were randomly assigned to the following groups: sham (n = 6) and I/R (n = 6). After 6 h of reperfusion, mice were reanesthetized, and the abdomen was opened via a longitudinal midline incision. Mice were then transferred to the microscope stage and placed in a left lateral position. Saline (0.9% NaCl)-soaked applicators were used to gently exteriorize a segment of the duodenum, the jejunum, or the ileum onto a mounted glass slide. Each segment was immediately covered with Saran wrap to avoid dehydration and exposure to ambient air and to minimize peristalsis. While on the microscope stage, animal temperature was maintained at 37°C with the use of overhead heating lamp, and the exposed segment of the intact small intestine was bathed in warm 37°C saline solution. After isolation of the small intestine, the preparation was allowed to equilibrate for 15 min. The outer layers of the small intestine were then identified, including serosa, muscularis, and submucosa, using an Eclipse TE200 inverted microscope (40× objective; Nikon, Tokyo, Japan). Submucosal postcapillary venules were visualized by identifying multiple underlying mucosal villi in cross section within the focal plane. Five randomly selected submucosal postcapillary venules per mouse (−40 μm in diameter) were identified, captured with a black-and-white camera (MTI VE1000; Dage-MTI, Michigan City, IN), projected onto a black-and-white monitor (WV-BM1410; Panasonic, Secaucus, NJ), and recorded with a video-cassette recorder (AG-1980; Panasonic).

Analysis of Leukocyte Rolling and Adherence. The flux of rolling and the number of adherent leukocytes were determined by offline video playback analysis as described previously (Scott et al., 2005). Vessel length and diameter were measured offline with ImageJ software. Leukocytes were quantified by defining their behavior along a 200-μm length of submucosal postcapillary venule (−40 μm in diameter) throughout a 30-s observation period. Leukocytes were classified as rolling if they were observed moving with a torsional motion along the endothelium across a central boundary line (expressed as cells per min), whereas those that remained stationary for 30 s within a given vessel were defined as adherent (expressed as cells per field; field is 200 × 40-μm vessel).

Immunohistochemistry. Paraffin-embedded sections were deparaffinized and treated with 0.3% H2O2 in methanol for 10 min to inhibit endogenous peroxidase activity. Subsequently, the samples were stained for iNOS protein by using rabbit anti-iNOS polyclonal antibody (BD Biosciences) at 1:100 dilution and Vectastain ABC (Vector Laboratories, Burlington, CA) peroxidase detection kit with the 3,3’-diaminobenzidine (Sigma-Aldrich) as a substrate for peroxidase.

Statistics. All results are presented as mean and S.E.M. The data were compared by one-way analysis of variance, and differences were considered to be significant if the p value was less than 0.05 based on Scheffé’s multiple comparison test. All analyses were performed using the StatView 5.0-J program (Abacus Concepts, Berkeley, CA).

Results

Permeability of the Small Intestine in Hindlimb I/R-Challenged Mice. Blood-to-lumen clearance of Texas Red-conjugated dextran (molecular mass, 3 kDa) was used as a marker of the small intestine dysfunction (permeability) in hindlimb I/R-challenged mice (Fig. 1). As shown in Fig. 1A,
induction of the hindlimb I/R resulted in an increase in Texas Red-dextran clearance in the small intestine of WT mice. It is important to note, however, that the above-mentioned response was a time-dependent phenomenon with the most profound increase in gut permeability seen at 30 min (but not 60 or 90 min) after Texas Red-dextran administration. Based on these findings, the experiments with respect to dysfunction (permeability) of the small intestine were carried out 30 min after Texas-Red dextran administration.

Further experiments addressing the mechanistic aspects of the above-mentioned phenomenon demonstrate that interfering with iNOS activity in WT mice by administration of iNOS inhibitor 1400W results in a significant reduction in Texas Red-dextran blood-to-lumen clearance. This result suggests that iNOS-derived NO plays a key role in hindlimb I/R-induced injury/dysfunction of the small intestine (Fig. 1B). The above-mentioned findings were supported by the experiments using a genetic approach. As shown in Fig. 1B, challenging iNOS-deficient mice with hindlimb I/R failed to induce an increase in Texas Red-dextran clearance in the small intestine compared with their wild-type counterparts.

Levels of NO$_2$/NO$_3$ and TNF-α in the Lumen of Small Intestine after Hindlimb I/R-Challenge. Dysfunction of the gut with respect to the increased blood-to-lumen clearance of Texas Red-dextran was accompanied by elevated levels of inflammatory markers in the lumen of the small intestine (Fig. 2). Challenging the WT mice with hindlimb I/R resulted in a significant increase in intraluminal levels of NO$_2$/NO$_3$ (Fig. 2A) and TNF-α (Fig. 2B). As in the previous experiments, interfering with iNOS-derived NO production by using pharmacological (1400W) or genetic (iNOS(−/−)) mice) approaches effectively reduced the intraluminal levels of NO$_2$/NO$_3$ (Fig. 2A) and TNF-α (Fig. 2B). Administration of 1400W to the sham-treated mice had no effect with respect to the above-mentioned parameters (data not shown).

Up-Regulation of the Mucosal Proinflammatory Phenotype (iNOS Protein, TNF-α Protein, and MPO Activity) in the Small Intestine of the Hindlimb I/R-Challenged Mice. Whether hindlimb I/R-induced dysfunction of the small intestine is associated with the alteration in the intestinal mucosal barrier function only, or is a consequence of a more complex inflammatory response remains to be tested. Thus, further experiments were designed to assess the up-regulation of the proinflammatory phenotype in the mucosa of the small intestine. To this end, the expression of iNOS (Western blot) and TNF-α (ELISA) proteins in the individual segments of the small intestine (i.e., duodenum, jejunum, and ileum) were assessed as the markers of inflammation. As shown in Fig. 3, challenging mice with hindlimb I/R resulted in a significant up-regulation of iNOS protein expression in each segment of the small intestine, with the most profound increase in iNOS expression found in jejunum and ileum. In parallel, similar responses with respect to the mucosal levels of the proinflammatory cytokine TNF-α were also found in the samples obtained from each segment assessed (Fig. 4). The latter changes were accompanied by increased polymorphonuclear leukocyte recruitment (an index of inflammation) to the mucosa of the small intestine as assessed by the mucosal levels of myeloperoxidase (MPO; data not shown). Interestingly, the most prominent up-regulation of the proinflammatory phenotype with respect to the above-mentioned parameters were found in jejunum and il-
were observed in hindlimb I/R-challenged mice (Fig. 5B). The latter changes were absent in hindlimb I/R-challenged animals treated with iNOS inhibitor 1400W (Fig. 5C). Administration of 1400W to the sham-treated animals did not produce any notable morphological changes in the small intestine (data not shown).

In parallel, expression of iNOS protein at the organ level was also assessed. Immunohistochemical analysis of iNOS protein (Fig. 6) indicate that induction of hindlimb I/R results in a marked expression of iNOS protein localized primarily to the epithelial cells lining the crypts and villi of jejunum, suggesting that intestinal epithelial cells may be the prime source of the gut mucosa-derived NO and contribute to the increased levels of NO\(_2\)/NO\(_3\) (Fig. 2A) in the lumen of the small intestine after hindlimb I/R-challenge.

**Leukocyte Recruitment to the Microvasculature of the Small Intestine after Hindlimb I/R-Challenge.** Leukocyte recruitment was directly assessed using intravital videomicroscopy. A significant increase in the flux of both rolling (Fig. 7A) and adherent (stationary) (Fig. 7B) leukocytes within submucosal postcapillary venules of jejunum and ileum were found in hindlimb I/R-challenged WT animals. However, no significant changes with respect to leukocyte rolling and adhesion were found in duodenum. It is important to note that interfering with iNOS activity (1400W) did not affect the flux of rolling leukocytes in the submucosal microvasculature of jejunum and ileum of hindlimb I/R-challenged WT mice (Fig. 7A). However, 1400W was very effective in reducing the number of adherent leukocytes under the same experimental condition (Fig. 7B). 1400W alone did not affect the numbers of rolling and adherent leukocytes in sham-treated mice (data not shown). The effects of pharmacological inhibition of iNOS (1400W) with respect to leukocyte recruitment were confirmed in the experiments using iNOS(−/−) mice. As shown in Fig. 7, A and B, no increase in the flux of rolling and adherent leukocytes was observed in the microvasculature of iNOS-deficient mice after hindlimb I/R. Interestingly, however, overall flux of rolling leukocytes under control (sham) condition was almost 2-fold higher in iNOS-deficient mice compared with their WT counterparts (Fig. 7A).

In parallel, mechanistic aspects of leukocyte recruitment

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**Fig. 4.** TNF-\(\alpha\) levels in mucosa of the small intestine after hindlimb I/R. TNF-\(\alpha\) (marker of inflammation) protein expression was assessed in the individual segments of the small intestine (duodenum, jejunum, and ileum) of WT and iNOS-deficient mice after hindlimb I/R. In some experiments, WT mice were treated with iNOS inhibitor 1400W (5 mg/kg) immediately after ischemia. Values are mean and S.E.M. \(n = 6, \# p < 0.05\) compared with sham-operated mice. *\(p < 0.05\) compared with hindlimb I/R-challenged mice.

**Fig. 5.** Histological appearance of the small intestine (jejunum) in hindlimb I/R-challenged mice. Histological samples obtained from jejunum of sham (A), hindlimb I/R (B), or hindlimb I/R + 1400W (C)-treated mice were stained with hematoxylin/eosin and analyzed for morphological appearance. Note that the jejunum of sham animals exhibited normal mucosal architecture (A), whereas formation of mild edema (arrowheads) and leukocyte infiltration (arrows) throughout the mucosa (without a notable erosion of the villi) was observed in hindlimb I/R-challenged mice (B). The latter changes were absent in hindlimb I/R-challenged animals treated with iNOS inhibitor 1400W (C). Shown are representative images from four experiments. Magnification, 40×.

**Fig. 6.** Immunohistochemical staining of iNOS protein in the small intestine (jejunum) of hindlimb I/R-challenged mice. Immunohistochemical analysis of iNOS protein in jejunum was assessed in WT mice after sham (A) and hindlimb I/R (B) challenges. Note a marked expression of iNOS protein localized primarily to the epithelial cells lining the crypts and villi of jejunum (arrows in B). Shown are representative images from three experiments. Magnification, 40×.
with regard to the expression of adhesion molecules was also investigated. To this end, expression of adhesion molecules E-selectin (molecule responsible for leukocyte rolling) and ICAM-1 (involved primarily in leukocyte adhesion) in the mucosa of the small intestine was assessed by using Western blot approach. As shown in Fig. 8, induction of hindlimb I/R resulted in up-regulation of both E-selectin and ICAM-1 protein expression in WT mice but not in iNOS(-/-) mice. Interfering with iNOS activity (1400W) in WT mice markedly reduced expression of ICAM-1 but not E-selectin. Shown is representative image from four experiments.

The levels of E-selectin (involved in leukocyte rolling) and ICAM-1 (involved in leukocyte adhesion) protein expression in WT mice but not in iNOS-deficient mice were obtained assessing the levels of E-selectin and ICAM-1 expression in the ileum but not duodenum (data not shown). The levels of E-selectin (involved in leukocyte rolling) and ICAM-1 (involved in leukocyte adhesion) protein expression in the absence or presence of iNOS inhibitor 1400W (or in iNOS-deficient mice), correlated with the number of leukocyte rolling/adhesion seen in the postcapillary venules of hindlimb I/R-challenged mice using intravital microscopy (compare Fig. 7, A and B, with the Fig. 8).

Finally, to address the mechanism(s) that can potentially be involved in regulation of TNF-α levels and proadhesive phenotype (i.e., ICAM-1 and E-selectin expression) by iNOS-derived NO, we assessed activation of the inflammationrelevant transcription factor NF-κB in the small intestine (jejunum) of the hindlimb I/R-challenged mice. It is known that the expression of many of the proinflammatory cytokines (e.g., TNF-α and interleukin-1β) and most of the adhesion molecules (e.g., E-selectin and ICAM-1) is tightly controlled by NF-κB. The results (EMSA assay; Fig. 9) indicate that hindlimb I/R results in activation of NF-κB in the small intestine.
intestine, an effect that can be significantly reduced by iNOS inhibitor 1400W or by iNOS-protein deficiency (iNOS\(-/\-\) mice). These findings indicate that iNOS-derived NO is involved in amplification of the inflammatory response in the gut at least in part through direct or indirect activation of transcription factor NF-\(\kappa\)B in the small intestine after hindlimb I/R.

**Discussion**

Previous studies indicate that inflammatory response and injury to remote organs (e.g., liver) can be caused by the systemic release of the proinflammatory mediators upon reperfusion of ischemic limbs (Lawlor et al., 1999; Yassin et al., 2002). Moreover, it has been demonstrated that SIRS leads to intestinal injury/dysfunction via the breakdown of gut barrier function (Hassoun et al., 2001; Fink, 2003). However, the mechanisms associated to the gut injury/dysfunction during systemic inflammation are largely unknown. The studies addressing the mechanistic aspect of intestinal epithelial hyperpermeability suggest that factors such as oxidative stress, NO, and cytokines (e.g., TNF-\(\alpha\)) can directly or indirectly contribute to the gut dysfunction (Lawlor et al., 1999; Fink, 2003; Han et al., 2004).

Our current findings in regard to the above-mentioned mechanisms indicate that induction of hindlimb I/R results in up-regulation of TNF-\(\alpha\) protein levels in both mucosa and lumen of the small intestine. The source of mucosal/luminal TNF-\(\alpha\) is not known at the moment; however, it could be associated to the hindlimb I/R-induced hyperpermeability of the intestinal microcirculation (i.e., TNF-\(\alpha\) from the circulation could enter mucosa of the small intestine). Alternatively, increase in TNF-\(\alpha\) levels could be a result of leukocyte recruitment or activation of the intestinal epithelial cells. Regardless of the source of TNF-\(\alpha\)-increased mucosal/luminal levels of TNF-\(\alpha\) can potentiate the remote intestinal inflammation/injury imposed by hindlimb I/R. In support of the above-mentioned information, it has been reported that up-regulation of iNOS protein (also found in this study) is a result of TNF-\(\alpha\)-mediated activation of the inflammation-relevant transcription factor NF-\(\kappa\)B (Leong and Karsan, 2000; Adams et al., 2002). It also has been demonstrated that interfering with iNOS ameliorates hemorrhagic shock- or sepsis-induced NF-\(\kappa\)B activation in the gut (Lush et al., 2003; Hierholzer et al., 2004).

NF-\(\kappa\)B, is a ubiquitous, rapidly acting transcription factor contributing to the amplification of inflammation through induction of vascular proadhesive phenotype (e.g., up-regulation of endothelial cell adhesion molecules ICAM-1 and E-selectin) and proinflammatory cytokine (e.g., TNF-\(\alpha\) and interleukin-1\(\beta\)) expression (Liu and Malik, 2006). NF-\(\kappa\)B is also known to be the key transcription factor contributing to the I/R-induced propagation of inflammatory response in the small intestine (Souza et al., 2005). Our current results are in line with the previous findings and indicate that hindlimb I/R results in activation of NF-\(\kappa\)B in the small intestine (jejunum), an effect that can be reduced by iNOS inhibitor 1400W or iNOS-protein deficiency (iNOS\(-/\-\) mice).

It is important to note that iNOS blockade has been shown to be beneficial in preventing I/R-induced gut injury/dysfunction (Suzuki et al., 2000; Naito et al., 2004). However, some deleterious effects of iNOS inhibition have also been reported. For example, side effects such as myocardial ischemia were caused by iNOS inhibition in an experimental model of sepsis (Avontuur et al., 1995). Moreover, elevation in tissue damage and organ dysfunction has been reported in septic animals after inhibition of iNOS (Lee et al., 2005).

Because there is a general agreement that NO derived from the constitutively expressed NO\(\gamma\)S (e.g., eNOS or nNOS) is “protective/anti-inflammatory,” the role of iNOS-derived NO seems to be rather controversial. How NO accomplishes its anti- or “proinflammatory” effects is a subject of intense investigation. However, it is obvious that even NO produced by the same NO\(\gamma\)S enzyme (i.e., iNOS) directly (e.g., through modification of mitochondrial respiratory chain enzyme(s) or interfering with antioxidant enzymes, e.g., glutathione or superoxide dismutase activities) or indirectly (e.g., through interaction with O\(_2^=\) and subsequent formation of highly reactive peroxynitrite) may possess different characteristics depending on the timing, location, and the rate of NO production (Grisham et al., 1999).

The role of NO in modulation of gut inflammation remains even more controversial because of the fact that the digestive tract has elevated basal levels of eNOS, nNOS, and even iNOS expression under physiological conditions (Kubes, 2000). The presence of iNOS in normal tissue of intestine, in contrast, should not be surprising, because the gut epithelium (particularly in the colon) is constantly exposed to the foreign antigens/noxious substances entering gastrointestinal tract either with the food or produced by the bacteria residing in the intestine as part of the normal intestinal milieu (Kubes, 2000).

The oppose effects of iNOS-derived NO in modulation of inflammation in the gut could also be attributed to the experimental model/conditions used. It has been demonstrated that iNOS offers “damaging” effects to the gut during systemic inflammation inflicted by endotoxin or burn (Chen et al., 2004; Han et al., 2004). Moreover, it seems that inhibition of iNOS within the first hour of endotoxia is detrimental to the gut, whereas inhibition of NO activity at 4 h results in protection of intestine against endotoxin-induced damage (Kubes, 2000). In parallel, remarkable proinflammatory effects of iNOS-derived NO have been demonstrated in various models of gut inflammation (e.g., trinitrobenzene sulfonic acid-induced colitis) (Kubes, 2000), whereas equally convincing data obtained from in vitro experiments involving human-derived mucosal microvascular endothelial cells isolated from IBD patients and monocyte-like cells indicate that iNOS offers anti-inflammatory and therefore protective effects (Binion et al., 2000). Finally, it has been shown that the cellular source of iNOS can be a key factor predisposing protective or damaging effects of NO (Poon et al., 2003).

Our data in regard to the above-mentioned information indicate that the most profound expression of iNOS protein occurs in the ileum and jejunum (but not duodenum) of the hindlimb I/R-challenged mice and that iNOS protein is primarily expressed in the mucosal epithelial cells rather than infiltrating leukocytes. It seems that remote injury/dysfunction to the gut inflicted by hindlimb I/R can be prevented interfering with iNOS activity, suggesting the proinflammatory nature of iNOS-derived NO under the experimental conditions used in this study.

The exact nature of the differential expression of iNOS in various segments of the small intestine found in this study is
not entirely clear. Recent evidence suggests, however, that during a severe condition (e.g., hemorrhagic shock), induction of iNOS in rat ileum (but not duodenum or jejunum) is a result of the suppressed expression of inducible heme oxygenase-1, an enzyme producing CO (Inoue et al., 2008). Whether the latter mechanism could account for differential expression of iNOS in the small intestine under the experimental conditions used remains to be determined.

Leukocyte recruitment is considered as an essential component of an immune response to infection or injury. However, an overwhelming accumulation of leukocytes contributes to the dysfunction of the affected organs during sepsis/SIRS (Brown et al., 2006). In general, during inflammation leukocytes tether and roll along the endothelium, followed by firm adhesion and subsequent emigration from the vasculature, which are mediated by selectins and adhesion molecules expressed on vascular endothelium (e.g., E-selectin, ICAM-1, and platelet/endothelial cell adhesion molecule-1) or the surface of leukocytes (e.g., β2 integrin lymphocyte function-associated antigen-1 and macrophage-1 antigen).

In the present study, hindlimb I/R resulted in up-regulation of E-selectin and ICAM-1 expression and subsequent leukocyte recruitment to the small intestine. Interestingly, interfering with iNOS activity (1400W) or protein (iNOS(−/−) mice) significantly reduced the expression of ICAM-1 and subsequent leukocyte adhesion but had no effect on E-selectin expression and leukocyte rolling, suggesting that iNOS-derived NO differentially regulates the expression of E-selectin and ICAM-1. The latter findings are in line with the recently published observation indicating that iNOS inhibitors or iNOS deficiency prevents sepsis-induced microvascular leakage but not leukocyte rolling in the microvasculature of cremaster muscle (Hollenberg et al., 2007). In regard to the above-mentioned findings, however, it is worthwhile to mention that in the present study the basal levels of E-selectin expression and flux of rolling leukocytes in the microcirculation of the gut submucosa were markedly higher in iNOS-deficient mice (Figs. 7 and 8). The exact nature of the above-mentioned phenomenon is not entirely clear; however, previous studies indicate that the basal levels of leukocyte rolling in the microvasculature of the mesentery are significantly higher in eNOS- and nNOS-deficient mice compared with the wild-type mice and seem to be associated to the higher levels of P-selectin expression under basal conditions (Lefer et al., 1999). Interestingly, iNOS deficiency seemed to have no effect on the basal levels of leukocyte rolling under the same experimental condition. Conversely, it has also been demonstrated that the basal levels of leukocyte rolling in the mesentery and cremaster muscle microcirculation of eNOS- and nNOS-deficient mice do not differ from their wild-type counterparts (Sanz et al., 2001). Moreover, some studies indicate that although the basal levels of leukocyte rolling in cremasteric and hepatic microcirculation of endotoxemic WT mice versus iNOS-deficient mice do not differ, the leukocytes obtained from endotoxemic iNOS-deficient mice roll more efficiently over an E-selectin-coated surface in vitro (Hickey et al., 1997). The latter findings suggest that leukocytes (predominantly polymorphonuclear leukocytes) deficient in iNOS are prone to be more “sticky” under septic (lipopolysaccharide stimulation) conditions. Whether increased leukocyte rolling in our experiments is associated with leukocyte activation and up-regulation of the proadhesive phenotype remains to be determined.

In summary, the present study indicates that circulating proinflammatory mediator(s) released by the skeletal muscle after bilateral hindlimb I/R lead to up-regulation of the proinflammatory phenotype in the small intestine with respect to activation of transcription factor NF-κB, increased production of TNF-α, and up-regulation of vascular proadhesive phenotype leading to the leukocyte recruitment and increased intestinal permeability, the phenomenon largely dependent on iNOS-derived NO.

References
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Address correspondence to: Dr. Gediminas Cepinskas, The Centre for Critical Illness Research, Lawson Health Research Institute, Victoria Research Laboratories, 6th Floor, Rm A6-136, 800 Commissioners Rd., East, London, ON N6A 4G5, Canada. E-mail: gediminas.cepinskas@lhsc.on.ca