PAPER

Gut and Liver

The Organs Responsible for Increased Nitric Oxide Production After Trauma-Hemorrhage and Resuscitation

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Objective: To determine which organs produce the increased levels of nitric oxide (NO) seen after hemorrhage and resuscitation.

Animals and Interventions: Adult male rats underwent laparotomy (ie, trauma induced) and were bled to and maintained at a mean arterial pressure of 40 mm Hg until 40% of the maximum bleedout volume was returned in the form of Ringer lactate. The rats were then resuscitated with Ringer lactate, 4 times the maximum bleedout volume for 1 hour. Sham-operated animals underwent only the surgical procedure.

Main Outcome Measures: Plasma levels of nitrate/nitrite (NO_3^-/NO_2^-, stable products of NO) were measured by colorimetric assay at the maximum bleedout volume; at the end of hemorrhage; at the end of resuscitation; and 1.5, 4, 8, and 24 hours after resuscitation. In additional rats, the heart, liver, small intestine, kidneys, and spleen were harvested 4 hours after resuscitation for the measurement of NO_3^-/NO_2^- levels. Moreover, tissue perfusion was determined in the above-mentioned organs by radioactive microspheres 4 hours after resuscitation in other groups of animals.

Results: Plasma levels of NO_3^-/NO_2^- were similar to those of sham-operated animals during hemorrhage and at the end of resuscitation. One and a half hours after the end of resuscitation, however, NO production increased significantly. The peak levels of plasma NO_3^-/NO_2^- occurred at 4 hours, and the levels remained elevated even 24 hours after resuscitation. Tissue NO_3^-/NO_2^- levels were significantly increased in the liver, small intestine, and spleen 4 hours after resuscitation. In contrast, the levels of NO_3^-/NO_2^- were similar to those of sham-operated animals in the heart and kidneys at all times. Blood flow in the heart was maintained after hemorrhage, whereas hepatic, intestinal, splenic, and renal perfusion decreased significantly.

Conclusions: The gut and liver seem to be the sites responsible for the increased NO production seen after trauma and hemorrhage. The overproduction of NO is most likely caused by up-regulation of inducible NO synthase. Thus, attempts to reduce NO production using specific inhibitors for inducible NO synthase might be helpful for improving hepatic and intestinal functions after trauma and hemorrhagic shock.

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SEVERE HEMORRHAGIC shock is characterized by circulatory failure with hypotension and loss of vascular reactivity to adrenergic agonists. This leads to tissue damage, multiple organ failure (MOF), and finally death. However, the precise mechanism underlying cardiovascular dysfunction and the subsequent MOF remains unknown. Based on a variety of studies, overproduction of nitric oxide (NO) caused by activation of the L-arginine–NO synthase (NOS) pathway is involved in the sequence of events leading to MOF after hemorrhagic shock.[1-5] Cuzzocrea et al[4] suggest that overproduction of NO may be responsible for the occurrence of MOF after zymosan-induced peritonitis. Two types of NOS have been characterized. Under physiologic conditions, NO release from vascular endothelial cells (through constitutive NOS [cNOS]) regulates vascular tone, blood pressure, and tissue perfusion.[6] After various pathophysiological conditions such as prolonged hemorrhage and sepsis, however, an inducible form of NOS (iNOS) may produce a large amount of NO, which has been implicated in producing cell and organ dysfunctions seen in those circumstances.[4,7-9] It has been shown that tumor necrosis factor and interleukin 1 (IL-1) can stimulate iNOS expression, leading to an overproduction of NO in vascular smooth muscle cells, endothelial cells, and macrophages in vitro[10-12] and in various organs in vivo.[1] Moreover, proinflammatory cytokines such as tumor necrosis factor, IL-1, and IL-6 were elevated after trauma-hemorrhage as early as 45 minutes after the induction of hypotension.[13] These cytokines are involved in pathophysiological responses after trauma-

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MATERIALS AND METHODS

EXPERIMENTAL MODEL

The nonheparinized model of trauma-hemorrhage and resuscitation used in this study was described in detail previously.[14] Briefly, male Sprague-Dawley rats (body weight, 275-340 g; Charles Rivers Labs, Wilmington, Mass) were fasted overnight before the experiment, but they were allowed water ad libitum. The animals were anesthetized with methoxyflurane inhalation, and a 5-cm ventral midline laparotomy was performed to induce tissue trauma before the onset of hemorrhage. The abdominal incision was closed in 2 layers. Both femoral arteries (for blood pressure monitoring and hemorrhage procedure) and a femoral vein (for fluid resuscitation) were cannulated using polyethylene 50 tubing (Clay Adams, Parsipany, NJ). Mean arterial pressure was monitored by attaching a femoral artery catheter to a strain gauge transducer (Micro-Med Inc, Louisville, Ky). All incisions were closed and bathed with a 1% lidocaine solution to provide analgesia throughout the experiment. Immediately after recovery from anesthesia, the animals were rapidly bled to a mean arterial pressure of 40 mm Hg within 10 minutes. This rapid bleeding, on awakening, put the animals in a state of depressed sensibility, thereby minimizing their distress. Mean arterial pressure was then maintained at 40 mm Hg by withdrawing additional blood until the rats could no longer maintain that pressure unless Ringer lactate was infused. This time was defined as maximum bleedout. The rats were maintained at a mean arterial pressure of 40 mm Hg until 40% of shed blood was returned in the form of Ringer lactate. At that time, the rats were resuscitated with 4 times the volume of maximum bleedout with Ringer lactate for 60 minutes. At the end of resuscitation, the catheters were removed, the incisions were closed with sutures, and the rats were returned to their cages. Food and water were allowed ad libitum. This experiment was performed in adherence to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. This project was approved by the Institutional Animal Care and Use Committee of Rhode Island Hospital, Providence.

NITRATE/NITRITE MEASUREMENT

Blood samples were collected by cardiac puncture at maximum bleedout; at the end of hemorrhage; at the end of resuscitation; and 1.5, 4, 8, and 24 hours after the completion of resuscitation. Because multiple sampling may affect various hemodynamic parameters, additional samples were not taken from the same animal at different time points. Thus, there was a cohort of 8 to 10 rats for each time point. Blood samples were collected at the corresponding time points in sham-operated animals. The blood specimens were drawn into a heparinized syringe, immediately placed on ice, and centrifuged at 3000 rpm at 4°C for 10 minutes; then plasma was frozen in aliquots and stored at −80°C until assayed. Some rats were killed by an overdose of anesthesia 4 hours after the completion of resuscitation, and the heart, liver, spleen, kidneys, and entire small intestine were harvested, weighed, and frozen at −70°C for subsequent nitrate/nitrite (NO_3^-/NO_2^-) level determination.

Plasma levels of NO_3^-/NO_2^- were determined colorimetrically (Cayman Chemical Co Inc, Ann Arbor, Mich). Briefly, plasma samples were thawed and then filtered

hemorrhage, possibly through up-regulation of NO production. Although inadequate perfusion has been postulated to produce cell and organ dysfunctions and although splanchnic circulation is thought to be at particularly high risk because of its tendency to be selectively hypoperfused under the shock state, it remains unknown whether altered tissue perfusion after hemorrhage has any effects on NO production. The aim of this study was to determine whether NO production is up-regulated after trauma-hemorrhage and resuscitation and, if so, which organs contribute to the increased NO production.

RESULTS

PLASMA LEVELS OF NO_3^-/NO_2^-

The results shown in **Figure 1** indicate that NO production was similar to that of sham-operated animals during hemorrhage and immediately after the completion of fluid resuscitation. One and a half hours after resuscitation, plasma levels of NO_3^-/NO_2^- increased significantly compared with sham values (23.9±3.2 vs 2.9±1.0 nmol/mL). The peak NO production occurred 4 hours after the completion of fluid resuscitation (82.2±11.8 vs 4.4±1.3 nmol/mL), and the production of NO remained elevated 8 and 24 hours after resuscitation (19.7±3.4 vs 3.5±1.0 and 22.4±5.2 vs 4.0±1.3 nmol/mL, respectively; Figure 1).

TISSUE LEVELS OF NO_3^-/NO_2^-

The results shown in **Figure 2** indicate that 4 hours after the completion of fluid resuscitation, NO_3^-/NO_2^- concentrations were significantly increased in the liver (63.2±15 vs 19.6±14 nmol/g), small intestine (43±12 vs 8±4 nmol/g), and spleen (38±7 vs 14.4±6.7 nmol/g) compared with sham values. The levels of NO_3^-/NO_2^- were similar to sham levels in the heart and kidneys. By multiplying the NO_3^-/NO_2^- concentration per gram of tissue with the weight of each organ (indicated in **Table 1**), we calculated the total content of NO_3^-/NO_2^- in each organ to determine which organ contributes to the increased plasma level of NO_3^-/NO_2^-. Although NO_3^-/NO_2^- concentrations remained significantly increased in the spleen, the results in **Figure 3** indicate that the liver (686±158 vs 210±98 nmol) and small intestine (258±91 vs 51±13 nmol) are the major sources of the increased plasma levels of NO_3^-/NO_2^-.

ORGAN BLOOD FLOW

The results in **Table 2** indicate that blood flow in the liver, kidneys, spleen, and small intestine decrease significantly 4 hours after hemorrhage and fluid resuscitation compared with that of sham-operated animals. The decreased hepatic perfusion was caused by the

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through 30-kd molecular weight cutoff filters (Centricon 30, Amicon Inc, Beverly, Mass). Nitrate in the sample was first converted to nitrite by the addition of nitrate reductase. The presence of nitrite was detected by the addition of Griess reagents and was quantitatively measured by analysis with a spectrophotometer (Bio-Tek EL 311 Microplate Autoreader, Bio-Tek, Winooski, Vt) at 550 nm. Tissue samples were homogenized in phosphate-buffered saline solution and centrifuged at 10 000*g* for 20 minutes. The supernatant was ultracentrifuged at 42 000*g* for 45 minutes at 4°C in a Beckman Avanti J-25I centrifuge (Beckman Instruments Inc, Fullerton, Calif) and then filtered through a 30-kd molecular weight cutoff filter. The presence of nitrite was then determined as described above.

BLOOD FLOW DETERMINATION

In additional groups of animals (n = 8 per group), blood flow in the heart, kidneys, liver, spleen, and small intestine was determined by radioactive microsphere technique. Four hours after the completion of fluid resuscitation, hemorrhaged or sham-operated rats were anesthetized with methoxyflurane, the left carotid artery was cannulated, and the catheter was inserted into the left ventricle by following the left ventricle pulse pressure tracing. The exact position of the catheter in the ventricle was verified at the autopsy. Microspheres labeled with strontium-85 (diameter, 15 µm; specific activity, 4.6×10^8 Bq; Du Pont/NEN, Billerica, Mass) were suspended in 10% dextran solution containing 0.05% polyoxyethylene sorbitan 80 surfactant to prevent aggregation and dispersed with a Vortex shaker for 3 minutes before injection. A 0.3-mL suspension of microspheres was injected into the left ventricle for 20 seconds. A reference blood sample was withdrawn from the femoral artery starting 10 seconds before the onset of microsphere injection and continuing for 90 seconds at a rate of 0.7 mL/min. After the microsphere injection, the left ventricular catheter was flushed with 1 mL of isotonic sodium chloride solution for 60 seconds. Blood pressure was monitored before and after the microsphere injection to ensure that this procedure did not affect mean arterial pressure. At the end of the experiment, the rats were killed with an overdose of pentobarbital sodium. After death, the organs were harvested, rinsed with isotonic sodium chloride solution, blotted, and weighed, and the radioactivity level was counted on a gamma counter (model 1470 Wizard, Wallac, Gaithersburg, Md). The reference sample was transferred to a vial and counted. Organ blood flows (in milliliters per minute per 100 g tissue) were calculated using the following equation:

$$\text{Organ Blood Flow} = (\text{RBF} \times \text{Ct})/\text{Cr} \times 100,$$

where RBF is reference blood sample withdrawal rate (0.7 mL/min); Ct, count per minute per gram of tissue; and Cr, count per minute in the reference blood sample. Portal blood flow was calculated as the sum of the blood flow to the stomach, spleen, pancreas, small intestine, large intestine, and mesentery. Total hepatic blood flow was calculated as the sum of portal blood flow and hepatic arterial blood flow.

STATISTICAL ANALYSIS

All data are expressed as mean±SEM. Differences between experimental groups were considered to be statistically significant if $P<.05$ as determined by 1-way analysis of variance and Tukey or unpaired Student *t* tests.

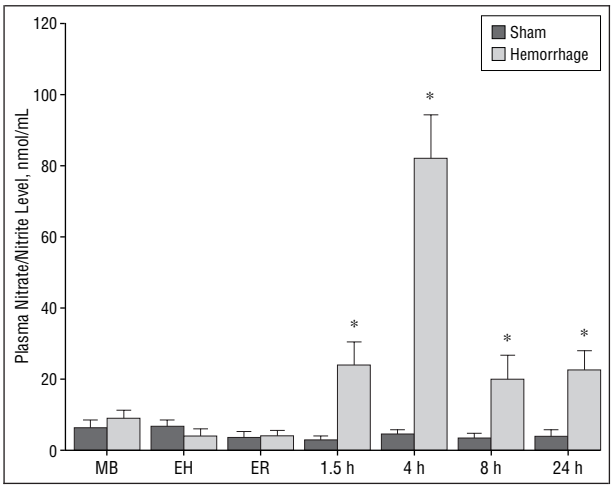

Figure 1. *Alterations in plasma nitrate/nitrite levels after trauma-hemorrhage and fluid resuscitation. Plasma levels of nitrate/nitrite were determined at maximum bleedout (MB); at the end of hemorrhage (EH); at the end of resuscitation (ER); and 1.5, 4, 8, and 24 hours after the completion of fluid resuscitation. There were 8 to 10 animals in each group at each time point. Data are represented as mean±SEM and compared by 1-way analysis of variance and Tukey tests. Asterisk indicates* P<*.05 compared with sham-operated animals.*

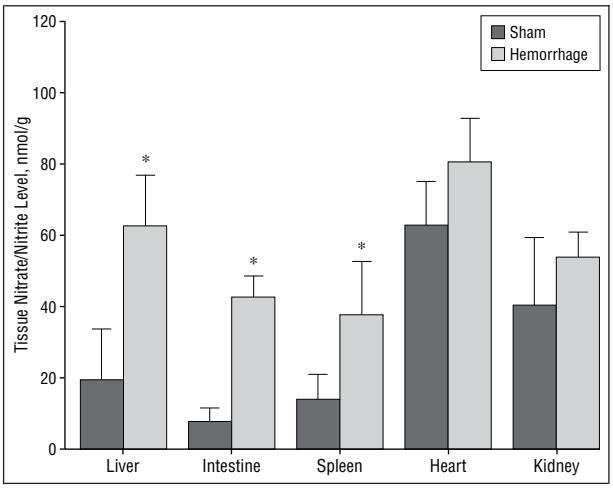

Figure 2. *Alterations in tissue nitrate/nitrite levels 4 hours after the completion of trauma-hemorrhage and fluid resuscitation. There were 8 to 10 animals in each group at each time point. Data are represented as mean±SEM and compared by unpaired Student* t *test. Asterisk indicates* P<*.05 compared with sham-operated animals.*

decreased portal blood flow. In contrast, the myocardial perfusion in hemorrhaged rats was similar to the sham value.

COMMENT

The only known endogenous source of NO_3^-/NO_2^- in mammalian tissues is through the conversion of L-arginine to NO by NOS. Nitric oxide degrades to NO_2^-,

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Table 1. Wet Weight of Various Organs After Sham Operation or Hemorrhage and Resuscitation*

	Organ Wet Weight, g	
	Sham	Hemorrhage
Heart	1.01 ± 0.03	1.03 ± 0.02
Kidneys	2.44 ± 0.11	2.47 ± 0.13
Liver	10.71 ± 0.18	10.88 ± 0.19
Small intestine	6.37 ± 0.21	6.43 ± 0.21
Spleen	0.75 ± 0.03	0.76 ± 0.04

*Organ wet weight was determined 4 hours after the completion of fluid resuscitation in hemorrhaged animals and sham-operated animals. Data are presented as mean ± SEM, with 8 to 10 rats in each group. Analysis with unpaired Student t test indicated that there was no significant difference in organ wet weight between the 2 groups of animals.

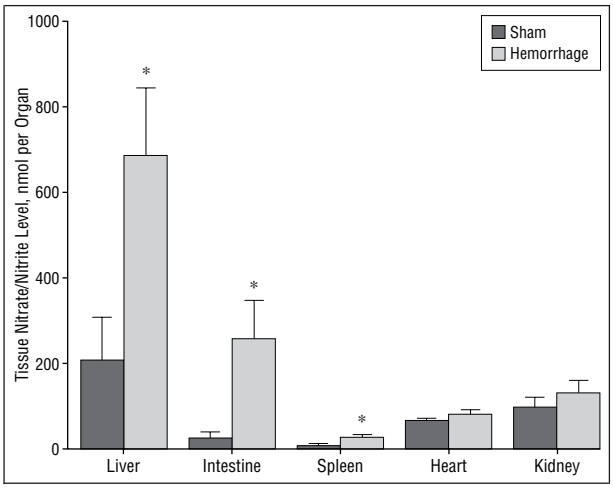

Figure 3. *Alterations in total content of nitrate/nitrite in each organ 4 hours after the completion of trauma-hemorrhage and fluid resuscitation. There were 8 to 10 animals in each group at each time point. Data are represented as mean±SEM and compared by unpaired Student t test. Asterisk indicates P<.05 compared with sham-operated animals. This figure is derived from the data in Figure 2 and Table 1.*

which is then converted to NO_3^- when it reacts with hemoglobin.[15,16] In this study, we examined circulating and tissue levels of NO_3^-/NO_2^-, the stable end products of NO, to estimate NO production after trauma-hemorrhage and resuscitation. Two types of NOS have been identified, the constitutive form and the inducible form. Through the constitutive pathway (ie, via the activation of cNOS), a small amount of NO is generated by vascular endothelial cells in a regulated manner, which seems to play a critical role in the control of local perfusion and blood pressure.[17,18] Previous studies[1] demonstrated a significant increase in the plasma level of NO_3^-/NO_2^- within the first 2 hours of onset of hemorrhage, and it has been postulated that the early increase of NO after hemorrhagic shock might be caused by activation of cNOS. However, this issue remains controversial because several authors[2,3,19-21] reported an impairment in the release of NO from endothelial cells after hemorrhage and resuscitation. Results of studies by Thiemermann et al[1] indicate that approximately 4 hours after the onset of hemorrhage, there was increased iNOS messenger RNA (mRNA) expression in the aorta, lung, liver, and

Table 2. Alterations in Organ Blood Flow After Trauma-Hemorrhage and Resuscitation*

	Organ Blood Flow, mL/min per 100 g	
	Sham	Hemorrhage
Heart	544 ± 63	436 ± 50
Kidneys	985 ± 60	731 ± 71†
Hepatic arterial blood flow	22 ± 2	30 ± 5
Portal blood flow	134 ± 5	90 ± 7†
Total hepatic blood flow	156 ± 6	121 ± 11†
Small intestine	122 ± 8	81 ± 6†
Spleen	172 ± 27	46 ± 4†

*Organ blood flow was determined by radioactive microsphere technique 4 hours after the completion of trauma-hemorrhage and resuscitation. Results are presented as mean ± SEM, with 8 animals in each group. The results were compared by unpaired Student t test.

†P<.05 compared with sham-operated animals.

spleen and that aortic segments isolated from rats after hemorrhage were associated with hyporeactivity to adrenergic agonists and increased iNOS activity in vitro. Kelly et al,[22] using a preheparinized model of severe hemorrhagic shock without subsequent resuscitation, showed that iNOS expression increased in the lungs and liver 5 hours after the onset of hemorrhage. Because the intestine was not examined for the expression of iNOS mRNA in that study,[22] it remains unclear whether the intestine is also involved in producing NO after hemorrhagic shock. Although the previously mentioned studies indicate that iNOS mRNA expression is increased in certain tissues after hemorrhage, such studies did not examine whether tissue levels of NO_3^-/NO_2^- also increased under those conditions. The aim of our study, therefore, was to determine not only the time course of NO production but also whether the peak production of NO was associated with increased tissue levels of NO_3^-/NO_2^-.

A large amount of NO can be produced by cells via the inducible pathway. Inducible NOS has been identified in macrophages, endothelial cells, and several other cell populations, such as vascular and nonvascular smooth muscle cells, cardiomyocytes, and fibroblasts. Expression of iNOS can be stimulated by bacterial products such as endotoxin or by various proinflammatory cytokines such as interferon gamma, IL-1, and tumor necrosis factor.[10,18,19] Because vascular decompensation occurs because of increased NO production, NO released from iNOS might be involved in the delayed peripheral vascular failure seen after severe hemorrhagic shock.[1,23]

Our results demonstrated an increase in plasma levels of NO_3^-/NO_2^- starting 1.5 hours after the completion of fluid resuscitation (ie, approximately 4 hours after the onset of hemorrhagic shock). The peak NO production occurred 4 hours after the completion of fluid resuscitation (ie, approximately 6.5 hours after the onset of hemorrhagic shock) and remained elevated at 24 hours. No change in plasma levels of NO_3^-/NO_2^- was detected during hemorrhagic shock or immediately after the completion of resuscitation. Results of previous studies[19,20] have demonstrated an early endothelial cell dysfunction (ie, reduced release of NO from vascular cNOS) after trauma-

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hemorrhage and resuscitation. Therefore, considering the delayed but prolonged increase in plasma levels of NO_3^-/NO_2^-, we suggest that the increase in NO production in the present study may be caused by up-regulation of iNOS. Four hours after the completion of resuscitation, concentrations of NO_3^-/NO_2^- were significantly increased in the liver, small intestine, and spleen compared with those of sham-operated animals. In view of the small weight of the spleen (approximately 0.75 g), it is unlikely that this organ was a major source for the increase in plasma NO_3^-/NO_2^- levels. In contrast, the liver (approximately 11.00 g) and small intestine (approximately 6.50 g) may represent the major organs responsible for the increased circulating levels of NO_3^-/NO_2^- after hemorrhagic shock and resuscitation. Tissue levels of NO_3^-/NO_2^- were determined 4 hours after the completion of resuscitation only. The reason for selecting this time point was that peak circulating levels of NO_3^-/NO_2^- were observed at this interval after hemorrhage and resuscitation. Whether tissue levels of NO_3^-/NO_2^- remain markedly elevated at subsequent intervals after hemorrhage and resuscitation requires future investigation.

The precise mechanism and cell populations responsible for the increased NO production after trauma-hemorrhage and resuscitation remain unknown. One possible mechanism could be the decreased perfusion of the small intestine because it has been shown that hypoxia induced by low flow conditions augments proinflammatory cytokine release from the liver and gut.[24-28] Moreover, preliminary results indicate that splenic and peritoneal macrophages from rats subjected to hypoxia express iNOS and release a large amount of NO_3^-/NO_2^-. Thus, the decreased blood flow in the liver, small intestine, and spleen after hemorrhage, as demonstrated in the present study (Table 2), may induce a local increase of cytokines that could, in turn, up-regulate iNOS expression, leading to a prolonged increase in tissue and plasma levels of NO_3^-/NO_2^-. Further support for this hypothesis comes from previous studies[29] that demonstrate that plasma levels of tumor necrosis factor significantly increase as early as 45 minutes after the onset of hypotension and remain elevated up to 6 hours after hemorrhage. Therefore, the increased circulating levels of tumor necrosis factor and IL-1 could stimulate iNOS, leading to the increased plasma level of NO_3^-/NO_2^-. It is thus possible that the enhanced formation of NO might be involved in vascular decompensation, organ injury, and the pathophysiological origin of the systemic inflammatory response. Additional support for this hypothesis comes from the fact that many of the cellular elements of the bowel (endothelium, smooth muscle, macrophages, and enterocytes) are capable of expressing iNOS in vitro.[30-32] Furthermore, studies[30-32] have demonstrated up-regulation of iNOS in the gut of rats exposed to infection or proinflammatory cytokines. Similarly, hepatocytes and Kupffer cells represent a large tissue mass with the potential to produce massive amounts of NO after specific stimulation.[33] Further studies are required to determine which cell populations in the liver and small intestine are responsible for the up-regulated NO production after trauma-hemorrhage and resuscitation.

Statement of Clinical Relevance

Hemorrhagic shock and MOF are the leading causes of postinjury death. The postulated mechanism is a "malignant" systemic inflammatory response. The liver and intestine have been identified as etiologic factors in MOF. It has been demonstrated that overproduction of NO by iNOS is one of the important mediators responsible for the cardiovascular dysfunction and inflammatory responses experienced after trauma and hemorrhage. The data presented here show that NO production increased significantly after trauma hemorrhage and resuscitation. In addition, the liver and small intestine seem to be the major organs responsible for NO production under such conditions. Because it is likely that the increased NO production after hemorrhage and resuscitation is caused by up-regulation of iNOS activity, pharmacological agents that inhibit iiNOS might be useful adjuncts for improving hepatic and gut functions and thereby decrease the incidence of MOF and death after trauma and hemorrhage.

Although studies[34] have shown that alveolar and interstitial macrophages produce NO during acute endotoxemia, and this molecule has been implicated as a mediator of acute lung injury in animal models of endotoxemia,[35-37] it remains unknown whether the lung plays any significant role in producing NO during and after trauma and hemorrhagic shock. Because the lung was not examined in our study, whether pulmonary tissues are the major source of the elevated levels of NO_3^-/NO_2^- under such conditions remains to be determined. In addition, although several studies[1,6,9] have suggested that overproduction of NO after various adverse circulatory conditions may be detrimental, studies by Harbrecht et al[21] have indicated that inhibition of NO synthesis by a nonspecific NOS inhibitor after hemorrhagic shock increases hepatic damage. In line with this observation, several studies[2,19,20] have demonstrated that acetylcholine-stimulated (ie, endothelium-dependent) vascular relaxation is depressed after hemorrhagic shock. Because acetylcholine-induced relaxation is mediated by endothelium-derived NO (ie, cNOS-derived NO), it seems that reduction of cNOS-derived NO is detrimental. In light of this, we propose that enhancing endothelial cNOS activity and reducing iNOS activity by specific pharmacological agents may be a useful adjuvant for the management of traumatized hosts. Tissue levels of NO_3^-/NO_2^- may not be the precise marker of local production of NO; further studies are needed to confirm that the increased tissue levels of NO_3^-/NO_2^- are solely caused by up-regulation of iNOS by using Western blot analysis, immunohistochemistry, or enzymatic assay.

In summary, our study demonstrates that NO production increased significantly after trauma-hemorrhage and resuscitation. The liver and small intestine seem to be the major sources of the up-regulated NO production by a mechanism that likely involves the up-regulation of iNOS. The increased production of NO might be responsible for the delayed vascular failure and the organ injury seen after hemorrhagic shock.

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The present study raises important questions for future research, such as whether attempts to decrease the level of up-regulated NO after trauma hemorrhage would be helpful for improving hepatic and intestinal functions under such conditions.

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DISCUSSION

Mitchell Fink, MD, Boston, Mass: You are probably quite aware that NO is a remarkably versatile and potent signaling and effector molecule produced by many different kinds of mammalian cells. I want to emphasize that we are speaking about NO, which is actually nitrogen monoxide, one of the simplest stable molecules in nature, and not nitrous oxide, the gas commonly employed as an adjunct to other volatile anesthetics in the operating room.

In the past few years, severe hemorrhage has been shown to induce increased expression of one of the enzymes responsible for NO production, which, as you have heard, is iNOS, the inducible isoform of nitric oxide synthase. Indeed, it has been proposed by some investigators that so-called irreversible shock following prolonged hemorrhagic hypotension is due to excessive production of the potent vasodilator NO. Other investigators actually have called this notion into question. The purpose of the present study from Dr Chaudry's group was to determine which organs contribute to increased NO production following hemorrhage and resuscitation in rats. Nitric ox-

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ide, because it is quite reactive in biological systems, is difficult to measure directly. Accordingly, the investigators in the study measured levels of nitrite plus nitrate, stable end products derived from NO.

You've heard the results, and I won't summarize those again. I will simply raise a couple of points about the methodology of the paper and raise another point about the implications of the results.

First, the method used to identify the sources of increased NO production is, I think, unusual and possibly open to criticism. In most similar sorts of studies, the sites of inducible NO synthase up-regulation have been identified in one of several mutually compatible ways, including performing Northern analyses seeking to identify the presence of mRNA for inducible NO synthase, performing Western blots looking for evidence of iNOS protein, or, recognizing that the enzymatic activity of the inducible isoform of NO synthase is calcium independent, measuring calcium-dependent and calcium-independent NO synthase activity in tissue homogenates. Both nitrite and nitrate, the products that were measured in this particular study, are hydrophilic, small ions capable of rapidly diffusing from the interstitium of tissues into plasma. One must wonder, therefore, whether changes in tissue nitrite and nitrate levels accurately reflect alterations in local NO production or maybe are simply reflective of changes in regional perfusion. Indeed, the organs showing increased levels of the ions in the present study also showed decrements in perfusion, and so one wonders whether the findings simply reflect diminished washout of NO metabolites that were being produced. Thus, I must ask the presenter whether there are any data showing that tissue nitrite plus nitrate levels actually correlate with measures of tissue NO synthase activity? By the same token, what happens to tissue nitrite and nitrate levels in experimental animals when you artificially raise circulating nitrate levels by infusion of the ion? The ion is nontoxic and that should be a fairly straightforward experiment.

The second point is that the inducible isoform of NO synthase is very tightly controlled in the human system but is on a hair trigger in rats. The iNOS promoter in rats appears to be different in a structural way from the iNOS promoter in humans. For example, following the injection of endotoxin in rats, circulating nitrite plus nitrate levels increase to very high levels. In contrast, in septic humans, although nitrite and nitrate levels are elevated, the changes are much more modest. Therefore, one is prompted to ask, "Do you think the results in this present study in rats actually apply to hemorrhage shock in human beings?"

Dr Catania: To respond to some of the points raised and some of the questions asked, in terms of Northern and Western analysis of the tissues involved, that work is actually under way as we speak. Unfortunately, the results were too preliminary at the time of the abstract deadline to include them. Some of our preliminary results do now in fact indicate that Northern and Western blot show up-regulation of the inducible NO synthase.

In terms of functional production, we were attempting to look at a measure of functional NO production. It has been shown in several different cytokine models that sometimes induction of mRNA expression does not necessarily correlate with functional expression of the active cytokine, and that was why we chose to look at the nitrite and nitrate. We could have also employed the cellular lysate (?) preparations and looked for calcium-dependent and calcium-independent function. We simply chose what we thought was a quicker and easier method.

In terms of correlation between levels of activity of the enzyme and tissue levels, nothing in the literature at this point shows a correlation. We feel that this is one of the first studies that actually shows elevated tissue levels of nitrite and nitrate. I think a very reasonable follow-up study would be to check iNOS activity and correlate that with tissue levels.

And we also did not infuse the ion and simply check to see if it accumulates in tissues. One wouldn't expect that it would accumulate in different tissues at different levels; however, it has never actually been shown that is not the case.

And finally, in terms of applicability to the human organism, NO has been studied quite extensively. In fact, in the last 4 years, there have been over 8000 publications using models as simple as tissue culture all the way through animal models and human models. I think at this point in terms of nitric oxide and its effects on hemorrhage and resuscitation, we are still trying to get a handle on exactly how much of a role it plays, and this basic science work is just intended to demonstrate that there are organs that are adversely affected by trauma and hemorrhage and NO is present in these organs.

Surgical Anatomy

Skeletal or voluntary muscles mostly pass from one bone across a joint (or joints) to another bone, and by contracting they approximate their sites of attachment. Skeletal muscles are under the control of the will and form about 42% of the total body weight.

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