Gut mucosal damage during endotoxic shock is due to mechanisms other than gut ischemia

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Gut mucosal damage during endotoxic shock is due to mechanisms other than gut ischemia. J Appl Physiol 95: 2047–2054, 2003. First published August 15, 2003; 10.1152/japplphysiol.00925.2002.—Whether the gut alterations seen during sepsis are caused by microcirculatory hypoxia or disturbances in cellular metabolic pathways associated with mitochondrial respiration remains controversial. We hypothesized that hypoperfusion or hypoxia and local production of nitric oxide might play an important role in the development of gut mucosal injury during endotoxic shock and investigated their roles by using differing levels of fluid resuscitation and occlusion of the superior mesenteric artery (SMA). Anesthetized New Zealand rabbits were allocated to group I (sham, n = 8); group II [low-dose endotoxin (LPS, Escherichia coli-055:B5, 150 μg/kg/fluid resuscitation (12 ml·kg⁻¹·h⁻¹); n = 8]; group III [high-dose LPS (1 mg/kg)/fluid resuscitation (12 ml·kg⁻¹·h⁻¹); n = 8]; group IV [high-dose LPS (1 mg/kg)/hypovolemia (4 ml·kg⁻¹·h⁻¹ fluids); n = 8]; and group V [SMA ligation/fluid resuscitation (12 ml·kg⁻¹·h⁻¹); n = 4]. Luminal gut lactate concentrations and P O₂ increased in groups IV and V (P < 0.05), reflecting alterations in gut perfusion. Interestingly, significant histological alterations were observed in all LPS groups but not in group V. Blood and luminal gut nitrate/nitrite concentrations increased only in group IV. The mechanism of gut injury in endotoxic shock seems unrelated to hypoxia and release of nitric oxide. Gut dysfunction may occur as a result of so-called “cytopathic hypoxia.”

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cells (11). A number of different biochemical mechanisms or mediators have been suggested to account for cellular dysfunction and cytopathic hypoxia in sepsis, including inactivation of pyruvate dehydrogenase, reversible inhibition of cytochrome oxidase by nitric oxide (NO), inhibition of mitochondrial respiratory complexes by peroxynitrite, and activation of the nuclear enzyme poly(ADP-ribosyl) polymerase (3, 13, 28, 29, 43, 51). We developed a rabbit model to investigate the role of blood flow alterations, tissue hypoxia, and NO release in the development of gut mucosal injury during endotoxic shock.

MATERIALS AND METHODS

Animal model. Thirty-six specific pathogen-free New Zealand rabbits (2.7–3.4 kg body wt) were handled according to the rules of the local Animal Care Committee after institutional approval for animal investigations was obtained. Induction doses of ketamine (20 mg/kg) and xylazine (4 mg/kg) were given intramuscularly for sedation and anesthesia, and were followed by a continuous intravenous infusion of ketamine (15–35 mg·kg⁻¹·h⁻¹) started 3 h after induction. An intravenous catheter was inserted into an ear vein for venous access (Surflo IV catheter, 18 gauge × 2 in.), and 20 ml of saline solution were given as a bolus. Cefazoline (50 mg/kg) and chloramphenicol (12.5 mg/kg) were administered intravenously. Tracheotomy was performed, and the animals were ventilated (Servo ventilator, Siemens, Solna, Sweden) with 40–60% of inspired oxygen fraction, tidal volume of 7–10 ml/kg, and a respiratory rate of 40 breaths/min further adjusted to maintain an arterial P O₂ of >80 Torr and an arterial P CO₂ (P ACO₂) between 35 and 45 Torr.

Surgical procedure. A 16-gauge polyethylene catheter was inserted into the right carotid artery and connected to a pressure transducer to enable continuous recording of arterial pressure. Another catheter (Surflo IV catheter, 22 gauge × 2 in.) was placed in the jugular vein for recording of right atrial pressure and venous access. A midline laparotomy was performed, and ultrasonic tonometry. The bowel segment was carefully rinsed with heated (37°C) physiological saline before a perfusate solution (RPMI 1640 medium, Sigma Chemical, St. Louis, MO) was infused at a rate of 6 ml/h. In addition, 10,000 KIU/ml of aprotinin (Trasylol, Bayer) were added per 10 ml of perfusate solution to inhibit proteolytic activity. The lumen of the proximal catheter was used for continuous infusion, and the lumen of the distal catheter was used to recover the perfusate every hour. GLP samples were taken hourly, centrifuged at 3,000 rpm, filtered through a 0.22-μm filter (Millex, Bedford, OR) to eliminate bacteria, and stored at −80°C.

Experimental protocol. The animals were randomized to four groups, and a fifth group (group V) was added after preliminary analysis of the results of the original four groups (Table 1). Group I ( sham) received placebo and 12 ml·kg⁻¹·h⁻¹ fluids (Ringer lactate). Group II (low-dose LPS/fluid resuscitation) received intravenous 150 μg/kg LPS (Escherichia coli-055:B5, DIFCO Laboratories, Detroit, MI) and 12 ml·kg⁻¹·h⁻¹ fluids. Group III (high-dose LPS/fluid resuscitation) received 1 mg/kg LPS + 12 ml·kg⁻¹·h⁻¹ fluids. Group IV (high-dose LPS/hypovolemia) received 1 mg/kg LPS + 4 ml·kg⁻¹·h⁻¹ fluids. Group V (gut ischemia/fluid resuscitation) received ligation of superior mesenteric artery (SMA) and 12 ml·kg⁻¹·h⁻¹ fluids. All animals received intravenous lactated Ringer solution as fluid resuscitation throughout the experimental protocol and a 20-ml intravenous bolus of hydroxyethyl starch (6%, molecular weight 200,000; D/0.5, Haes-Steril) at the end of the surgical instrumentation. A continuous intravenous infusion of hydroxyethyl starch was started after the bolus in all animals except group IV, to provide a total volume of 20 ml/kg over 5 h. Immediately after baseline measurements, LPS was diluted in normal saline (1 mg/ml) and administered intravenously over 3 min to groups II–IV. Group V had the peritoneum re-opened for ligation of the SMA at this time point.

Analytical methods. Blood-gas analyses were performed on arterial samples (ABL-30, Radiometer, Copenhagen, Denmark). Samples for lactate analysis were stored on ice and analyzed (ABL-30, Radiometer) within 10–30 min. Blood samples were centrifuged immediately (3,000 rpm, 60 min), and plasma was stored at −80°C. NO. Stable end products of NO metabolism, nitrate (NO₃⁻), and nitrite (NO₂⁻) concentrations were determined spectrophotometrically from serum and GLP by using the Griess reaction. Briefly, for NO₂⁻ measurements, the absorbance was measured at 540 nm in a microplate ELISA reader (Titertek multiscan MCC/340, MKII, Eflab, Finland). NO₃⁻ level was determined after stoichiometrical reduction to NO₂⁻.

Tonometry. For tonometry measurements, 1 ml of 0.9% saline was placed in the silicone balloon of the tonometer and allowed to equilibrate for 30 min. The first 0.7 ml aspirated was discharged, and analysis was immediately performed in the remaining 0.3 ml in a blood-gas analyzer (ABL 500 radiometer). Ileum P CO₂ was corrected for incomplete equilibration time during the 30-min sampling periods by multiplying P CO₂ by 1.24 (5). Ileal mucosal-P ACO₂ gradient (P ACO₂ gap) was calculated as the difference between ileal P CO₂ and P ACO₂.

Grading of mucosal damage. At the end of the experiment, each animal received a lethal injection of pentobarbital so-

Table 1. Characteristics of rabbits

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Rabbits</th>
<th>Endotoxin Dose, μg/kg iv</th>
<th>Crystalloids, ml</th>
<th>Colloids, ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1</td>
<td>265(234–296)</td>
<td>68(60–76)</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>2</td>
<td>261(234–289)</td>
<td>67(60–74)</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>3</td>
<td>265(250–273)</td>
<td>67(64–70)</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>4</td>
<td>250(242–273)</td>
<td>64(62–70)</td>
<td></td>
</tr>
</tbody>
</table>

Values are medians (minimum–maximum). *P < 0.05 vs. all groups.
dium, and the ileum was immediately removed. A segment was taken for optical microscopy and fixed in 10% formaldehyde saline followed by sectioning and staining with hematoxylin and eosin. Mucosal histology was graded as previously described (4) by using the following scale: grade 0, normal mucosa; grade 1, subepithelial space formation; grade 2, extension of the subepithelial space with moderate lifting of the epithelial layer from the lamina propria; grade 3, massive epithelial lifting down the sides of the villi; grade 4, denuded villi with lamina propria and dilated capillaries exposed; and grade 5, digestion and disintegration of the lamina propria, hemorrhage, and ulceration.

Data analysis. Results are presented as means ± SD. Significance was tested by repeated-measures ANOVA. Bonferroni adjustment was used for multiple comparisons. Linear regression was used to test the relation between gut lactate release and \( \Delta P_{\text{CO}_2} \) and \( Q_{\text{SMA}} \). The grade of histological damage was evaluated by using a Mann-Whitney rank sum test. A \( P \) value of <0.05 was considered statistically significant.

RESULTS

All groups received a similar amount of fluids, except the hypovolemic group (group IV) (Table 1). Body temperatures were maintained between means of 37.8 and 38.5°C.

Hemodynamics. After LPS administration, mean arterial pressure decreased significantly in group II (low LPS, high fluid; from 76 ± 11 to 52 ± 12 mmHg at 3 h; \( P < 0.05 \), group III (high LPS, high fluid; from 69 ± 6 to 52 ± 7 mmHg at 3 h; \( P < 0.05 \), and especially in group IV (from 68 ± 8 to 38 ± 8 mmHg at 3 h; \( P < 0.05 \) (Fig. 1). At 1, 2, and 3 h, group III had significantly lower mean arterial pressure than group I (56 ± 9 vs. 72 ± 7 mmHg at 1 h; 51 ± 9 vs. 80 ± 14 mmHg at 2 h; and 52 ± 7 vs. 77 ± 18 mmHg at 3 h; \( P < 0.05 \) for all). \( Q_{\text{aorta}} \) was significantly lower at 1 h in group III (49 ± 9 ml/min) and group IV (45 ± 10 ml/min) than in group I (82 ± 37 ml/min) (\( P < 0.05 \) for all). After this transient decline, \( Q_{\text{aorta}} \) returned to baseline in all groups (\( P = \) not significant). \( Q_{\text{SMA}} \) increased significantly above baseline in group III (from 52 ± 9 to 84 ± 12 ml/min at 2 h, 88 ± 17 ml/min at 3 h, and 81 ± 17 ml/min at 4 h; \( P < 0.05 \) for all) (Fig. 1).

Tonometry-derived measurements. \( P_{\Delta CO_2} \) gap increased significantly in group IV (from 1.7 ± 6.6 to 17.2 ± 11.0 Torr at 3 h and to 19 ± 9 Torr at 4 h) and group V (from 6.2 ± 4.0 to 62 ± 38 Torr at 1 h, 64 ± 35 Torr at 2 h, and 69 ± 22 Torr at 4 h) (\( P < 0.05 \) for all) (Fig. 1). \( P_{\Delta CO_2} \) gap was significantly higher in group V compared with all other groups at 1, 2, 3, and 4 h (\( P < 0.05 \) for all).

Lactate measurements. An increase in arterial lactate concentrations occurred in group III (from 2.2 ± 0.6 to 4.3 ± 2.0 meq/l at 4 h; \( P < 0.05 \)) and group IV (from 2.0 ± 0.7 to 6.7 ± 1.3 meq/l at 4 h; \( P < 0.05 \)) (Fig. 2). Lactate concentrations were considerably higher in group IV than in the other groups at all time points (Fig. 2). Luminal gut concentrations increased significantly in group IV (from 0.2 ± 0.1 to 1.1 ± 1.3 meq/l at 4 h; \( P < 0.05 \)) and, especially, in group V (from 0.3 ± 0.3 to 5.6 ± 0.7 meq/l at 4 h; \( P < 0.05 \)) (Fig. 2). In group \( V \), but not in groups II–IV, there was a significant relation between gut lactate release and \( Q_{\text{SMA}} \) (\( r = 0.47, P = 0.0008 \)) and between gut lactate and \( P_{\Delta CO_2} \) gap (\( r = 0.62, P = 0.0001 \)) (Fig. 3).

Serum and gut concentrations of NOx. After LPS administration, \( \text{NO}_2^-/\text{NO}_3^- \) (NOx) concentrations significantly increased in serum and decreased in the GLP in group IV (from 149 ± 32 to 223 ± 55 \( \mu \text{mol/l} \), and from 675 ± 161 to 422 ± 224 \( \mu \text{mol/l} \), respectively;
Both P < 0.05 (Fig. 4). These levels were unaltered in all other groups.

**Histological damage.** All ileum specimens obtained in control animals were normal (degree 0). Mild to severe alterations were observed in all endotoxin groups. No histological alterations were detected after prolonged occlusion of the SMA (group V; Fig. 5).

**DISCUSSION**

Cellular dysfunction and injury secondary to different types of shock are explained usually by a diminished cellular oxygen availability resulting from decreased arterial oxygen tension (hypoxic hypoxia), hemoglobin concentration (anemic hypoxia), or blood flow (stagnant hypoxia). In sepsis, however, a number of studies have suggested that tissue injury can occur despite adequate tissue oxygenation (11), and our findings show convincingly that endotoxemia can induce histological lesions by mechanisms other than tissue hypoxia. Our observations are consistent with the development of so-called "cytopathic hypoxia," i.e., altered oxygen utilization despite adequate oxygen availability in the vicinity of the cell mitochondria. Hypoxia is unlikely to have played a major contribution to the histological lesions observed in our study for several reasons. First, hypoxemia and anemia were absent, and mesenteric blood flow was even increased after generous fluid resuscitation. Even though LPS administration induced an early hypotensive state, mesenteric oxygen delivery was well preserved. Second, mucosal PCO₂ did not increase, and this can reasonably exclude a reduction in regional blood flow (5). Finally, the absence of luminal lactate release in fluid-resuscitated animals suggests the absence of gut ischemia. It was only when hypovolemia was purposely maintained (group IV) or when ischemia was induced by ligation of SMA (group V) that increases in PaCO₂ gap and luminal gut release of lactate occurred.

Our observations are in accord with results from other studies. Fink et al. (13) showed that gut permeability alterations occurred after endotoxin administration but not after a similar mechanically induced reduction in mesenteric flow, suggesting that hypoperfusion by itself is not responsible for increased permeability. In a feline ileum preparation, epithelial necrosis occurred early after LPS administration, despite unaltered ileal tissue oxygen content, blood volume, and blood flow (8). Revelly et al. (33) reported that marked decreases in mucosal ATP content related to permeability and translocation alterations induced by LPS were not improved by massive resuscitation. In an isolated pig hindlimb preparation, the LPS-induced decrease in the skeletal muscle cytochrome aa₃ redox status did not depend on oxygen delivery (14). Finally,

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**Fig. 2.** Serum lactate (A) and luminal gut lactate (B) measurements at baseline (time 0) and hourly. Values are means ± SD. ■, Group I; ○, group II; △, group III; ●, group IV; ◌, group V. *P < 0.05 vs. baseline.

**Fig. 3.** Comparison between Q̇SMA (A) and PCO₂-gap (B) with luminal gut lactate release. ■, Group I; ○, group II; △, group III; ●, group IV; ◌, group V.
in vitro studies indicate that cell dysfunction occurs within minutes of exposure to endotoxin and that a major functional derangement involves the inhibition of pyruvate dehydrogenase activity, pyruvate and lactate accumulation in the cell, and deranged oxidative phosphorylation (11).

Our observations indicate that these changes can occur as early as 4 h after LPS administration. Other studies have also reported that LPS or bacteria can rapidly induce tissue injury. Hersch et al. (20) reported that tissue injury, including the gut, antedated multiple-organ dysfunction in a normotensive sheep model with cecal ligation and perforation. Yu and Martin (49), using the same scoring system for mucosal damage (4) that we used, demonstrated gut mucosal injury in normotensive septic rats 24 h after tracheal instillation of Pseudomonas aeruginosa. Apoptosis is likely to be involved in histological lesions in association with necrosis as a result of cytotoxic mediators. Crouser et al. (8) reported that ileal necrosis and apoptosis could occur as early as 2 and 4 h, respectively, after LPS administration. Hotchkiss et al. (21) found extensive focal crypt epithelial and lymphocyte apoptosis in intestinal tissues obtained intraoperatively from patients with acute traumatic injuries.

A key finding in our study was the absence of histological lesions after prolonged mechanical occlusion of the SMA. In this group, gut hypoxia must have been present and was reflected by the marked rise in PaCO₂ gap in gut lactate production, which was known to be secondary to gut ischemia (40). The well-maintained integrity of the gut mucosal morphology in this group after prolonged ligation of the SMA may be explained by several factors. Initially, the collateral vessels from the caudal mesenteric and celiac axis were not ligated so that some flow could be maintained. Indeed, we confirmed this finding by infusing 5 ml of methylene blue into the right atrium 2 and 3 h after SMA ligation in another rabbit; there was a small amount of methylene blue distributed in a heterogeneous manner in the jejunum and ileum, showing that the collateral vessels maintained some flow. The vascular bed can also compensate for the flow reduction by increasing the oxygen extraction rate. Indeed, blood flow can be decreased to <50% without significantly altering gut oxygen consumption (25). In this range, ileal oxygen consumption could be maintained independent of blood flow. Finally, there was no reperfusion possible, and much histological damage actually occurs during reperfusion. In addition, other cell protective mechanisms, such as induction of heat shock proteins, may have taken place (23).

The involvement of cytopathic hypoxia in the pathogenesis of multiple organ failure may account for the poor results of studies on optimization of oxygen delivery in patients with septic shock (16, 19), except for when the intervention takes place very early to correct other factors such as hypovolemia and cardiac dysfunction (34). This is in contrast with high-risk surgical patients in whom these therapeutic goals are more likely to improve survival (26, 38, 47). Indeed, all efforts to increase cellular oxygen availability may be in vain in the presence of derangements in mitochondrial oxygen metabolism (31).

Even though luminal gut lactate release was not observed, serum lactate concentrations significantly increased after LPS administration, suggesting that

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Fig. 4. Levels of serum (A) and gut luminal perfusate (B) nitrate/nitrite (NOx) at baseline and 4 h after LPS. Values are mean ± SD. ■, Group I; ▼, group II; ■, group III; ●, group IV; ○, group V. aP < 0.05 vs. baseline.

Fig. 5. Column scatterplots represent grades of histological injury in groups. Within-group comparisons: aP < 0.05 vs. group I. bP < 0.05 vs. group II.
blood lactate comes primarily from other sources. Bellomo et al. (2) reported that lactate is even taken up by the gut in early endotoxemia in dogs. Other important sources of lactate in sepsis may be the lung (2, 9) or leukocytes (17). Lactate production in sepsis may arise from sites of inflammation rather than areas of poor perfusion and is related to augmented glycolytic metabolism by inflammatory cells and mitochondrial dysfunction. As in other studies in endotoxic shock, we showed that gut ischemia was present only when fluid replacement was insufficient (13, 50). Adequate fluid replacement was essential to maintain splanchnic perfusion but was unable to prevent endotoxin-induced mucosal injury. In the group with fluid restriction, there was more severe hyperlactatemia compared with the other groups, indicating that in this circumstance, due to hypovolemia, the liver clears lactate less efficiently. In contrast, despite severe mesenteric hypoperfusion, animals in group V had significantly lower lactate concentrations due to better resuscitation.

The pathophysiological mechanisms underlying mucosal gut injury during septic shock remain poorly understood. LPS either by itself or via the release of various cytokines is able to alter cellular function by a NO-dependent pathway (3, 28, 29, 42). Studies with well-controlled conditions of oxygenation and perfusion such as in vitro enterocyte monolayers or Ussing chamber models have shown that NO mimics the action of LPS on permeability to both probe and bacteria (15, 28, 36). Salzman et al. (36) have documented that NO donors dilate tight junctions, cause cytoskeletal disruption, and deplete cellular ATP. Changes in the dynamics of NO deplete intracellular levels of ATP, cause marked derangements in the structure of the actin-based cytoskeleton, and increase intracellular ionized calcium concentration (28). In the present study, plasma or gut luminal NO\textsubscript{2} and NO\textsubscript{3} concentrations did not correlate with LPS-induced ileal mucosal injury, and there was a significant decrease in ileal luminal NOx concentrations compared with baseline during hypovolemia. Crouser et al. (8) also reported that inducible NO synthase induction was not involved in the early ileal epithelial necrosis induced by LPS in a feline model. In pigs with septic shock induced by live bacteria, Snygg et al. (39) noted that jejunal mucosal NO production decreased markedly during severe hypovolemia, whereas stable production was observed after E. coli sepsis. The mechanisms behind the inhibition of ileal mucosal NOx production are not known. Possible mechanisms include the presence of diminished flow and tissue dyoxia secondary to hypovolemia leading to a state of low availability of the substrate L-arginine and/or oxygen, or direct inhibition of cellular enzymes in the intestinal mucosa (39). Thus a very complex relationship between NO and hypoxia or flow may exist, and it is possible that constitutively formed NO plays a role in these changes.

Much of the oxidative injury associated with NO production is mediated by peroxynitrite, a toxic oxidant derived from the reaction of NO and superoxide (29). NO-induced increases in intestinal monolayer permeability can be prevented by peroxynitrite scavengers (28). Agents that interfere with the formation of peroxynitrite, such as superoxide dismutase mimetics and peroxynitrite decomposition catalysts, can also reduce endotoxin-induced cellular injury. In rats, Salvemini et al. (35) found that superoxide dismutase mimetics were able to reduce the LPS-induced increase in microvascular leakage, lipid peroxidation, and epithelial cell injury seen in duodenum and jejunum, suggesting that superoxide and peroxynitrite play a significant role in the pathogenesis of gut injury during endotoxemia. In sepsis or shock, production of peroxynitrite is increased, and it acts as a potent cytotoxic agent producing breaks in cellular DNA. DNA strand breaks activate the repair enzyme poly(ADP-ribose) synthase (PARS), which is able to induce cell energy depletion and increase gut mucosal permeability. There is now increasing evidence that exaggerated PARS activation contributes to cellular injury in sepsis. Jagtap et al. (22) reported that a phenanthridinone inhibitor of PARS was able to ameliorate LPS-induced gut injury in rats. There are few data on other mechanisms involved in LPS-induced gut injury. The naturally occurring protein lactoferrin, by its binding activity to the lipid A portion of LPS, prevented villus atrophy, edema, and vacuolation induced by LPS in the gut of mice; in this model, lactoferrin attenuated the lethal effects of LPS (24).

We acknowledge that the technique used to isolate a closed segment of the gut has some limitations. First, the inflation of the balloons inside the bowel may induce local ischemia and pressure damage. However, histological analysis of normal bowel segment and isolated bowel segment did not show major differences. Second, the gut mucosa could have been affected by the continuous washout by the gut perfusate. We do not believe that luminal perfusion affected the gut mucosa because no histological lesions were detected in control animals and the preparation was able to detect an increase in lactate release and mucosal CO\textsubscript{2} in the presence of a decreased mesenteric blood flow.

In summary, this study indicates that LPS can induce early ileal mucosal injury by mechanisms other than tissue hypoxia and NO release. Fluid resuscitation is fundamental to maintain intestinal blood flow but does not prevent mucosal damage.

REFERENCES


