Selective inhibition of iNOS attenuates trauma-hemorrhage/resuscitation-induced hepatic injury

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Although trauma-hemorrhage produces tissue hypoxia, systemic inflammatory response and organ dysfunction, the mechanisms responsible for these alterations are not clear. Using a potent selective inducible nitric oxide (NO) synthase inhibitor, N-[3-(aminomethyl)benzyl]acetamidine (1400W), and a nonselective NO synthase inhibitor, N(G)-nitro-L-arginine methyl ester (L-NAME), we investigated whether inducible NO synthase plays any role in producing hepatic injury, inflammation, and changes of protein expression following trauma-hemorrhage. To investigate this, male Sprague-Dawley rats were subjected to midline laparotomy and hemorrhagic shock (mean blood pressure 35–40 mmHg for ∼90 min) followed by fluid resuscitation. Animals were treated with either vehicle (DMSO) or 1400W (10 mg/kg body wt ip), or L-NAME (30 mg/kg iv), 30 min before resuscitation and killed 2 h after resuscitation. Trauma-hemorrhage/resuscitation induced a marked hypotension and increase in markers of hepatic injury (i.e., plasma α-glutathione S-transferase, tissue myeloperoxidase activity, and nitrotyrosine formation). Hepatic expression of iNOS, hypoxia-inducible factor-1α, ICAM-1, IL-6, TNF-α, and neutrophil chemoattractant (cytokine-induced neutrophil chemoattractant-1 and macrophage inflammatory protein-2) protein levels were also markedly increased following trauma-hemorrhage/resuscitation. Administration of the iNOS inhibitor 1400W significantly attenuated hypotension and expression of these mediators of hepatic injury induced by trauma-hemorrhage/resuscitation. However, administration of L-NAME could not attenuate hepatic dysfunction and tissue injury mediated by trauma-hemorrhage, although it improved mean blood pressure as did 1400W. These results indicate that increased expression of iNOS following trauma-hemorrhage plays an important role in the induction of hepatic damage under such conditions.

hypoxia-inducible factor-1α; myeloperoxidase activity; cytokine; chemokine

TRAUMA-HEMORRHAGE INITIATES severe hypotension (12), hypoxia, and inflammatory response, characterized by the release of proinflammatory mediators as well as activation and sequestration of neutrophils (18). In addition, free reactive radicals are produced following trauma-hemorrhage, including nitric oxide (NO) generated by NO synthase (NOS) (1). At least two isoforms of NOS are found in the liver: endothelial NOS (eNOS) and inducible NOS (iNOS). NO generated by eNOS plays a vital physiological role in maintaining appropriate microvascular tone and blood flow and exhibits protective effects (30). Studies have shown that nonspecific NOS inhibition or genetic deletion of eNOS results in reduced microvascular perfusion and aggravated liver injury (10, 17). Nonetheless, it remains unclear whether NOS plays a major role following trauma-hemorrhage and whether it contributes to the prevention or induction of trauma-hemorrhage-induced inflammatory response and subsequent hepatic dysfunction (6). Previous studies have demonstrated that iNOS expression is upregulated during hemorrhagic shock and that the resulting increased production of NO produces a range of deleterious responses, including the release of the inflammatory cytokines/chemokines, the formation of peroxynitrite (ONOO−/nitrotyrosine, alterations in the expression or function of proteins, and end-organ damage (16). Recent studies have shown that hypoxia-inducible factor-1α (HIF-1α) plays an important role in hypoxia-induced inflammation and inflammatory diseases. Moreover, a number of inflammatory mediators can activate HIF-1 even under normoxic conditions (15). It is well known that HIF-1α can mediate iNOS expression under hypoxia conditions. On the other hand, some studies revealed that NO is a key factor sustaining HIF-1α activation during hypoxia and inflammation. Several reports showed that NO could both promote and inhibit the activity of HIF-1 (14, 26, 29, 40, 42); however, the in vivo response of HIF-1α to iNOS inhibition has not been examined following trauma-hemorrhage and resuscitation.

Initial results have varied from protective to detrimental with NOS inhibitors following injury (22). This variability in the results may be due to differences in the parameters measured, the experimental models employed, and, most important, the selectivity of NOS inhibitors used (19, 32). Studies have shown that N(G)-nitro-L-arginine methyl ester (L-NAME), a nonspecific NOS inhibitor, increases hepatic and intestinal injury following ischemia-reperfusion (I/R) (24, 31). A clinical study was recently discontinued because treatment with N(G)-monomethyl-L-arginine showed higher mortality (25). Thus interest has focused on selective inhibition of iNOS, while preserving eNOS activity. With regard to specific inhibitors, 3-[aminomethyl]benzylacetamidine [(1400W)] is at least 5,000-fold more selective for iNOS than eNOS, and its potency and selectivity for iNOS inhibition were far greater than any previously used NOS inhibitors (13). Although potent selective iNOS inhibitors 1400W have been described (12), so far no reports used this type of drug treatment after trauma-hemorrhage to determine...
the effect of a highly selective inhibition of iNOS on organ injury. The aim of this study was to examine the role of iNOS and HIF-1α in the development of hepatic damage following trauma-hemorrhage and resuscitation.

METHODS

Trauma-hemorrhagic model. A nonheparinized model of trauma-hemorrhage and resuscitation was used as described (18). Briefly, adult male (275–325 g) Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were fasted overnight and anesthetized with isoflurane (Abbott, Minrad, Bethlehem, PA) inhalation before soft tissue trauma was induced via a 5-cm midline laparotomy. The abdominal incision was closed, and polyethylene catheters (PE-50, Becton Dickinson, Sparks, MD) were placed in both femoral arteries and the right femoral vein. The wounds were bathed with 1% lidocaine (Elkins-Sinn, Cherry Hill, NJ) to minimize postoperative pain. The rats were then placed into a Plexiglas box (21 × 9 × 5 cm) in a prone position and allowed to awaken, after which they were rapidly bled to a mean blood pressure (MBP) of 35–40 mmHg within 10 min. Hypotension was maintained until the animals could no longer maintain MBP of 35–40 mmHg unless additional fluid was added. The rats were then resuscitated with 40% of the MBO volume was returned in the form of RL (400 ml/kg). The rats were maintained at MBP of 35–40 mmHg until the animals could no longer maintain MBP of 35–40 mmHg unless additional fluid in the form of Ringer lactate (RL) was administered. This time was defined as maximum bleed out (MBO), and the amount of withdrawn blood was recorded. The rats were maintained at MBP of 35–40 mmHg until 40% of the MBO volume was returned in the form of RL (~90 min from the onset of bleeding). The animals were then resuscitated with four times the volume of the shed blood over 60 min in the form of RL. Sham-operated animals underwent the same soft tissue trauma, which included the ligation of the right femoral artery and vein, but neither hemorrhage nor resuscitation was performed.

iNOS inhibitor 1400W (10 mg/ml) was dissolved in 1 ml 10% DMSO and injected in the trauma-hemorrhage/resuscitation group (10 mg/kg body wt, Sigma). This dose was selected on the basis of previously published studies (13, 41). l-NAME (NOS inhibitor, 30 mg/kg iv, Sigma) or normal saline) was administered 30 min before resuscitation, which was after MBO time. Thus a 300-g rat received a volume of 0.33 ml (ip). Similarly, sham rats also received the same volume of 1400W, l-NAME, or vehicle. The animals were killed 2 h after the end of resuscitation or sham operation, and blood and tissues were collected for subsequent analysis.

All experiments were performed in adherence with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (NIH publication 85-23, 1996) and approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

Assessment of hepatic injury. α-Glutathione S-transferase (α-GST) is a highly sensitive and specific biomarker of hepatic injury (4, 35). At 2 h after resuscitation, plasma samples were obtained. Plasma α-GST levels were determined using a commercially available enzyme immunoassay kit according to the manufacturer’s instructions (Biotrin International, Dublin, Ireland). Myeloperoxidase (MPO) activity is an indicator of neutrophil infiltration and free radical generation, which was determined as described previously (18). Formation of nitrotyrosine in the liver was evaluated as one of the hepatic injury markers using a commercially available nitrotyrosine ELISA kit (Cell Science, Canton, MA) according to the manufacturer’s instructions.

Determination of hepatic and plasma nitrate/nitrite levels. NO concentration in the liver tissues and plasma was evaluated by measuring nitrate/nitrite levels using a commercially available colorimetric assay kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer’s instructions. At 2 h after the end of resuscitation or sham operation, plasma liver tissues were obtained. Tissue samples (100 mg wet weight) were homogenized in 1 ml of lysis buffer (pH 7.4) containing 50 mM HEPES, 10 mM sodium pyrophosphate, 1.5 mM MgCl2, 1 mM EDTA, 0.2 mM sodium orthovanadate, 0.15 M NaCl, 0.1 M NaF, 10% glycerol, and 0.5% Triton X-100. Samples (250 µl) were further centrifuged using a 30-kDa molecular mass cutoff filter (Fisher Scientific, Pittsburgh, PA) at 14,000 g for 50 min at 4°C to remove proteins before analysis.

Western blot analysis. Approximately 100 mg of snap-frozen liver tissue was homogenized in 1 ml of lysis buffer containing 50 mM HEPES, 10 mM sodium pyrophosphate, 1.5 mM MgCl2, 1 mM EDTA, 0.2 mM sodium orthovanadate, 0.15 M NaCl, 0.1 M NaF, 10% glycerol, 0.5% Triton X-100, and protease inhibitor cocktail. The lysates were centrifuged by clarification. Samples were separated on 4–12% SDS-polyacrylamide gels (Invitrogen) and transferred onto nitrocellulose membranes (Invitrogen). Membranes were blocked with 5% nonfat dried milk in Tris-buffered saline-Tween (TBST) and then immunoblotted with the following primary antibodies: iNOS (1:1,000); eNOS (1:1,000); phospho-eNOS (Ser1177) (1:1,000); β-actin (all from Cell Signaling Technology, Beverly, MA); and HIF-1α (Abcam, Cambridge, MA) (1:1,000) overnight at 4°C. After being washed with TBST three times, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG secondary antibody and developed by enhanced chemiluminescence (Amersham, Piscataway, NJ). Rabbit monoclonal β-actin was used as the loading control. Quantification of the blots was performed using ChemiImager 5500 imaging software (Alpha Innotech, San Leandro, CA), and density values obtained from six rats/group were pooled and presented as means ± SE.

Determination of hepatic cytokines, chemokines and ICAM-1 levels. The hepatic cytokines, IL-6 and TNF-α, chemokine cytokineduced neutrophil-1 (CINC-1), and ICAM-1 levels were determined using ELISA kits (R&D, Minneapolis, MN) according to the manufacturer’s instructions. The chemokine macrophage inflammatory protein-2 (MIP-2) was measured using Rat MIP-2 CytoSet TM kit (BioSource Cytokines & Signaling, Invitrogen).

Statistical analysis. The data are presented as means ± SE (n = 6 rats/group). The Western blot analyses were performed with at least four animals per group. Statistical differences among groups were determined by one-way ANOVA followed by Tukey’s test. A value of P < 0.05 was considered to be significant.

RESULTS

Effects of iNOS inhibitor 1400W on blood pressure. The parameters of blood pressure, including diastolic and systolic pressure, in sham-operated and trauma-hemorrhaged animals are shown in Table 1. In sham-operated animals receiving vehicle (DMSO or normal saline), 1400W (10 mg/kg ip), or

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<td>MBP, mmHg</td>
<td>125 ± 19</td>
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<td>118 ± 19</td>
<td>77 ± 14*</td>
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<td>Systolic, mmHg</td>
<td>150 ± 18</td>
<td>146 ± 17</td>
<td>143 ± 26</td>
<td>102 ± 16*</td>
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<td>Diastolic, mmHg</td>
<td>111 ± 18</td>
<td>104 ± 19</td>
<td>98 ± 16</td>
<td>60 ± 9*</td>
<td>79 ± 8*</td>
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Table 1. Effects of 1400W and l-NAME on blood pressure in sham or trauma-hemorrhage/resuscitation rats.
l-NAME (30 mg/kg, iv), the blood pressure remained in the physiological range during the entire observation period. In contrast, trauma-hemorrhage/resuscitation resulted in severe hypotension with diastolic and systolic pressure significantly lower than values observed in sham animals. If iNOS inhibitor 1400W or NOS inhibitor l-NAME were administered 30 min before resuscitation, attenuation of the trauma-hemorrhage-induced hypotension and the depressed diastolic and systolic pressure responses were observed.

**Plasma α-GST level, hepatic MPO activity, and nitrotyrosine formation.** To determine whether iNOS inhibition was associated with the attenuation of hepatic injury following trauma-hemorrhage/resuscitation, the liver injury markers (plasma α-GST, hepatic MPO activity, and nitrotyrosine formation) were measured. As shown in Fig. 1A, differences in plasma α-GST levels were not noted between vehicle-, 1400W-, or l-NAME-treated sham animals. In contrast, trauma-hemorrhage/resuscitation resulted in markedly increased plasma α-GST levels compared with the shams. Treatment with 1400W attenuated the increase in plasma α-GST levels in the trauma-hemorrhage/resuscitation rats. However, l-NAME could not attenuate trauma-hemorrhage/resuscitation-induced depressed hepatic function.

Trauma-hemorrhage induced a significant increase in hepatic MPO activity in vehicle-treated rats, which was attenuated by treatment with 1400W and not by l-NAME (Fig. 1B). No significant difference in liver tissue MPO activity was observed between vehicle-, 1400W- or l-NAME-treated sham animals. Sham-operated rats (irrespective of 1400W or l-NAME treatment) had little detectable hepatic nitrotyrosine levels. Trauma-hemorrhage/resuscitation increased hepatic nitrotyrosine levels that were significantly reduced by treatment with 1400W (Fig. 1C).

**Nitrate/nitrite levels in the liver tissue and plasma.** The levels of nitrate/nitrite in plasma (Fig. 2A) and liver tissues (Fig. 2B) among vehicle-, 1400W-, or l-NAME-treated sham animals were not statistically different. There was a significant increase in nitrate/nitrite levels of plasma and liver after trauma-hemorrhage compared with sham values. Treatment with 1400W or l-NAME prevented the increase in plasma nitrate/nitrite levels at 2 h after resuscitation.

**Hepatic iNOS, HIF-1α, and eNOS expression.** Hepatic iNOS (Fig. 3A) and HIF-1α (Fig. 3B) expression were significantly increased in the trauma-hemorrhage group treated with vehicle compared with the respective sham group. Furthermore, the changes in HIF-1 protein expression following trauma-hemorrhage were similar in both total and nuclear extracts (data not shown). Administration of 1400W following trauma-hemorrhage/resuscitation prevented the increase in iNOS and HIF-1α expression. No difference in iNOS and HIF-1α protein levels in sham animals treated with vehicle or 1400W was detectable. Trauma-hemorrhage/resuscitation did not significantly alter hepatic eNOS expression, even with 1400W treatment (Fig. 4). The eNOS phosphorylation at Ser1177 (Fig. 4) was decreased in trauma-hemorrhage/resuscitation rats, and this effect of trauma-hemorrhage/resuscitation was not influenced by 1400W treatment.

**Hepatic protein expression of cytokines, chemokines, and ICAM-1.** There were no significant differences in IL-6 (A) and TNF-α (B) levels in sham-treated groups (Fig. 5). Following trauma-hemorrhage/resuscitation, the protein expression of IL-6 and TNF-α in the liver was significantly increased. Treatment with 1400W normalized these cytokine levels, and the values were similar to the sham animals. As shown in Fig. 6, no change was observed in hepatic MIP-2 (A), CINC-1 (B), and ICAM-1 (C) protein levels between the vehicle-, 1400W- or l-NAME-treated sham groups. Trauma-hemorrhage/resuscitation produced a significant elevation in hepatic chemokines and ICAM-1 content that was reduced by 1400W treatment. However, l-NAME could not attenuate the expression of cytokines, chemokines, and adhesion molecules in liver tissues following trauma-hemorrhage/resuscitation.

**DISCUSSION**

This study was undertaken to evaluate the contribution of iNOS-derived NO in producing the inflammatory response and subsequent hepatic injury following trauma-hemorrhage/resuscitation. The results demonstrate that trauma-hemorrhage caused
severe hypotension and liver damage associated with increased plasma/GST levels. The increased MPO activity, nitrotyrosine formation, iNOS and HIF-1α expression, and proinflammatory cytokines/chemokines and ICAM-1 levels were observed in the liver following trauma-hemorrhage. Both systemic and hepatic levels of NO (nitrate/nitrite) were also elevated. Treatment with 1400W, a potent selective iNOS inhibitor, prevented the persistent hypotension and attenuated the hepatic injury, which was associated with the decreased hepatic MPO activity, nitrotyrosine formation, and expression of iNOS and HIF-1α. The attenuation of hepatic injury by 1400W was associated with the reduction of hepatic levels of IL-6, TNF-α, ICAM-1, MIP-2, and CINC-1. Although administration of the nonselective NOS inhibitor L-NAME attenuated the hypotension, it could not ameliorate hepatic injury and inflammation in the trauma-hemorrhage/resuscitation group. These data suggest that iNOS is responsible in part for producing organ injury after trauma-hemorrhage/resuscitation and also plays a key role in the activation of inflammatory cascades following resuscitation.

A number of investigators have suggested that manipulation of NOS activity may be a useful therapeutic approach following low flow conditions. NO derived from iNOS has been implicated in many pathological events (5, 20, 33) and in the development of tissue injury (28). Nonetheless, the precise role of NO in different physiological functions after injury is not entirely clear because most of the previous studies utilized nonspecific NOS inhibitors (9, 39).

Pharmacokinetic studies have demonstrated that 1400W is a specific inhibitor of iNOS that is active for up to 3 h after administration (13). Our results indicate that 2 h after trauma-hemorrhage/resuscitation, 1400W was effective in attenuating liver injury and the inflammatory response. Thus inhibition of iNOS in the first 3 h after trauma-hemorrhage/resuscitation appears to be critical and perhaps sufficient to prevent subsequent injury to the liver following trauma-hemorrhage/resuscitation. However, the mechanism by which iNOS inhibition decreases trauma-hemorrhage/resuscitation-induced liver injury is unclear. One possibility is that iNOS-mediated cytotoxicity increases cellular damage, leading to increased cytokine/chemokine release and recruitment of inflammatory cells.

Studies have examined the role of NOS inhibitors in septic models for treatment of systemic hypotension and reduction of plasma nitrate/nitrite levels (37). The capability of an iNOS inhibitor to stabilize blood pressure and attenuate hepatic injury suggests that this agent is a useful tool for treatment of hemodynamic instability following shock. Thus specific inhibition of iNOS may be a better alternative to nonspecific NOS inhibition for hemodynamic therapy of patients (25). Interestingly, our results indicate that although nonspecific NOS inhibitor L-NAME effectively restored systemic blood pressure and significantly decreased nitrate/nitrite levels, it could not...
attenuate liver injury following trauma-hemorrhage/resuscitation. However, the selective inhibition of iNOS with 1400W results in a decrease in hepatic damage. This beneficial effect of the iNOS inhibitor is in concordance with the protective effects of 1400W against liver injury in septic models (41). Although we did not observe an increase in liver blood flow (data not shown), another study demonstrated that 1400W improves the microcirculation in rat cremaster muscle during reperfusion (43). The difference between microcirculatory flow and hepatic blood flow suggests that iNOS-induced pathological alteration is not directly responsible for producing major alterations in blood flow in the early stage following hemorrhagic shock.

Trauma-hemorrhage/resuscitation shares some similarities with warm I/R. The cascade of events leading to hepatic injury in I/R includes the release of cytokines/chemokines and free radicals, leukocyte activation, and apoptosis (6, 38). Resuscitation following severe hemorrhage may exacerbate hepatic damage through the generation of reactive oxygen species (ROS), including peroxynitrite from activated neutrophils (6), and by excessive production of NO through iNOS (36). Neutrophils are also activated in the early phase of hemorrhagic shock (33), and hepatic injury is characterized by increased neutrophil accumulation in the liver. The activated neutrophils are a potential source of ROS, and such neutrophils infiltrate the liver; this occurs along with increased expression of P-selectin and ICAM-1 and with elevated local chemoattractants/chemokines levels (11, 28). All of the above agents appear to be the essential components of the inflammatory cascade and are associated with hepatic injury following trauma-hemorrhage (1). At 2 h after resuscitation, we observed increased MPO activity. Therefore, in addition to the hepatic resident cells such
as hepatocytes and Kupffer cells (34), activated neutrophils may also be a source of NO generation following resuscitation. NO can react with superoxide to form peroxynitrite, which is capable of nitrating tyrosine residues of proteins and enzymes to generate nitrotyrosine (27). Physiological concentrations of peroxynitrite have been reported to attenuate hepatic injury by inhibiting leukocyte-endothelial interactions (23). Therefore, similar to NO, peroxynitrite can have both beneficial and detrimental effects on hepatic tissues that are concentration dependent. We hypothesized that peroxynitrite during the early stage of hemorrhagic shock might contribute to liver injury. Therefore, an early blockade of NO-mediated oxidative/nitrosative damage from iNOS may decrease tissue damage (8). This study showed that the hepatic injury in trauma-hemorrhagic rats was accompanied by significant upregulation of iNOS protein expression and nitrotyrosine formation that was prevented by 1400W treatment.

Recent studies have shown that HIF-1α plays an important role in hypoxia-induced and inflammatory diseases. A number of inflammatory mediators can activate HIF-1α even under normoxic conditions (15). HIF-1α can also mediate iNOS expression under hypoxic conditions. However, previous studies have also shown that NO is a key factor in sustaining HIF-1α activation during hypoxia. Furthermore, NO has been reported to both promote and to inhibit the activity of HIF-1 (26, 29, 40, 42). Our results are consistent with a recent finding that suggested that specific iNOS inhibition not only decreased HIF-1α expression but also lowered VEGF expression (14). This study indicated that there must be an interactive mechanism between HIF-1α and iNOS (14). Although several mechanisms are likely involved in the iNOS regulation of HIF-1α expression, it is probable that administration of 1400W in rats following trauma-hemorrhage preserved blood pressure and presumably limited tissue hypoxia. Alternatively, administration of 1400W following trauma-hemorrhage attenuates the production of inflammatory mediators, which decreases hepatic HIF-1α levels. However, more studies are needed to confirm this notion.

It can be argued that because the effects of 1400W were only examined at a single time point, i.e., 2 h after the end of resuscitation, it remains unclear whether the salutary effects are sustained for longer periods of time after treatment. Although we have not examined whether the salutary effects of 1400W persisted for a prolonged period of time, previous studies have shown that if an agent such as estradiol, flutamide, or dehydroepiandrosterone produces salutary effects at 2 h after treatment, those effects are sustained for prolonged periods of time and they also improve the survival of animals (7, 18, 21).

It is well established that trauma-hemorrhage/resuscitation produces hypoxia and increased iNOS expression in the liver (21). Cells or tissues respond to low oxygen availability by initiating a series of adaptive responses through transcriptional activation and stabilization of HIF-1 (3). Regulation of HIF-1α by NO is an additional mechanism by which NO might modulate cellular responses to hypoxia (2). The modulation of the hypoxic and inflammation response by NO is believed to have widespread pathophysiological significance (15). Our results indicated that inhibition of iNOS prevented the accumulation of HIF-1α, thus revealing a major role for iNOS in regulating responses to hypoxia.

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GRANTS

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