Induced nitric oxide promotes intestinal inflammation following hemorrhagic shock

Christian Hierholzer,1 Jörg C. Kalff,1 Timothy R. Billiar,1 Anthony J. Bauer,2 David J. Tewardy,3 and Brian G. Harbrecht1

1Departments of Surgery and Medicine and 2Division of Gastroenterology, University of Pittsburgh, Pittsburgh, Pennsylvania 15213; and 3Department of Medicine, Baylor College of Medicine, Houston, Texas 77030

Submitted 18 October 2002; accepted in final form 15 August 2003

Hierholzer, Christian, Jörg C. Kalff, Timothy R. Billiar, Anthony J. Bauer, David J. Tewardy, and Brian G. Harbrecht. Induced nitric oxide promotes intestinal inflammation following hemorrhagic shock. Am J Physiol Gastrointest Liver Physiol 286: G225–G233, 2004; 10.1152/ajpgi.00447.2002.—In hemorrhagic shock (HS), increased cytokine production contributes to tissue inflammation and injury through the recruitment of neutrophils [polymorphonuclear cells (PMN)]. HS stimulates the early expression of inducible nitric oxide synthase (iNOS) that modulates proinflammatory activation after hemorrhage. Experiments were performed to determine the contribution of iNOS to gut inflammation and dysmotility after HS. Rats subjected to HS (mean arterial pressure 40 mmHg for 2.5 h followed by resuscitation and death at 4 h) demonstrated histological signs of mucosal injury, impairment of intestinal smooth muscle contractility, extravasation of PMN, and increased gut mRNA levels of ICAM-1, IL-6, and granulocyte colony-stimulating factor (G-CSF). In addition, DNA binding activity of NF-κB and Stat3, an IL-6 signaling intermediate, was significantly increased. In shocked rats treated with the selective iNOS inhibitor L-NAME-(1-iminoethyl)-lysine at the time of resuscitation, histological signs of intestinal injury and PMN infiltration were reduced and muscle contractility was almost completely restored. Selective iNOS inhibition in shocked animals reduced the binding activity of NF-κB and Stat3 and reduced mRNA levels of ICAM-1, IL-6, and G-CSF. The results of studies using iNOS knockout mice subjected to HS were similar. We propose that early upregulation of iNOS contributes to the inflammatory response in the gut wall and participates in the activation of signaling cascades and cytokine expression that regulate intestinal injury, PMN recruitment, and impaired gut motility.

nuclear factor-κB: cytokines; intestinal function; ileus

HEMORRHAGIC SHOCK IS COMMONLY viewed as a whole body ischemia-reperfusion (I/R) insult. The intestine is highly sensitive to I/R injury and experiences a marked reduction in blood flow during circulatory shock due to a disproportionate constriction of the splanchnic circulation (18). We have shown (11) that isolated I/R injury of the gut is associated with decreased gut motility and can result in postinjury atony and functional ileus. The intestine plays a critical role in the systemic inflammatory response due to its ability to secrete proinflammatory cytokines and release mediators into the systemic circulation following the breakdown of the mucosal barrier (25, 28, 32). These events may accentuate tissue damage in other organ systems and may contribute to organ failure and mortality (6). Despite the commonly held perception that hemorrhagic shock and I/R injury are equivalent, there are substantial differences in the body’s response to shock induced by loss of circulating volume compared with localized ischemia that suggest that hemorrhagic shock induces a unique physiological response (11).

Hemorrhagic shock stimulates the expression of the inducible nitric oxide (NO) synthase (iNOS), which is responsible for the sustained production of NO (10, 17). Recent studies (7, 15) provide evidence that iNOS plays a critical role in regulating smooth muscle contractility of the intestine. NO regulates intestinal smooth muscle function through iNOS-dependent upregulation of adhesion molecules and iNOS-dependent infiltration of polymorphonuclear cells (PMN) into the jejunal muscularis that subsequently affects intestinal smooth muscle function. We have previously shown (10, 20) that NO regulates intestinal smooth muscle function through iNOS-dependent upregulation of adhesion molecules and NO-dependent infiltration of polymorphonuclear cells into the jejunal muscularis, which subsequently affects intestinal smooth muscle function.

We have previously shown (10, 20) that NO regulates the magnitude of the inflammatory response after hemorrhage and participates in proinflammatory cell signaling. In addition to regulating the inflammatory response, NO produced by iNOS may contribute to organ damage through both direct cytotoxic effects and the interaction with reactive oxygen intermediates (ROIs) under conditions of redox stress, although the mechanisms responsible for tissue injury may be organ specific (29, 30). NO has also been shown to directly impair intestinal barrier function at the cellular level (34). There are therefore several potential mechanisms through which NO may modulate intestinal function after hemorrhage.

We have previously demonstrated that NO from iNOS regulates proinflammatory activation, gene expression, and tissue injury in the liver after hemorrhage (10, 22). For this study, we hypothesized that excess NO derived from iNOS expression was, in part, responsible for the increased inflammatory response, impaired smooth muscle function, and structural damage of the intestine produced by hemorrhage. We report here that in hemorrhagic shock, iNOS inhibition with L-NAME-(1-iminoethyl)-lysine (L-NIL) (23) or genetic deletion of the iNOS gene reduces the intestinal inflammatory response measured by PMN infiltration, cytokine production, and activation of proinflammatory transcription factors NF-κB and Stat3. Downregulation of inflammation is accompanied by improved intestinal smooth muscle function and reduced intestinal damage.

MATERIALS AND METHODS

Hemorrhagic shock protocol and organ isolation and preparation. This study was approved by the University of Pittsburgh Animal Care and Use Committee and conformed to National Institutes of Health

Address for reprint requests and other correspondence: B. G. Harbrecht, Univ. of Pittsburgh Medical Center, F1264-200 Lothrop St., Pittsburgh, PA 15213 (E-mail: harbrechtbg@msx.upmc.edu).

http://www.ajpgi.org 0193-1857/04 $5.00 Copyright © 2004 the American Physiological Society
guidelines for the care and use of laboratory animals. Fasted male Sprague-Dawley rats (Charles River Breeding Laboratory, Cambridge, MA) were used (mean weight 274 ± 3.7 g). Animals were randomly subjected either to the shock or sham protocol. In brief, after initial anesthesia with methoxyflurane, the animals were orally intubated and the right carotid artery and left jugular vein were cannulated. Arterial blood pressure was monitored continuously, and animals were ventilated by administration of 2.5-ml tidal volume of room air at 72 strokes/min. After vascular cannulation, the animals received intravenous anesthesia (pentobarbital sodium, 50 mg/kg) as needed.

The hemorrhage protocol has been previously described (13). After an initial bleed of 2.25 ml/100 g body wt over 10 min, blood was withdrawn or returned as needed to maintain a mean arterial blood pressure (MAP) of 40 mmHg. At the point in time at which 35% of the shed blood had been returned (total shed blood volume 7.8 ± 0.5 ml), the animals were resuscitated to a MAP of 80 mmHg by administration of the remaining shed blood plus two times the shed blood volume in lactated Ringer solution. Total shock time averaged 157 ± 2.3 min. Animals were killed 4 h after the initiation of resuscitation. Control animals underwent cannulation and anesthesia for an identical period of time as shock animals but were not bled. One group of animals (n = 5) received l-NIL (Alexis, Laufelfingen, Switzerland) at 50 μg·kg−1·h−1, whereas the control group (both sham and shock animals) received saline infusion. l-NIL was dissolved in 1 ml of sterile saline and was infused at the initiation of resuscitation for a period of 1 h. For experiments in mice, pair-mated iNOS knockout and wild-type mice were anesthetized with methoxyflurane. The femoral artery and vein were cannulated, and the mice were hemorrhaged to maintain a MAP of 30 mmHg. After 3 h of shock, the mice were resuscitated and monitored for 4 h, at which time they were killed (10).

After death, the carcasses were flushed with cold (4°C) isotonic saline solution. The entire small bowel was then removed and placed in cold preoxygenated Krebs-Ringer buffer (KRB). A standard KRB (Sigma, St. Louis, MO) was used (in mM): 137.4 Na+, 5.9 K+, 2.5 Ca2+, 1.2 Mg2+, 134 Cl−, 15.5 HCO3−, 1.2 H2PO4−, and 11.5 glucose. This physiological solution was gassed with 97% O2-3% CO2 to establish a pH of 7.4. Midjejunal segments were cut from the bowel and immersed in KRB in a Sylgard-fi fluff chamber. The chamber was filled with gas-permeable fi fluff (Sigma), 10 ml KRB, and 100 μM H2O2. All histochemically stained whole mounts were covered with a coverslip and counted. 10 min. The reaction was stopped with cold KRB. All histochemically stained whole mounts were immersed in a mixture of 10 mg Hanker-Yates reagent (Sigma), 10 ml KRB, and 100 μM H2O2. All histochemically stained whole mounts were covered with a coverslip and counted. Leukocytes were counted in stained whole mounts were covered with a coverslip and counted.

EMSA. EMSA was performed using whole tissue extracts from the experimental groups as described (10). Binding reactions were performed using 20 μg extracted protein and radiolabeled DNA-binding elements. The activation of NF-κB was determined using the duplex oligonucleotide based on the NF-κB binding site upstream of the murine iNOS promoter (5). Activation of Stat3 was assessed using the high-affinity serum-inducible element (hSIE) duplex oligonucleotide that preferentially binds Stat3 and Stat1 (33). EMSA was performed on a 4% polyacrylamide gel as described (4). Supershift analysis and cold competition assay were performed to confirm the identity of the protein-DNA complex and to demonstrate specific NF-κB binding. Antibodies specific for NF-κB p50, NF-κB p65, and Stat3β were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and were generated against amino acids 313–363 of human NF-κB p50 and amino acids 531–550 of human NF-κB p65 or in rabbits against the COOH-terminal amino acids of murine Stat3. The Stat3β-specific antibody was obtained at Charles River Pharmaservices (South-
bridge, MA) by immunizing chickens with the COOH-terminal 10-amino acid residues of human Stat3β conjugated to thyroglobulin. The level of transcription factor activation was quantitated using Phospholimager analysis of gel shift band intensities.

Statistics. Unless otherwise indicated, data are presented as means ± SE. Comparisons of means were performed using ANOVA followed by comparison of individual pairs of means using Scheffé’s test. Both tests were contained within the StatView 4.1 program (Abacus Concepts, Berkeley, CA).

RESULTS

Hemorrhagic shock results in increased expression of iNOS in the jejunum. In previous studies (17), we have shown that iNOS (or NOS2) is upregulated in both the lungs and liver during shock. In this study, we measured iNOS mRNA levels in the jejunum of rats following resuscitation from hemorrhagic shock to determine whether iNOS was also generated at this site. With the use of semiquantitative RT-PCR, we found that levels of iNOS mRNA were increased 8.7-fold (P < 0.03) over sham controls 4 h following resuscitation from hemorrhage (Fig. 1). Treatment with the selective enzyme inhibitor L-NIL had no effect on iNOS mRNA levels in shock or sham animals.

Selective iNOS inhibition attenuates shock-induced morphologic injury of the intestine. We have shown that iNOS inhibition reduced lung injury and pulmonary PMN infiltration in shocked rats (10). Furthermore, in a rat model of I/R injury, the infiltration of PMN into the muscularis of jejunum was a critical mediator of intestinal smooth muscle dysfunction (11). We hypothesized that induced NO would contribute to jejunal damage and infiltration of PMN into the muscularis. Intestinal injury and muscularis PMN infiltration were assessed by histology using staining with hematoxylin and eosin and for MPO. Cross-sections of midjejunum from shocked animals demonstrated a broadening of the intestinal villi, edema, and infiltration of inflammatory cells (Fig. 2). Isolates of the muscularis demonstrated an 8.3-fold increase in MPO-positive PMN in shock animals compared with sham control animals. Jejunal cross sections of NIL-treated shock animals demonstrated a restoration of mucosal architecture and inhibition of PMN infiltration in the muscularis (Fig. 2).

Selective iNOS inhibition attenuates shock-induced muscular dysfunction of the intestine. Smooth muscle contractility of the intestine during sepsis or after surgical manipulation is regulated, in part, by iNOS activity (7, 15). To test the hypothesis that induced NO contributes to the impairment of smooth muscle function following hemorrhagic shock, we generated dose-response curves of intestinal smooth muscle contractility in vitro using control (KRB) and bethanechol-stimulated (0.1–300 μM) preparations. Circular muscular strips from the midjejunum of rats subjected to shock demonstrated a 66% reduction in contraction amplitude in response to bethanechol (Fig. 3) 4 h after hemorrhage compared with control specimens (P = 0.01). With the use of the iNOS inhibitor L-NIL, the contractile response of the circular muscle following shock was almost completely restored (P = 0.03).

iNOS regulates expression of ICAM-1. PMN recruitment and diapedesis from the vasculature into the tissues requires adhesion of PMN to endothelial cells, and ICAM-1 regulates the PMN influx into the muscularis of the jejunum (14). To determine whether iNOS was involved in the regulation of intestinal ICAM-1 expression following shock, we measured ICAM-1 mRNA levels in shock rats treated without and with iNOS inhibition. With the use of semiquantitative RT-PCR, ICAM-1 mRNA levels from the jejunum of rats subjected to hemorrhagic shock (HS) and killed 4 h following resuscitation (A and B). RT-PCR reactions were performed using total RNA (2.5 μg) from the jejunum of shock and sham animals receiving L-NIL (5, gray bars) or saline (n = 5, black bars). Reaction products were separated on polyacrylamide gels, dried, and exposed to Phosphorimager (A). In B, the radioactive signal in the region corresponding to the predicted amplified fragment of rat iNOS mRNA was quantitated with correction to β-actin (C) using laser densitometry and ImageQuant software and plotted. Values shown represent means ± SE. #P < 0.03 compared with sham.
iNOS modulates the activation of transcription factors important for cytokine expression. Activation of transcription factors NF-κB and STAT contributes to the expression of cytokines such as TNF, IL-6, and G-CSF. There is iNOS-dependent activation of NF-κB and Stat3 DNA binding in the lungs following hemorrhage (10). To evaluate whether NF-κB activation in the jejunum of shock rats was iNOS dependent, we measured NF-κB activation after hemorrhage without and with iNOS inhibition. NF-κB activation was determined by EMSA using the duplex oligonucleotide based on a consensus NF-κB binding sequence. The jejunum of shock animals demonstrated a 3.4-fold increase in NF-κB activation at 4 h following resuscitation compared with sham controls (Fig. 7). Binding activity was completely inhibited by an excess of unlabeled oligonucleotide, and supershift analyses demonstrated the binding complex to consist of p50 and p65 subunits (data not shown). After L-NIL treatment, levels of NF-κB activation decreased 57% in shocked animals.

IL-6 and G-CSF both signal through the activation of STATs, proteins that serve the dual function of signal transducers and activators of transcription. IL-6 and G-CSF predominantly activate Stat3, one of seven STAT proteins identified and cloned so far (1, 3). To determine whether IL-6 and G-CSF expression were regulated by NO-mediated changes in Stat3 activation, EMSA was performed using protein extracts from the jejunum of shock and sham animals with and without L-NIL treatment and the hSIE, a duplex oligonucleotide probe known to form a complex with Stat3 and Stat1. Jejunal extracts from sham shock animals demonstrated low levels of activated SIF-A (Stat3 homodimer) complex. SIF-B (Stat3/Stat1 het-

Fig. 3. Smooth muscle contractility of the jejunum is almost completely restored following selective NOS inhibition in shock. The contractility of the muscularis (g·mm⁻²·section L-NIL) was measured in response to the cholinergic agonist bethanechol in doses of 0.1–0.300 μmol. Smooth muscle contractility of animals subjected to shock (●) was decreased by 66% compared with sham control animals (▲, P = 0.01) and was almost completely restored following treatment with L-NIL (●, shock + L-NIL).
erodimer) and SIF-C (Stat1 homodimer) complexes also were detected. Jejunal extracts from shock animals demonstrated a marked increase in all complexes, especially SIF-A. Binding of protein to the labeled hSIE binding element was completely inhibited by 25-fold excess of unlabeled hSIE oligonucleotide but not by up to 100-fold excess of unlabeled nonspecific oligonucleotide (data not shown). Incubation with the Stat3\textsuperscript{H9251} or Stat3\textsuperscript{H9252}-specific antibody resulted in supershift, but incubation with both antibodies did not completely supershift the SIF-A complex with the residual, demonstrating mobility similar to Stat3\textsuperscript{H9253}. DNA-affinity puriﬁcation of whole tissue extracts of shock gut followed by immunoblotting with Stat3-speciﬁc monoclonal antibody that recognizes all Stat3 isoforms conﬁrmed the activation of all Stat3 isoforms including Stat3\textsuperscript{H9253} (data not shown). Quantitation of the SIF-A complex (Stat3 homodimer; Fig. 8) revealed a 3.4-fold increase 4 h following resuscitation in shock animals compared with sham controls. Treatment with the iNOS inhibitor L-NIL resulted in a 46% reduction of SIF-A binding activity.

Studies using iNOS knockout mice. To conﬁrm that the results observed following L-NIL treatment were the consequence of selective inhibition of the inducible form of NOS, studies using iNOS-deﬁcient mice were employed. We measured the activation of NF-κB and Stat3 in the jejunal of iNOS-deﬁcient and wild-type mice 4 h following hemorrhagic shock. Analogous to the results detected in rats, hemorrhagic shock followed by resuscitation in wild-type control mice resulted in increased binding activity, 3.8-fold for NF-κB ($P < 0.01$) and 3.6-fold for Stat3 ($P = 0.02$), in the jejunal of sham shock mice (Fig. 9). The results of competition assays with unlabeled oligonucleotides and supershift analysis were similar.
to those seen in the rat model described above (data not shown). In mice lacking the iNOS gene, binding activity of NF-κB in the jejunum following hemorrhagic shock was reduced by 61% (P < 0.02) and 56% (P < 0.03), respectively, compared with wild-type animals.

**DISCUSSION**

The intestine is highly sensitive to I/R injury and experiences a marked reduction in blood flow during circulatory shock due to a disproportionate constriction of the splanchnic circulation. Intestinal injury and loss of gut barrier function have been implicated in systemic inflammation and may promote distant organ failure (6). The intestinal injury following shock is associated with an inflammatory response within the gut wall as well as impairment of smooth muscle contractility. Recent studies provide evidence that iNOS is involved in the regulation of intestinal smooth muscle function. With the use of rat models of smooth muscle dysfunction induced by sepsis (7) or surgical manipulation (15, 16), iNOS inhibition or iNOS deficiency resulted in a marked attenuation of the contractile response. We therefore investigated whether iNOS played a critical role in the impairment of smooth muscle function after hemorrhage. In this study, with the use of the selective iNOS inhibitor L-NIL and iNOS knockout mice, we demonstrate that induced NO plays a critical role in regulating the inflammatory response as well as morphological and functional damage in the jejunum after hemorrhagic shock. NO from iNOS decreased intestinal smooth muscle contractility and regulated the infiltration of PMN into the muscularis. We found that iNOS inhibition after hemorrhage restored intestinal contractility and reduced PMN influx into the muscularis. These results indicate that iNOS is involved in regulation of intestinal smooth muscle function after hemorrhagic shock. Inhibition of iNOS activity in our study not only resulted in amelioration of gut dysfunction but also dramatically reduced shock-induced intestinal injury.

We demonstrated that iNOS regulates the increased expression of the adhesion molecule ICAM-1 that regulates PMN influx into the intestine. Furthermore, the iNOS-dependent increase in the expression of the proinflammatory cytokines IL-6 and G-CSF after hemorrhage was associated with an iNOS-dependent increase in DNA binding activity of NF-κB.
and Stat3 in the jejunum. These data indicate not only an association between NO-dependent inflammation and organ injury but also suggest an important role for NO in cell signaling in the intestine after hemorrhage. These results demonstrate that the induction of iNOS in hemorrhagic shock is a key event that initiates the inflammatory response and results in organ injury in the intestine as well as in the lung and liver (10).

The activation of Stat3 is a critical step in the signaling cascade of several cytokines including IL-6 and G-CSF. G-CSF and IL-6 bind to their respective receptors and activate protein tyrosine kinases resulting in phosphorylation of the receptor complex. The receptor complex then phosphorylates STAT proteins causing their dimerization and translocation into the nucleus where they bind to specific DNA binding sequences in the promoter regions of genes and facilitate their transcription. STAT protein activation has been implicated in the association between NO-dependent inflammation and smooth muscle dysfunction in a rat model of intestinal ischemia (11). In this study, we demonstrate that the increased expression of ICAM-1 after hemorrhagic shock that regulates the PMN influx is iNOS dependent. The intestinal macrophage cell population has been shown to be a significant source of iNOS in the gut, and leukocytes infiltrating into the intestine as a result of iNOS upregulation may cause damage through the release of granular and lysosomal contents (15). Our data demonstrate that reduced PMN infiltration into the intestine is associated with decreased histological evidence of intestinal injury after hemorrhage. However, it remains unclear whether NO is capable of directly damaging the muscularis layer. Under conditions of redox stress, induced NO may also mediate organ damage through interaction with reactive radicals present during reperfusion, and this mechanism may also occur in the intestine after hemorrhage (29).

The effects of NO on intestinal function and injury range from beneficial to detrimental and may be dose dependent (34). Because intestinal blood flow is diverted to essential organs such as the brain and heart during shock, NO may play a beneficial role by facilitating oxygenation through vasodilation. Studies have shown that at lower concentrations, NO may protect the intestinal mucosa by maintaining perfusion and inhibiting adhesion of PMN and platelets (19). In support of this mechanism, nonselective blockade of NOS has been shown to increase I/R-induced intestinal injury, whereas L-arginine administration and NO donors improved I/R-induced injury (15, 16). However, at high concentrations, NO promotes intestinal injury and loss of gut barrier function (34). Recent studies (34) demonstrate that increased iNOS activity is di-
rectly involved in endotoxin-induced gut injury by reducing cellular viability, causing villous injury, and contributing to loss of intestinal barrier function by increasing intestinal permeability and promoting bacterial translocation. Increased permeability of cell monolayers produced by excessive NO was accompanied by ATP depletion and decreased tight junction integrity (26). In accordance with these data, in our study, selective iNOS inhibition was protective to the intestine and restored muscle contractility. Whether mucosal barrier function was altered in this model remains unresolved and will require further study.

An important contributor to tissue damage following gut ischemia is the generation of ROIs. Inducible NO contributes to intestinal injury not only through its direct effect on intestinal ischemia is the generation of ROIs. Inducible NO contributes to intestinal injury not only through its direct effect on intestinal ischemia but also through oxidative stress, which can lead to cellular damage. Studies using peroxynitrite scavengers demonstrated reduced NO-mediated permeability of intestinal monolayers, suggesting that the NO-induced increase in mucosal permeability may be mediated by peroxynitrite (31). ROIs can be produced by resident macrophages, infiltrating neutrophils, and monocytes. A decrease in peroxynitrite produced by iNOS inhibition may also contribute to the reduction of intestinal injury seen in these experiments.

The stimuli resulting in increased iNOS expression after hemorrhagic shock have not yet been defined. We have demonstrated that hemorrhagic shock results in activation of the transcriptional factor NF-κB and that some of the increased NF-κB binding after shock is iNOS independent (Fig. 7). Increased binding activity of NF-κB is important in the expression of iNOS. iNOS may potentially be induced after hemorrhage by the activation of NF-κB through redox-sensitive mechanisms or potentially through hypoxia-sensitive transcription factors that are NO independent (9). The resulting increased production of NO from iNOS may then produce intestinal injury and dysfunction directly or through the recruitment of leukocytes into the intestine. In conclusion, this study demonstrates that the induction of iNOS contributes to the initiation of a complex inflammatory response in the gut wall, including activation of signaling proteins and cytokine expression, ultimately leading to morphological and functional intestinal injury. These results suggest that reducing bioavailability of induced NO in hemorrhagic shock by selective iNOS inhibition may be beneficial to the gut.

**REFERENCES**


