Hypertonic Saline and Pentoxifylline Attenuates Gut Injury After Hemorrhagic Shock: The Kinder, Gentler Resuscitation

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Background: **We have previously demonstrated that postshock resuscitation with Hypertonic saline and Pentoxifylline (HSPTX) attenuates pulmonary and histologic gut injury when compared with Ringer's lactate (RL). In this study, we hypothesized that the decrease in gut injury observed with HSPTX is associated with the attenuation of inducible nitric oxide synthase (iNOS) activity and production of ileal proinflammatory mediators after hemorrhagic shock.**

Methods: **In a rat model of hemorrhagic shock, resuscitation was conducted with RL (32 mL/kg; n = 7) or HSPTX (4 mL/kg 7.5% NaCl + PTX 25 mg/kg; n = 7). Sham animals that did not undergo shock were also studied. Four hours after resuscitation, the terminal ileum was collected for evaluation of nitrite, tumor necrosis factor (TNF)-α, Interleukin (IL)-6, and cytokine-induced neutrophil chemoattractant (CINC) by enzyme immunoassay. Heme oxygenase-1 (HO-1), iNOS, cytoplasmic inhibitor of kappa B (Iκ B) phosphorylation, and nuclear factor (NF)κ B p65 nuclear translocation were determined by Western blot.**

Results: **HSPTX resuscitation resulted in a 49% decrease in iNOS when compared with RL ($p < 0.05$). Similar results were obtained when examining nitrite (882 ± 59 vs. 1,435 ± 177 μmol/L; $p < 0.01$), and HO-1 content ($p < 0.05$). RL resuscitation resulted in markedly higher levels of TNF-α (83 ± 27 vs. 9 ± 5 pg/mL; $p < 0.01$), IL-6 (329 ± 58 vs. 118 ± 43 pg/mL; $p < 0.05$), and CINC (0.43 ± .06 vs. 0.19 ± .08 ng/mL; $p < 0.05$) than HSPTX. The increase in cytokines observed with RL was also associated with an increase in I-κB phosphorylation ($p < 0.01$) and NF-κB p65 nuclear translocation ($p < 0.001$).**

Conclusion: **The attenuation in gut injury after postshock resuscitation with HSPTX is associated with downregulation of iNOS activity and subsequent proinflammatory mediator synthesis. HSPTX has the potential to be a superior resuscitation fluid with significant immunomodulatory properties.**

Key Words: **Hypertonic saline, Pentoxifylline, NF-κB, Nitric oxide, Hemorrhagic shock.**

J Trauma. 2007;62:818–828.

Hemorrhagic shock is the most common cause of early death after trauma. Patients who survive this initial insult undergo a global ischemia-reperfusion phase that may culminate in the development of acute lung injury (ALI), multiple organ failure (MOF), and death.[1,2] Although the exact mechanism(s) by which shock and reperfusion produce host organ injury are yet to be elucidated, laboratory and clinical evidence strongly suggest that splanchnic hypoperfusion plays a central role in the initiation of this process.[3–6] Under normal circumstances the human gastrointestinal tract receives approximately 20% of the cardiac output. During shock however, blood is preferentially shunted toward the heart and brain at the expense of the gastrointestinal tract resulting in intestinal mucosal ischemia. With reperfusion, the gut becomes center for neutrophil priming and a generator of proinflammatory mediators such as tumor necrosis factor (TNF)-α and Interleukin (IL)-6 that contribute to both local and distant organ injury.[7–10]

With the onset of ischemia-reperfusion injury, reactive radicals such as nitric oxide (NO) are generated and participate in the initiation and propagation of the inflammatory cascade. Normally, constitutive nitric oxide synthases are responsible for the production of intestinal NO at picomolar concentrations. This basal level of NO protects gut mucosa by maintaining splanchnic perfusion and inhibiting neutrophil and platelet adhesion.[11] In contrast, the inducible form of nitric oxide synthase (iNOS) has been shown to increase after ischemia-reperfusion, and its activity produces sustainable quantities of NO in nanomolar concentrations.[12,13] The excessive production of NO by iNOS increases gut barrier dysfunction through direct effects on cell signaling and through the production of cytotoxic mediators, such as peroxynitrite, that are generated by the interaction between NO and oxygen species.[14] The proinflammatory nature of iNOS is supported by studies demonstrating that both iNOS knockout mice and animals administered selective pharmacologic iNOS inhibitors were more resistant to intestinal injury and pulmonary inflammation after ischemia-reperfusion injury.[13,15,16]

Submitted for publication September 13, 2006.

Accepted for publication October 31, 2006.

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Supported by the Department of Surgery, Division of Trauma and Surgical Critical Care, University of California School of Medicine, San Diego, California.

Presented at the 65th Annual Meeting of the American Association for the Surgery of Trauma, September 28–30, 2006, New Orleans, Louisiana.

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DOI: 10.1097/TA.0b013e31802d9745

There is recent evidence suggesting that the choice of postshock resuscitation strategy can also modulate the inflammatory response. Therefore, in terms of the inflammation, the type of fluid used to restore perfusion may be just as important as restoring perfusion itself. Racemic Ringer's lactate (RL), the current standard resuscitative fluid, has been shown to increase neutrophil oxidative burst, adhesion molecule expression, endothelial dysfunction, and cellular apoptosis in animal models of ischemia-reperfusion.[17–20] The proinflammatory properties of RL have been attributed to the D-isomer of lactate, because neutrophil exposure does not occur upon exposure to RL strictly composed of the L-isomer.[20,21] This modification, however, does not circumvent the second major disadvantage of isotonic fluid resuscitation that is the large infusion volumes necessary to achieve organ perfusion and normotension. Consequently, there has been increased interest in alternative resuscitation strategies that could either attenuate the inflammatory response or reduce the amount of fluid needed for resuscitation.

Recently, our laboratory has proposed a therapeutic resuscitative fluid that combines the small volume resuscitation of hypertonic saline (HS; 7.5% NaCl, 4 mg/kg) with the anti-inflammatory properties of the nonspecific phosphodiesterase inhibitor Pentoxifylline (PTX). HSPTX has been observed, in vitro, to attenuate neutrophil oxidative burst and CD11b expression more efficiently than HS infusion alone.[22] Additionally in a rat model of hemorrhagic shock, HSPTX has been shown to markedly reduce histologic gut injury, lung injury, and pulmonary inflammation characterized by diminished matrix metalloproteinase-2 and -9 activity, cytokine-induced neutrophil chemoattractant (CINC) concentration, and neutrophil infiltration.[23,24] In this series, we hypothesized that the attenuation in postshock gut injury observed with HSPTX resuscitation is associated with attenuation of intestinal iNOS activity, oxidative stress, and local proinflammatory cytokine production when compared with RL in vivo.

MATERIALS AND METHODS

The experiment was approved by the University of California Animal Subjects Committee and in accordance with guidelines established by the National Institutes of Health.

Experimental Model

Male Sprague-Dawley rats (300–400 g) were purchased from Harlan Sprague-Dawley (San Diego, Calif.). A 12-hour light and dark cycle was instituted, and food and water were provided ad libitum. Animals were anesthetized with ketamine and xylazine by intraperitoneal injection. A right inguinal incision was performed, and the femoral artery and vein were cannulated with polyethylene catheters (PE50). The venous catheter was utilized for injection of resuscitative fluids, and the arterial catheter was used to withdraw blood and monitor the mean arterial pressure (MAP). Blood was withdrawn during a period of 10 minutes until a MAP of 35 mm Hg was obtained. Controlled hypotension was then maintained at 35 ± 5 mm Hg for 1 hour by withdrawal or reinfusion of blood as necessary.

The animals were randomly divided into three groups according to the treatment received. Sham animals (n = 5) underwent cannulation without shock or resuscitation and served as negative controls. RL resuscitated animals (n = 7) received 32 mL/kg of racemic RL. HSPTX treated animals (n = 7) received 4 mL/kg of 7.5% NaCl + 25 mg/kg of PTX (Sigma, St. Louis, Mo.). PTX was dissolved in HS without the infusion of any additional fluid, and its dose was chosen based on studies from our laboratory demonstrating its safety and lack of hypotension at this concentration.[24] The volume of RL infusion was calculated to give equivalent sodium loads to both the RL and HSPTX treated animals. After resuscitation with the respective fluid, all shed blood was reinfused and there were no differences in the amount of shed blood reinfused between the two groups. As seen in our prior study, there was no significant difference between preshock, shock, and resuscitation MAP or the shed blood volume between the groups (11.9 mL and 11.4 mL for the RL and HSPTX groups, respectively).[24] The body temperature of the animals was maintained at 37°C throughout the experiment with a heating blanket.

Tissue Procurement and Protein Extraction

At the end of volume resuscitation, the catheters were removed, the incision was closed, subcutaneous bupivacaine was administered, and the animals were returned to their cages. The animals were killed via cardiac puncture 4 hours after the completion of shock and resuscitation. This time point was chosen based on our previous experiments in which the early signs of end organ injury and neutrophil activity were observed at 4 hours.[23] Immediately after being killed, the terminal ileum was harvested and snap frozen in liquid nitrogen and stored at −70°C.

The terminal ileum was homogenized in 1 mL of ice-cold T-PER Tissue Protein Extraction Reagent containing 1% protease inhibitor cocktail (Pierce Biotechnology, Rockford, Ill.). Homogenates were centrifuged at 10,000× *g* for 5 minutes, and the supernatant was aliquoted and stored at −70°C for analysis of TNF-α, IL-6, CINC, nitrite, Heme oxygenase-1 (HO-1), and iNOS expression. To analyze inhibitor of kappa B (I-κB) and nuclear factor (NF)-κB p65 subunit phosphorylation, the extraction of cytoplasmic and nuclear proteins, respectively, was performed using nuclear and cytoplasmic extraction reagents (NE-PER, Pierce) supplemented with 1% protease inhibitor cocktail (Pierce) per the manufacture's instructions. The protein concentration of all extracts was determined using the bicinchoninic acid protein assay (Pierce). Absorbance was measured on a standard curve for albumin with a microplate reader (Molecular Devices, Sunnyvale, Calif.).

Western Blot Analysis (iNOS, HO-1, phosphorylated I-κB, phosphorylated p65 NF-κB)

In separate experiments, total cell (HO-1 and iNOS), cytoplasmic (I-κB), or nuclear extracts (NF-κB) containing 10 μg of protein per sample were suspended in sodium dodecyl sulfate (SDS) sample buffer (Invitrogen, Carlsbad, Calif.) and collected by boiling the samples at 100°C for 5 minutes. Proteins were separated by SDS-polyacrylamide gel electrophoresis using 8% to 16% tris-glycine polyacrylamide gradient gels and transferred to nitrocellulose membranes (Invitrogen). The membranes were blocked with 5% milk in tris-buffered saline/Tween 20 for 1 hour. In separate experiments, primary antibodies specific for HO-1 (1:2,000 Cell Signaling, Beverly, Mass.), iNOS (1:500 BD Biosciences, San Diego, Calif.) phosphorylated I-κBα (1:250 Cell Signaling) or phosphorylated NF-κB p65 (1:500 Cell Signaling) were incubated with the membranes overnight at 4°C in tris-buffered saline (Tween) supplemented with 5% Bovine serum albumin. The membranes were washed and incubated for 1 hour at room temperature with horseradish peroxidase-linked anti-rabbit IgG (1:2,000) prepared in blocking solution. After thorough washing, the Pierce Supersignal West Pico Chemiluminescent Kit was applied for antibody detection with X-ray film (Amersham Biosciences, Buckinghamshire, UK). Band quantification for Western blot analysis was performed with UN-SCAN-IT Gel Digitizing software (Silk Scientific, Orem, Utah). Data are expressed at the mean band pixel total ($n \geq 4$ per group).

Nitrite Levels

In aqueous solution, NO rapidly degrades into its stable metabolites, nitrate and nitrite. Using a commercially available kit (Oxis Research, Portland, Ore.) nitrate is first reduced to nitrite via nitrate reductase for total nitrite measurements. Nitrite quantification ($n \geq 4$ per group) using Greiss reagent was performed and expressed as the mean concentration (μmol/L).

Proinflammatory Cytokine Levels (TNF-α, IL-6, CINC)

TNF-α and IL-6 were measured from whole cell protein extracts ($n \geq 5$ per group) using commercially available enzyme linked immunosorbent assays (ELISA), R&D Systems, Minneapolis, Minn.). Results are expressed as pg/mL.

The CINC quantification was also determined through ELISA. CINC is a member of the IL-8 family present in rats whose functions resemble that of human IL-8. The wells of a 96-well immunoplate (NUNC Brand, Rochester, N.Y.) were coated with capture antibody (goat anti-CINC, Peptide Institute, Osaka, Japan) diluted 1:396 in coating buffer, and incubated overnight at 4°C. Nonspecific binding sites were blocked with a buffer comprised of 5% nonfat dry milk (Sigma) in sterile phosphate buffered saline (PBS, Irvine Scientific, Santa Ana, Calif.). The wells of the plate were washed with 0.05% Tween 20 (Fischer Scientific, Pittsburgh, Penn.) in PBS. Whole cell extracts (100 μL; $n > 4$ per group) were incubated on the plate at room temperature for 2 hours. After washing, the secondary antibody (rabbit anti-CINC) was diluted 1:2,000 in blocking buffer and dispensed onto the plate for incubation at room temperature. The conjugate, horseradish peroxidase-linked anti-rabbit IgG (Cell Signaling) was added and incubated at room temperature for an additional 30 minutes. Immunopure TMB substrate kit (Pierce) was used for detection, and absorbance was read at 450 nm. Data are presented as ng/mL.

Statistical Analysis

All values are expressed as the mean + the SEM of n observations, where n represents the number of animals in each group. Each assay was performed in duplicate or triplicate where appropriate. Statistical significance of differences among groups was determined by analysis of variance (ANOVA) with Bonferroni correction. A p value <0.05 was considered statistically significant.

RESULTS

Gut iNOS Content

Resuscitation with RL significantly upregulated ileal iNOS content when compared with HSPTX (96,403 ± 8,359 vs. 49,336 ± 14,998; $p < 0.05$). No difference in iNOS induction was observed between the Sham group (63,166 ± 10,580) and those who underwent postshock resuscitation with HSPTX (Fig. 1).

Gut Total Nitrite Levels

In vivo, NO is rapidly metabolized to nitrite (NO_2^-) and nitrate (NO_3^-) through an interaction with the heme group in hemoglobin.[25] To determine whether the modulation in iNOS expression correlated with NO production after ischemia and reperfusion, ileal nitrite levels were determined. Nitrite levels declined by 38% when HSPTX was utilized for resuscitation versus RL (882.5 ± 58.9 vs. 1,435 ± 177 μmol/L, respectively; $p < 0.01$; Fig. 2). No statistically significant difference was seen between animals treated with HSPTX and the negative control (627.4 ± 168.7 μmol/L).

Gut NF-κB Expression

In its inactive state, NF-κB remains quiescent in the cytoplasm associated with the inhibitory protein, I-κB. Reactive oxygen species generated through reperfusion is one stimulus capable of activating the cascade responsible for I-κBα phosphorylation, which, in turn, allows for ubiquitination and proteolytic degradation of this inhibitory subunit and results in nuclear translocation of the transcriptionally active p50-p65 NF-κB heterodimer.[26–28] Resuscitation with RL led to an 88% increase in ileal cytoplasmic I-κBα phosphorylation over the Sham group (55,881 ± 13,680 vs. 6,719 ± 177; $p < 0.01$). This marked increase in I-κBα phosphorylation was not observed after HSPTX resuscitation

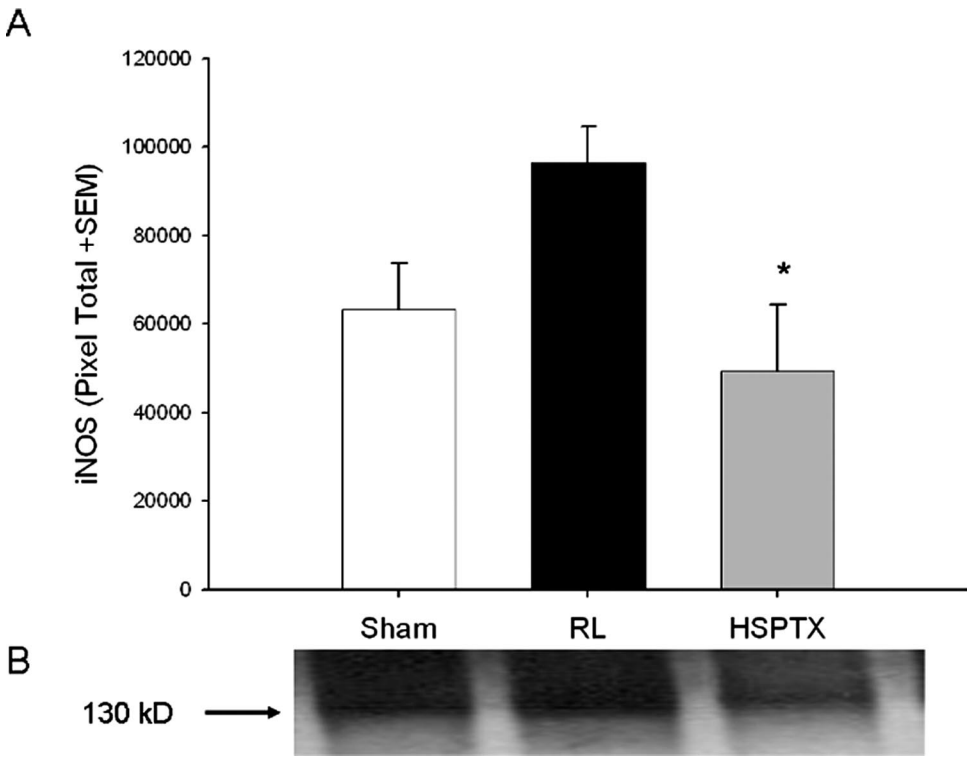

Fig. 1. (A) *Ileal iNOS expression after hemorrhagic shock and resuscitation with RL (32 mL/kg) or HSPTX (4 mL/kg of 7.5% NaCl + 25 mg/kg of PTX). Results are expressed as the mean ± SEM ($n \geq 4$ per group). The HSPTX-treated animals had significantly decreased iNOS levels when compared with RL (*$p < 0.05$ vs. RL).* (B) *Representative Western blot of iNOS expression.*

(11,333 ± 1,127) and remained comparable to that seen in the negative control (Fig. 3).

To assess whether the attenuation I-κBα phosphorylation observed with HSPTX after hemorrhagic shock affected nuclear translocation of NF-κB, we analyzed the relative amounts of NF-κB p65 subunit phosphorylation in gut nuclear extracts. As expected, RL-treated animals had a significantly higher degree of nuclear translocation over that of the Sham group (51,692 ± 1,741 vs. 14,005 ± 4,596; $p < 0.001$). There was 65% less p65 phosphorylation in the HSPTX treated group (18,499 ± 6,168) when compared with their RL-treated counterparts ($p < 0.01$; Fig. 4).

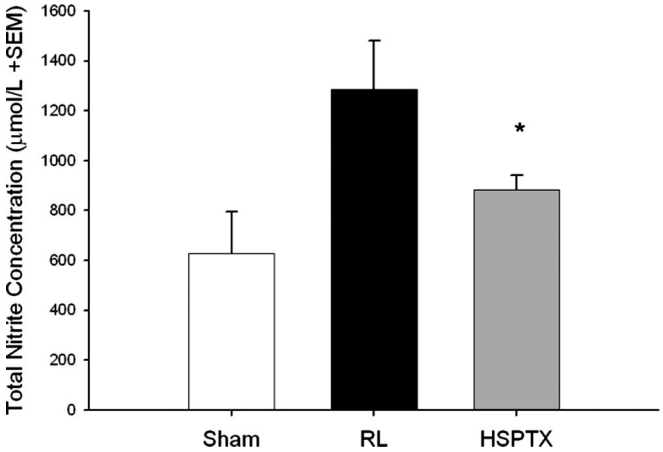

Fig. 2. *Gut nitrite levels. Ileal nitrite levels were quantified as described in Material and Methods. Data are presented as the mean ± SEM in μmol/L ($n \geq 4$ per group). HSPTX resuscitation (4 mL/kg of 7.5% NaCl + 25 mg/kg of PTX) after hemorrhagic shock resulted in a 32% decrease in nitrite levels when compared with RL resuscitation (32 mL/kg; *$p < 0.01$ vs. RL).*

Gut TNF-α Levels

TNF-α is considered a proximal mediator in the initiation and potentiation of the inflammatory cascade. Animals resuscitated with HSPTX had levels of TNF-α similar to that of the Sham group (9.1 ± 5.6 vs. 18.5 ± 9.4 pg/mL, respectively). Additionally, resuscitation with RL led to a significant 89% increase in ileal TNF-α when compared with HSPTX (82.6 ± 28.6 vs. 9.1 ± 5.6 pg/mL; $p < 0.01$; Fig. 5).

Gut IL-6 Levels

IL-6 is an important cytokine produced by endothelial cells that increases gut permeability and contributes to gut barrier dysfunction after hemorrhagic shock and resuscitation.[29,30] Ileal IL-6 production was 65% greater in animals administered RL versus those who received HSPTX after shock (329.4 ± 58.2 vs. 118.4 ± 42.7 pg/mL; $p < 0.01$; Fig. 6). IL-6 levels did not differ between the Sham and HSPTX group (142.3 ± 44.2 vs. 118.4 ± 42.7 pg/mL, respectively).

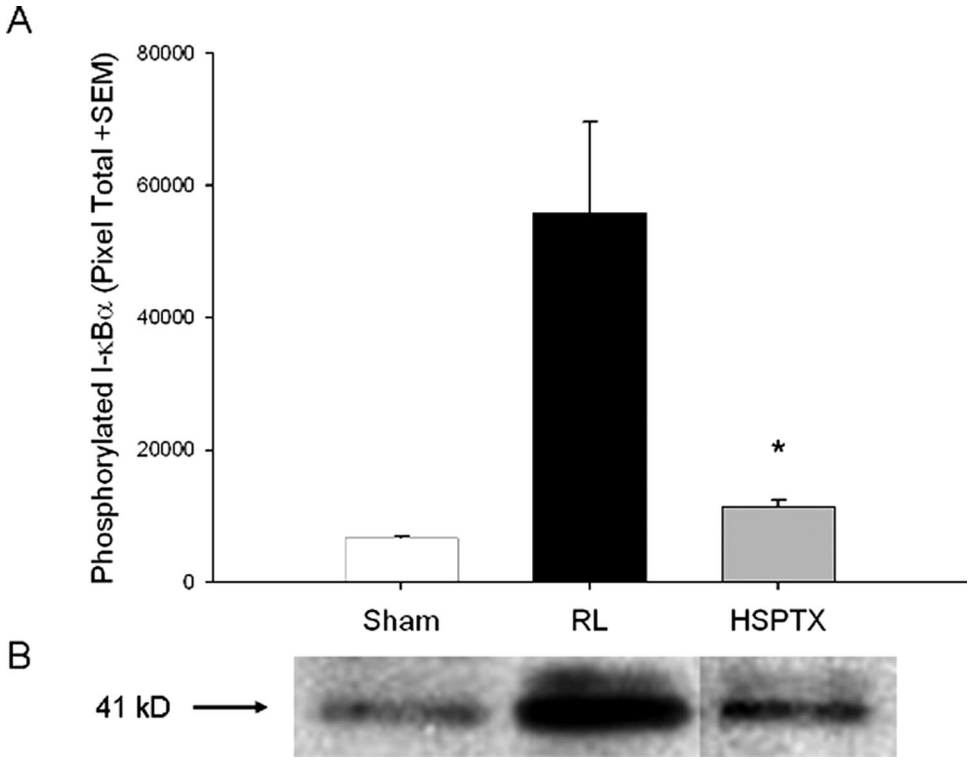

Fig. 3. (A) *Cytoplasmic I-κBα phosphorylation was determined after shock and resuscitation with either RL (32 mL/kg) or HSPTX (4 mL/kg of 7.5% NaCl + 25 mg/kg of PTX). Results are expressed as the mean ± SEM (n ≥ 4 per group). The HSPTX-treated animals had 80% less I-κBα phosphorylation than their RL-treated counterparts (*p < 0.01 vs. RL).* (B) *Representative Western blot of phosphorylated I-κBα phosphorylation.*

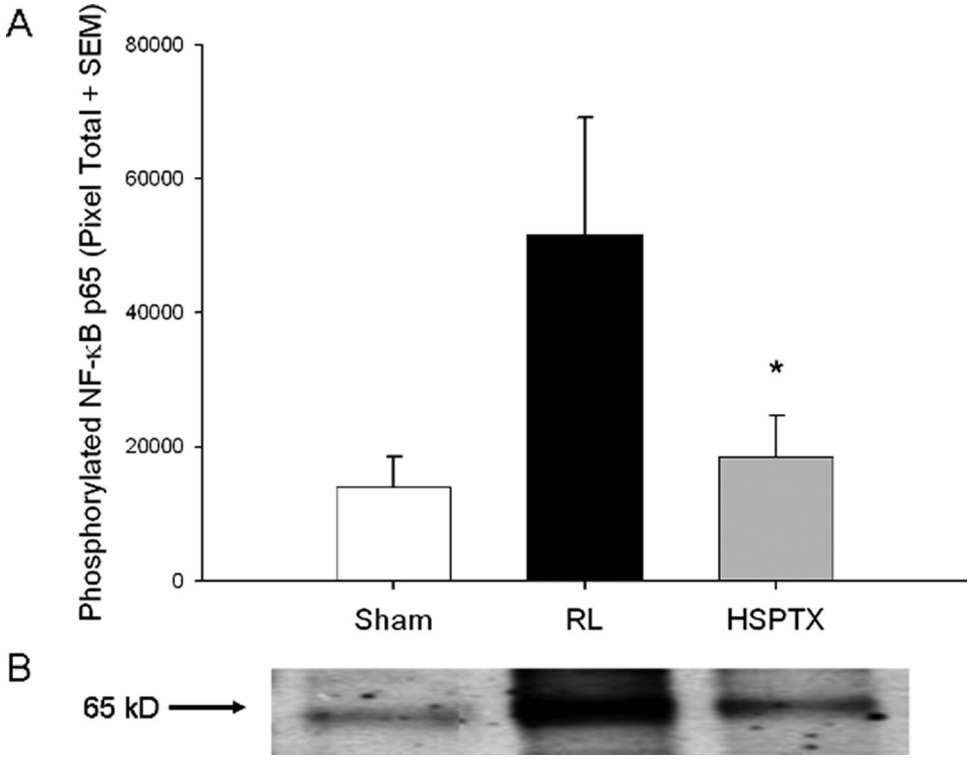

Fig. 4. (A) *Nuclear NF-κB p65 subunit phosphorylation was determined after shock and resuscitation with either RL (32 mL/kg) or HSPTX (4 mL/kg of 7.5% NaCl + 25 mg/kg of PTX). Results are expressed as the mean ± SEM (n ≥ 4 per group). The HSPTX-treated animals had 65% less p65 phosphorylation than their RL-treated counterparts (*p < 0.001 vs. RL).* (B) *Representative Western blot of NF-κB p65 subunit phosphorylation.*

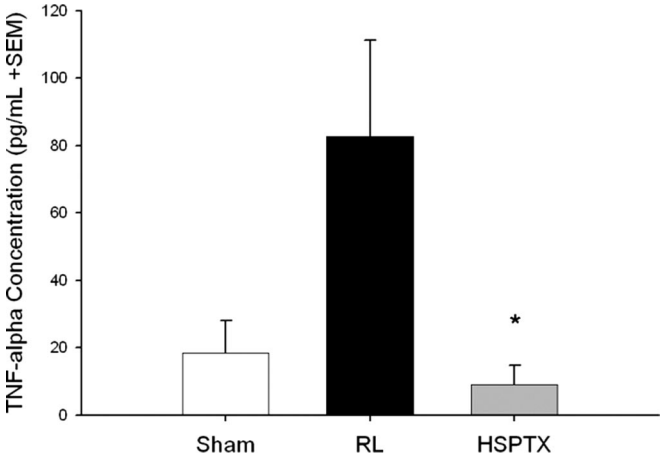

Fig. 5. *Effects of resuscitation strategy on gut TNF-α levels. Ileal TNF-α concentration was determined by ELISA as described in Materials and Methods. Data are presented as the mean ± SEM in pg/mL (n ≥ 5 per group). HSPTX resuscitation after hemorrhagic shock (4 mL/kg of 7.5% NaCl + 25 mg/kg of PTX) resulted in an 89% decrease in TNF-α levels when compared with the RL resuscitation group (32 mL/kg; *p < 0.01 vs. RL).*

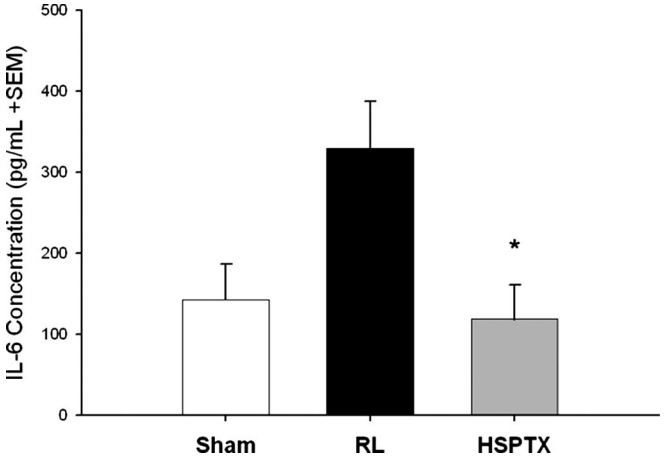

Fig. 6. *Effects of resuscitation strategy on gut IL-6 concentration. The ileal concentration of IL-6 was determined by ELISA as described in Materials and Methods. Data are presented as the mean ± SEM in pg/mL (n ≥ 5 per group). HSPTX resuscitation after hemorrhagic shock (4 mL/kg of 7.5% NaCl + 25 mg/kg of PTX) resulted in a 65% decrease in TNF-α levels when compared with the RL resuscitated group (32 mL/kg; *p < 0.01 vs. RL).*

Gut Cytokine-Induced Neutrophil Chemoattractant (CINC) Levels

In humans, IL-8 regulates chemotaxis and aids in neutrophil recruitment and activation in end organs during ischemia-reperfusion.[31] Because IL-8 does not exist in rodent species, other chemokines such as CINC that fulfill the same biological functions must instead be measured. Similar to the results observed with both TNF-α and IL-6, the concentration of gut CINC did not differ between the Sham group and those treated with HSPTX (0.18 ± 0.03 vs. 0.19.5 ± 0.08 ng/mL).

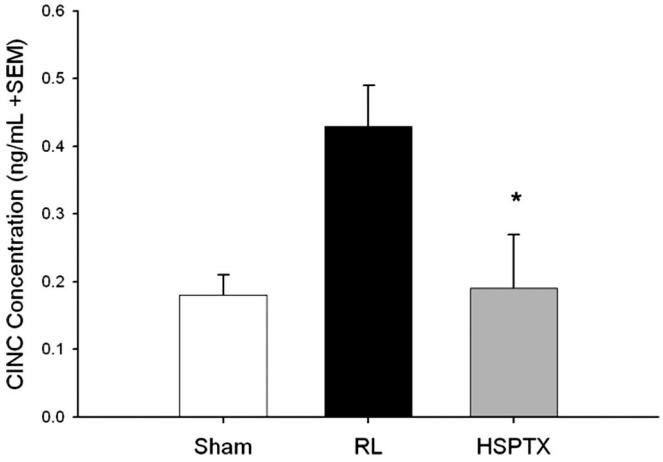

Fig. 7. *Effects of resuscitation strategy on gut CINC levels. The ileal concentration of CINC was determined by ELISA as described in Materials and Methods. Data are presented as the mean ± SEM in ng/mL (n ≥ 5 per group). HSPTX resuscitation after hemorrhagic shock (4 mL/kg of 7.5% NaCl + 25 mg/kg of PTX) resulted in a 55% decrease in TNF-α levels when compared with RL resuscitation (32 mL/kg; *p < 0.05 vs. RL).*

Administration of RL resulted in a marked increase in ileal CINC (0.43 ± 0.06 ng/mL) over both the Sham group and the HSPTX counterparts ($p < 0.05$; Fig. 7).

Gut HO-1 Content

HO-1 serves as a marker of the relative degree of oxidative stress within tissues, and its expression is induced by the presence of reactive oxygen species generated during reperfusion.[32,33] In animals resuscitated with RL, a marked increase in HO-1 expression was seen in ileal extracts (72,057 ± 353; $p < 0.05$; Fig. 8). HSPTX infusion did not result in induction of HO-1, and levels paralleled those demonstrated in the Sham group (50,815 ± 4,460 vs. 51,141 ± 8,955, respectively).

DISCUSSION

The morbidity and mortality associated with MOF after trauma remain high despite major advances in the care and management of critically injured patients during the past two decades. It has been established that hyperactivity of the host's own immune system plays a central role in the development of postinjury organ dysfunction. The role of the gut as "motor of MOF" has also been recognized for many years.[34,35] The initial hypothesis that distant organ injury was because of the systemic translocation of gut-derived bacteria and endotoxin via the portal vein was disproved by studies in which portal vein diversion had no effect on the development of resuscitation-induced lung injury after hemorrhagic shock.[36] More recently, Deitch and colleagues have presented extensive evidence implicating postshock mesenteric lymph as the conduit for gut-derived inflammatory mediators capable of endothelial cell injury, adhesion molecule expression, and neutrophil acti-

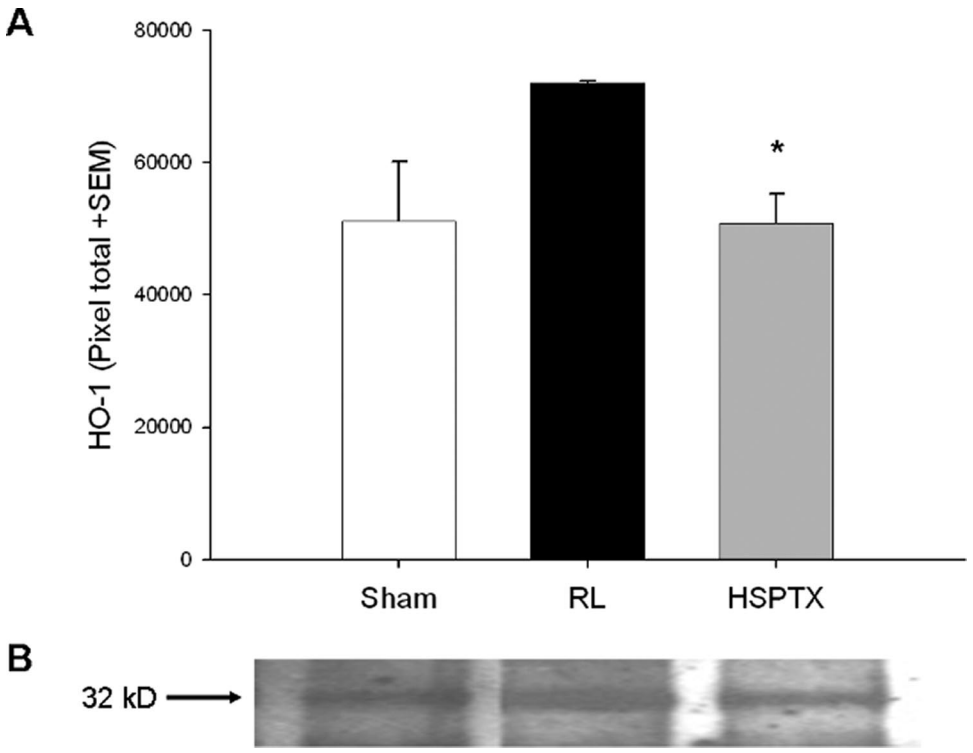

Fig. 8. (A) *Ileal HO-1 content after hemorrhagic shock and resuscitation with RL (32 mL/kg) or HSPTX (4 mL/kg of 7.5% NaCl + 25 mg/kg of PTX). Results are expressed as the mean ± SEM in ($n \geq 4$ per group). The HSPTX-treated animals had significantly decreased HO-1 levels when compared with those animals who received RL (32 mL/kg; *$p < 0.05$ vs. RL).* (B) *Representative Western blot of HO-1 expression.*

vation across species.[37–39] Although lymphatic duct ligation has been shown to abrogate the development of lung injury after shock,[8,9] it is not a clinically applicable technique. Therefore, alternative strategies aimed at diminishing ischemia-reperfusion and resuscitation-induced gut injury immediately after trauma may ultimately reduce the incidence of associated systemic inflammatory response syndrome and MOF.

HS and the addition of PTX to conventional RL resuscitation have both been proposed as unique reperfusion regimens with significant anti-inflammatory properties. Both strategies have been shown to independently ameliorate gut permeability, bacterial translocation, neutrophil activation, endothelial dysfunction, and lung injury after hemorrhagic shock.[40–46] However, only PTX has the capacity to downregulate proinflammatory cytokines such as TNF-α,[47,48] indicating that despite their numerous similarities, PTX and HS modulate different steps in the inflammatory cascade and have the potential to act synergistically when administered simultaneously.[22]

Our laboratory has previously demonstrated complete abrogation of villus flattening, mucosal sloughing, and cellular infiltration when HSPTX was administered for post-shock resuscitation.[24] In the present study, we examined the effects of fluid strategy on the induction of NO-dependent pathways responsible for gut injury after ischemia-reperfusion and determined that HSPTX, in contrast to RL, was not associated with activation of iNOS, production of nitrite, induction of NF-κB, and the generation of the proinflammatory cytokines TNF-α, IL-6, and CINC in the intestine after shock.

In this series of experiments, fluid resuscitation was determined by a volume rather than pressure driven model to more closely simulate the clinical practice of 3:1 crystalloid resuscitation currently recommended in the Advanced Trauma Life Support (ATLS) guidelines.[49] On average, 11.9 mL of blood were withdrawn from the RL-treatment arm, and, by the ATLS protocol, those animals should receive an infusion of RL at 33 mL/kg. This closely resembles the actual 32 mL/kg administered to the RL-treatment arm which resulted in an equivalent sodium load to the HSPTX group. Therefore, over-resuscitation in the RL group is unlikely in this model and is supported by our previous study of hemorrhage in which the base deficits between treatment groups did not differ both during and after resuscitation.[24] It should also be noted that a volume control group in which animals were administered 4 mL/kg of crystalloid was not included in this study for comparison because of the fact that in preliminary experiments, animals resuscitated with Ringer's lactate at this low volume experienced inconsistent under-resuscitation and high mortality rates (unpublished observation-R.C.).

Previous studies by Hierholzer et al. have demonstrated that iNOS induction is one of the key events initiating the inflammatory response in the gut after hemorrhage and that activation of the proinflammatory transcription factors

NF-κB and signal transducer and activator of transcription 3 (STAT3) is iNOS-dependent.[12,13] In similar animal models, selective inhibition of iNOS prevented up-regulation of IL-6 and CINC transcription and production.[13,15] These findings are not surprising because numerous proinflammatory mediators, including TNF-α, IL-1β, IL-6, and IL-8, contain regulatory binding sites for NF-κB in their promoter regions. Despite the fact that we have demonstrated a strong association between HSPTX resuscitation and the attenuation NO-mediated gut injury, the underlying mechanism(s) as to how the administration of HSPTX blunts iNOS induction was not determined. From what is known about the actions of both HS and PTX, it is postulated that blood flow-dependent and -independent factors are responsible.

The primary goal of fluid resuscitation is the rapid restitution of circulatory blood flow and normalization of hemodynamic parameters. Interestingly, studies have shown that gastric acidosis persists after restoration of blood pressure to normal levels, indicating continued splanchnic hypoperfusion.[50] Therefore, strategies aimed at restoring the mesenteric circulation should limit mucosal ischemia and iNOS induction. Recently, Rocha e Silva and colleagues have demonstrated significant improvements in gastric oxygenation when HSPTX resuscitation was compared with either RL or HS in an animal model of severe hemorrhagic shock.[51] The explanation for the synergistic effect of HSPTX on the microcirculation is because of multiple factors. Through its hemorrheologic properties, PTX can increase the deformability of red blood cells, and by the same token, HS has been shown to reduce red blood cell fragility after hemorrhagic shock.[52] This increase in flexibility allows red cells to pass through the microcirculatory bed more easily and restore perfusion. Second, HS is superior to RL in decreasing endothelial edema and restoring the diameter of ileal vessels.[53] Finally, the adhesion of neutrophils to arterioles and venules in the microcirculation can lead to vessel occlusion and the cessation of blood flow. Through intravital microscopy, both HS and PTX have each been shown to have less neutrophil rolling, margination, and adhesion when compared with RL.[54]

In addition to its effects on hemodynamics and perfusion, HSPTX can also directly attenuate the respiratory burst of primed neutrophils and the subsequent production of reactive oxygen species commonly seen after ischemia and reperfusion.[22,23] In this study we demonstrated a significant decrease in gut HO-1 expression in HSPTX-treated animals versus those who received RL. HO-1 is an inducible cytoprotective enzyme that serves as a reliable indicator of the degree of oxidative stress within tissues.[32,33] The reduction in intestinal oxidative stress associated with HSPTX is beneficial for two reasons: (1) reactive oxygen species and oxidative stress can produce direct cellular injury and neutrophil activation, and (2) oxygen radicals have the capacity to react with NO and yield a variety of cytotoxic oxidizing agents including peroxynitrite. Peroxynitrite is capable of initiating multiple deleterious effects on the gut including lipid peroxidation, deoxyribonucleic acid (DNA) strand breakage, and mitochondrial enzyme inhibition.[14,55]

Another potential mechanism by which HSPTX may reduce NO-mediated gut injury is through modulation of intracellular signaling cascades. Both HS and PTX are known to cause rapid, dose-dependent increases in intracellular adenosine 3′5′-cyclic monophsophate (cAMP) and activation of protein kinase A (PKA).[44,56] In a study conducted by Chong et al., it was determined that cAMP decreases microvascular permeability in rat mesenteric postcapillary venules, whereas guanosine 3′5′-cyclic monophosphate (cGMP), a mediator if NO signaling, had the opposite effect.[57] Furthermore, it has been demonstrated that the inhibition of extracellular signal-regulated kinase (ERK), a well documented effect of PKA activation,[58] can suppress iNOS synthesis in human colonic epithelial cells in vitro.[59]

Although the effects observed with the utilization of HSPTX as an anti-inflammatory resuscitative fluid are promising, validation in a large animal model and further extended studies evaluating the effects of HSPTX on mortality after trauma must be completed before human use. In summary, the attenuation of gut injury observed with HSPTX in an animal model of hemorrhagic shock is associated with down-regulation of iNOS activity, ileal cytokine production, and oxidative stress when compared with standard RL resuscitation. This novel resuscitation strategy may have future therapeutic potential in the fight against ischemia-reperfusion injury and MOF.

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DISCUSSION

Dr. Gregory P. Victorino (Oakland, California): Dr. Coimbra's group found that resuscitation of hypertonic saline and pentoxifylline decreased several inflammatory mediators in a hemorrhagic shock model. The authors have established themselves on the forefront of resuscitation strategy research, and this study is part of their ongoing work with PTX, which is a phosphodiesterase inhibitor.

I have several questions. No. 1, do you have controls for the volume of your resuscitation fluid? In other words, are your observations a result of a difference in volume from the two resuscitation protocols?

No. 2, you showed a decrease in several markers of inflammation, but what we really want to know, as trauma surgeons, is whether this resuscitation strategy will decrease the incidence of multi-organ failure and death? I was wondering if you had any data on how this strategy affects these important outcome measures.

No. 3, I'm sure that you are thinking long term in wanting to get this resuscitation into the clinical realm. In review, I had some concern about a recent publication in the *British Journal of Pharmacology*, which showed that the combination of pentoxifylline and thiopental actually decreased the survival in their studies. I was wondering if your group was experiencing the same things in your animal model?

And the last question is a theoretical one in that this resuscitation protocol decreased all the inflammatory markers you looked at, and several of these markers were decreased back to normal values. But if I get shot, I don't want a normal immune system. I want an immune system that is up-regulated to fight infection and heal all of my injuries. In other words, we want some immune response, but we don't want over-exaggerated or hyperactivity of the immune response. Will this resuscitation regime enable us to titrate our therapy in order to obtain an appropriate immune response?

Dr. Jessica Deree (San Diego, California): To answer the first question about the volume control, in this series, we used Ringer's lactate at a concentration similar to what would be given during a 3:1 crystalloid resuscitation recommended by ATLS guidelines. Fortunately, this amount of fluid also contained an equivalent sodium load to what was administered to the HSPTX animals. These animals were proven to equal resuscitation according to their base deficit, lactate, and mean arterial pressure readings. Previously, we have done experiments where a group of animals receive 4 mL/kg of Ringer's lactate as a volume control; however, these animals were consistently under-resuscitated and sustained extremely high mortality rates. Therefore, it was not included here.

In this study, we did not use hypertonic saline resuscitation group, either. These studies have been previously completed by our lab, and our main focus with this series was to examine our proposed low volume, anti-inflammatory resuscitation with the current clinical standard. Additionally, in a recent study by Rocha e Silva, it has been demonstrated that HSPTX increases gastric oxygenation and mucosal flow in a large animal model when compared to hypertonic saline alone.

To answer your question regarding the effects of HSPTX on organ failure and death, we have published two articles looking at lung injury and have demonstrated attenuation in histologic lung injury, myeloperoxidase levels, matrix metalloproteinase expression, and the pro-inflammatory cytokine, TNF-α, IL-1, and CINC. We also have preliminary data examining the beneficial effects of HSPTX in the liver as it relates to histology and enzyme levels. We have not yet established differences in mortality because our current model of shock is rarely lethal. We have extended time-course studies to look specifically at this.

With regard to the third question regarding thiopental, in our animal models, we've used a combination of ketamine and xylazine but recently switched to pentobarbital and have never had this experience. In that article you mention, they are studying a model of pancreatitis in rats and demonstrate that the combination of thiopental and PTX results in a significant amount of pulmonary edema and increased mortality. Our lab also has our own model of pancreatitis in which we used pentobarbital, and we have never seen this effect. There have been other papers that have shown no adverse effect with the combination of thiopental and pentoxifylline. One in particular examines the development of lung injury secondary to high-frequency ventilation in the same species of rodent; however, it is something worth noting and is probably important to examine in the future.

Lastly, with the immune system, I agree with you that attenuation of the immune response is good, and inhibition is bad. When we look at immune function, there are basically two major phases seen after trauma, a peak and a lull, so to speak. There is the initial neutrophil mediated phase, which is the exaggerated response we were examining here. And later on, this suppression that we're talking about is usually a T-cell mediated effect that helps to fight off infection. In our original studies from our lab looking at hypertonic saline, we focused on the T-cell mediated effects. When these animals received a second hit of cecal ligation and puncture after

shock and resuscitation, there was actually a decrease in mortality when hypertonic saline was compared to traditional resuscitation. Hopefully, we will have similar findings when these experiments are performed with HSPTX.

Dr. Rao R. Ivatury (Richmond, Virginia): Is this dose-dependent and time-dependent? Why did you choose one-hour post injury? Is there any evidence that ischemia-reperfusion injury is maximal at that time?

Dr. Jessica Deree: Well, organ injury is definitely dependent on the duration and degree of shock. But from our experience, animals at four hours after resuscitation have already developed end organ injury. In addition, this time point is early enough to allow us the opportunity to examine transcription-factor activation and cell signaling to a degree. This is why we chose that time point specifically for this study.