HEMORRHAGIC SHOCK FOLLOWED by resuscitation induces a systemic inflammatory response that may cause multiple organ failure. This systemic inflammatory response appears due, in part, to an increased production of proinflammatory cytokines, especially those produced by the intestine.

In nonseptic shock, it is postulated that the major stimulus for cytokine production could be intestinal ischemia-reperfusion (3, 15). Indeed, using rat models of hemorrhagic shock, we (37) and others (11) have demonstrated that intestinal ischemia-reperfusion parallels cytokine gene expression. However, the exact role of intestinal ischemia-reperfusion in the systemic inflammatory response has not been directly demonstrated.

One possible approach for this evaluation is by assaying the effect on the systemic inflammatory response of interventions that selectively prevent ischemic injury at the level of the intestine. Among the known anti-ischemic interventions, preconditioning with brief transient episodes of ischemia is considered one of the most potent, at least at the level of the heart. Indeed, brief periods of ischemia, separated by reperfusion markedly limit myocardial infarct size after prolonged coronary artery occlusion (19). At the level of the heart, this protection occurs in two phases: early (classic) preconditioning, for which the protective effects are transient and disappear if the period of reperfusion separating brief ischemic episodes and prolonged ischemia extends over 3–6 h (20) and delayed preconditioning (or “second window of protection”), for which protection reappears after 12–24 h (10, 13, 16, 29).

Although not as extensively studied as in the heart, there is also evidence that preconditioning may protect the intestine against ischemia-reperfusion injury (6, 7, 9, 14, 23). Thus preconditioning may be used as a tool to assess the role of intestinal ischemia-reperfusion in systemic inflammatory response. Indeed, if intestinal preconditioning limits the systemic consequences of hemorrhagic shock, this will provide direct evidence that intestinal ischemia-reperfusion injury is a cause of systemic inflammatory response in shock. Moreover, identification of the protective mechanisms of intestinal preconditioning might lead to the discovery of new targets for the treatment of shock states.

Mechanisms of the protective effects of preconditioning (at least in its delayed phase) involve the production of one or more protective proteins, including heat shock proteins (HSP), antioxidant enzymes, or nitric oxide synthase (8, 16, 45). Among the HSPs, recent evidence suggests that the heme-oxygenase-1 (HO-1), which is induced after ischemia or oxidant stress, may exert marked protective effects in ischemia-reperfusion, oxidant injury, or endotoxin shock (14, 24, 27, 36, 44). Indeed, we have shown recently that induction of HO-1 (by hemoglobin) induces a delayed, 24-h prevention of the inflammatory responses to hemorrhagic shock (36). Thus HO-1 may be one of the endogenous enzymes involved in the protective effects of preconditioning.
Thus the purposes of the present study were 1) to assess whether intestinal preconditioning reduces the systemic inflammatory response in a rat model of hemorrhagic shock followed by saline resuscitation, 2) to study the role of HO-1 induction in the anti-inflammatory effects of intestinal preconditioning, and 3) to compare the effect of HO-1 induction to that of bilirubin (40), one of the metabolites of HO-1.

**MATERIALS AND METHODS**

**Induction of Intestinal Preconditioning**

Adult male Wistar rats weighing 300–320 g were used. On day 1, they were anesthetized with 50 mg/kg ip pentobarbital sodium. A laparotomy was performed, and the superior mesenteric artery was exposed. The abdominal area was covered with gauze, soaked in saline at 37°C, and a plastic remnant with gauze, soaked in saline at 37°C, was added to minimize dehydration and evaporative heat loss of exposed tissues.

Preconditioning was induced by four cycles of 1-min mesenteric artery occlusion (arterial clamp) separated by 10 min of reperfusion. After induction of preconditioning, the occluder was removed and the abdominal area was closed using polyester sutures. The animals were allowed to recover from anesthesia (usually within 30 min) after which they were returned to their cage for 24 h. Sham animals were subjected to the same protocol, except that the mesenteric artery was not occluded.

Twenty-four hours after preconditioning or sham surgery (day 2), rats were reanesthetized, the carotid arteries were cannulated for the measurement of blood pressure (Millar 2-F catheter) and induction of hemorrhage, and the jugular vein was cannulated for administration of fluids. In some experiments, rats were subjected to laparotomy, and a Doppler probe (1–1.5 mm internal diameter) was placed around the mesenteric artery and connected to a Triton System 6 signal processor and a chart recorder, to measure mesenteric blood velocity and to calculate mesenteric blood flow.

Hemorrhage was induced by blood withdrawal over a period of 30 min to achieve a mean arterial blood pressure of 40 mmHg (37). This level of blood pressure was maintained for 60 min, and then animals were resuscitated over 20 min by first returning all shed blood, followed by administering warmed NaCl 0.9% in volumes necessary to maintain mitogen-activated protein within 90% of baseline levels. Fluid resuscitation was continued for 2 h, and cumulative fluid requirements were used as an indicator of microvascular injury. At the end of the resuscitation period, carotid and mesenteric blood samples were collected in syringes containing 5 units of heparin, and the intestine and lungs were taken out.

**HO-1 Gene Expression, Tumor Necrosis Factor-α Production, Pulmonary Injury, and Intestinal Lactate Production**

HO-1 gene expression was assessed by semiquantitative RT-PCR. For this purpose, total RNA was extracted from the entire intestinal wall according to a one-step method. RT protocols were performed with 2 μg of total RNA in 30 μl (final volume) of reaction buffer. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. Aliquots of the reverse transcription reaction were amplified with the following rat primer sequences: HO-1: upper 5’-TTGTCCGAGGCTTGAGG-3’, lower 5’-TCAT-GCGAGCAGTAGAGC-3’, size 524; GAPDH: upper 5’-CTGACACTTGGCAGTC-3’, lower 5’-ATGTCAGATCCAC-AAGGA-3’, size 262.

Plasma tumor necrosis factor (TNF-α) concentration was measured with an immunosassay kit (cytoscreen rat TNF-α ultrasensitive ELISA; Biosource International). Blood samples were collected in syringes containing 5 units of heparin. Samples were immediately centrifuged at 10,000 g for 20 min, and plasma was stored at −80°C until it was used for measurement of TNF-α. Plasma was filtered through a 0.22-mm filter before use. The minimal detectable dose of TNF-α was <0.7 pg/ml.

The lung wet weight-to-dry weight ratio was used as an index of pulmonary edema and microvascular injury (33). Lung tissue samples were dried at 80°C for 24 h. Dry tissue weight was then determined, and wet weight-to-dry weight ratios were calculated.

Intestinal lactate accumulation was assessed in intestinal samples homogenized in 2 ml of water/acetone. The homogenates were centrifuged at 15,000 g for 10 min, and the supernatants were vacuum-evaporated and lyophilized. The resulting dried residues were suspended in 1 ml of water for subsequent analysis. Lactate content was determined enzymatically.

**Experimental Protocol**

Assessment of mesenteric blood flow, lung injury, fluid requirement, and lactate and TNF-α production. Four groups of animals were used (n = 20 per group): 1) sham rats subjected to anesthesia and sham surgery on days 1 and 2; 2) untreated hemorrhagic shock, in which the animals were subjected to sham surgery on day 1 and hemorrhagic shock on day 2; 3) preconditioning + hemorrhagic shock (PC + HS), in which the animals were subjected to preconditioning on day 1 and hemorrhagic shock on day 2 (i.e., 24 h after preconditioning); and 4) preconditioning with the HO-1 inhibitor tin protoporphyrin (Sn-PP) plus hemorrhagic shock (PC + Sn-PP + HS), in which the animals were subjected to preconditioning on day 1 followed 24 h later by hemorrhagic shock in the presence of Sn-PP (20 μmol/kg sq) 5 min before hemorrhagic shock. In pilot experiments, we found that Sn-PP did not alter the effects of hemorrhagic shock in the absence of preconditioning (data not shown).

In each of the four groups, 10 rats were used for measurements of mesenteric flow, and 10 rats were used for lung injury as well as lactate and TNF-α production. In one additional group of rats (n = 10), the effect of bilirubin (40 μmol/kg ip) on TNF-α production was assessed.

At the end of hemorrhagic shock, tissue and blood samples were obtained, immediately frozen, and maintained at −80°C until analytical determination of lung injury and lactate and TNF-α production.

**HO-1 induction.** To study the effect of preconditioning on intestinal HO-1 induction, rats were assigned to seven experimental groups (n = 10/group). One group of rats was subjected to sham surgery both on days 1 and 2, whereas the other six groups were subjected to hemorrhagic shock without (time 0) or with 1 or 2 h of restitution 24 h after preconditioning or sham surgery. At the end of each experiment, intestine samples were isolated for the measurement of HO-1 mRNA expression.

**Statistics**

Data are presented as means ± SD. Results were compared using Student’s t-test or ANOVA when appropriate, and P < 0.05 was considered statistically significant.
RESULTS

Survival

All animals survived in the sham groups. One animal of 10 in the hemorrhagic shock group died within 3 h of resuscitation, whereas all animals survived in the hemorrhagic shock group with intestinal preconditioning (PC/H11001 HS).

Mesenteric Blood Flow

In sham rats, mesenteric blood flow (Fig. 1) was maintained around 5.6 ± 0.4 ml·min⁻¹·kg⁻¹ throughout the experiment. In hemorrhagic shock rats, mesenteric blood flow decreased significantly to 1.7 ± 0.4 ml·min⁻¹·kg⁻¹ after hemorrhage and returned to 4.1 ± 0.5 ml·min⁻¹·kg⁻¹ after saline restitution. This level was significantly below baseline. In PC + HS rats, mesenteric blood flow decreased to 1.6 ± 0.3 ml·min⁻¹·kg⁻¹ and returned to levels not significantly different from baseline (5.4 ± 0.5 ml·min⁻¹·kg⁻¹). This value of mesenteric flow was higher than that of the hemorrhagic shock group (P < 0.05). The beneficial effect of preconditioning was abolished by the HO-1 inhibitor Sn-PP; indeed, in this group, blood flow at the end of resuscitation (4.3 ± 0.4 ml·min⁻¹·kg⁻¹) was significantly below baseline and below that observed in the untreated preconditioned group, without significant difference from the control group.

![Fig. 1. Mesenteric blood flow. Effect of intestinal preconditioning on mesenteric blood flow before hemorrhage (time 0), during shock, and after 2 h of saline restitution in sham, hemorrhagic shock (HS), hemorrhagic shock with preconditioning (HS + PC), hemorrhagic shock with preconditioning plus tin protoporphyrin (PC + Sn-PP + HS). *P < 0.05 compared with sham values; n = 10 animals per group. Data represent means ± SD.](http://ajpgi.physiology.org/)

![Fig. 2. Lactate production and lung and vascular injury induced by hemorrhagic shock. A: intestinal lactate concentration (mmol/mg protein) in the intestine at the end of the 2-h restitution period in the groups HS, PC + HS, and PC + Sn-PP + HS. B: cumulative fluid resuscitation volumes during the 2-h restitution period in the 4 groups of rats. C: wet weight-to-dry weight ratios in the lungs isolated from the 4 groups of rats. Data represent means ± SD of 10 animals per group. *P < .05 compared with sham rats; prot, protein.](http://ajpgi.physiology.org/)
Intestinal Lactate Accumulation, Fluid Requirements, and Lung Edema

As compared with sham rats, intestinal lactate accumulation increased in rats subjected to hemorrhagic shock (11 ± 2 vs. 4.0 ± 0.2 mmol/mg protein) (Fig. 2). This increase was prevented by preconditioning (4.2 ± 0.3), whereas the effect of preconditioning was prevented by the HO-1 inhibitor Sn-PP (12 ± 3).

At the end of resuscitation, the cumulative volume of fluids required to maintain blood pressure to a value of ≤90% of baseline was significantly lower after preconditioning than in untreated hemorrhagic shock (untreated = 57 ± 3, preconditioning = 40 ± 2 ml/kg). It must be noted that the mean value from the untreated group (hemorrhagic shock) is underestimated because of the death of one animal before the end of the experiment. The effect of preconditioning on fluid requirement was abolished by the HO-1 inhibitor Sn-PP (55 ± 4 ml/kg).

Hemorrhagic shock increased lung wet weight-to-dry weight ratio from 4.3 ± 0.2 to 6.2 ± 0.3 (P < 0.05). This index of lung edema was reduced by preconditioning (4.8 ± 0.3, P < 0.05), but this effect was abolished by Sn-PP (6.4 ± 0.2).

Plasma Levels of TNF-α

TNF-α was not detectable in the control group without hemorrhagic shock (data not shown). Preconditioning significantly reduced systemic and mesenteric concentrations of TNF-α. However, the decrease in TNF-α production induced by PC was abolished by Sn-PP. The effect of preconditioning on TNF-α could be mimicked by bilirubin (Fig. 3).

HO-1 Induction

As compared with sham rats, hemorrhagic shock without resuscitation did not significantly affect intestinal HO-1 mRNA expression. During saline resuscitation, an increased intestinal HO-1 mRNA expression was observed at 2 h. Compared with untreated rats, PC induced a more than fivefold increase in HO-1 mRNA expression that was present before resuscitation and did not further increase during resuscitation (Fig. 4).

DISCUSSION

The major findings of the present study are that selective prevention of intestinal ischemia-reperfusion injury, achieved by intestinal preconditioning, markedly reduced the systemic consequences of hemorrhagic shock, including systemic inflammatory responses and organ injury.

The protective effects induced by intestinal preconditioning were accompanied by a marked expression of HO-1 and were blocked by an HO-1 inhibitor, suggesting that they are mediated, at least in part, by HO-1.

Ischemic preconditioning refers to a phenomenon whereby a given tissue is rendered resistant to the deleterious effects of prolonged ischemia by previous...
exposure to brief periods of vascular occlusion. Although initially demonstrated at the level of the heart, this concept has been rapidly extended to other organs, including the intestine (6, 7, 9, 23, 30). In this context, the fact that selective prevention of intestinal injury by preconditioning reduces systemic inflammatory response and organ injury demonstrates that ischemia and/or reperfusion injury to the intestine could be a major cause of systemic inflammation in nonseptic shock. This hypothesis is in agreement with the data of Chang (4) who showed that in the absence of intestine, hemorrhagic shock is associated with both an improved outcome and higher hepatic ATP levels in rats.

Given the essential role of free radicals as mediators of systemic inflammatory response after hemorrhagic shock in our model (35), it is likely that the protective mechanisms of preconditioning involve a reduction in oxidative stress. In this regard, Sola et al. (30) showed that the protective effect of intestinal preconditioning was accompanied by a decreased accumulation of xanthine and a diminished conversion of xanthine dehydrogenase to xanthine oxidase, which is a major source of free radicals during reperfusion. Other studies (8, 45) have demonstrated that preconditioning increases expression and/or activity of various antioxidant enzymes, especially superoxide dismutase. There is also evidence that increased production of nitric oxide may be an important mediator of preconditioning, possibly through an interaction with free radicals (7, 9, 31).

In addition to the above-mentioned systems, accumulating evidence suggests that intracellular stress proteins might play a major role in the endogenous defense against oxidative stress. For example, Kume et al. (12) showed that HSP-72 is induced in the liver by short-term warm ischemic preconditioning and that this expression contributes to the reduction of the subsequent ischemia-reperfusion injury of the liver. HO-1 is one of these intracellular stress proteins (HSP-32) (38, 39). Indeed, HO-1 is induced not only by the substrate heme but also by a variety of nonheme substances such as heavy metals, endotoxin, heat shock, cytokines, or prostaglandins (5). Besides its role in heme degradation, including biliverdin/bilirubin, ferritin, and CO may potentially modulate oxidative stress. Indeed, ferritin may confer cytoprotection by sequestering iron, whereas bilirubin and biliverdin are efficient superoxide radical scavengers and may inhibit lipid peroxidation (2, 17, 18). Indeed, we found that treatment of the rats with bilirubin reduced TNF-α to the same extent as preconditioning, indirectly suggesting that this end product may be, at least in part, responsible for the protection induced by HO-1.

With regard to the potential role of CO, we observed that intestinal preconditioning prevents the decrease in mesenteric blood flow and intestinal lactate accumulation in the intestine after hemorrhagic shock. When HO-1 activity was inhibited by Sn-PP, the effect of intestinal preconditioning on mesenteric blood flow and lactate content were abolished. This vascular effect may be due, in part, to CO. CO is a gas molecule that shares some of the properties of NO. It may induce vasodilatation that could be protective in situations of organ injury (34, 43) and also has antiapoptotic and anti-inflammatory properties. Such actions, arising from induced HO-1, would be beneficial to cells or organs under oxidative stress.

In summary, our work indicates that intestinal preconditioning decreases inflammatory response after hemorrhagic shock followed by resuscitation, by using a mechanism involving HO-1 overexpression. This demonstrates directly that ischemia-reperfusion injury of the intestine is a central contributor of the systemic inflammatory response in hemorrhagic shock. Furthermore, identification of the exact mechanisms responsible for HO-1 induction during preconditioning may lead to the development of new pharmacological interventions that mimic this potent endogenous protective pathway and that could be protective in many situations in which oxidative stress has been implicated as a cause for organ injury, such as shock state or vascular surgery.

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REFERENCES

4. Chang TW. Improvement of survival from hemorrhagic shock by enterecstasy in rats: finding to implicate the role of the gut for
