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

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Gut mucosal damage during endotoxic shock has been associated with the development of organ dysfunction and multiple-organ failure (10, 27). However, the pathophysiological mechanisms underlying sepsis-induced gut injury are unclear, and whether it is caused by microcirculatory hypoxia or disturbances in cellular metabolic pathways associated with mitochondrial respiration remains controversial. Indeed, blood flow redistribution may result in regional ischemia in the gastrointestinal tract during sepsis despite a globally increased systemic oxygen delivery (D\(_{O2}\)). Gut alterations, including ileal mucosal acidosis (1, 12, 37, 45), increase in mucosal permeability (13, 37), and gut mucosal injury (6, 20, 30, 49), have often been reported after administration of lipopolysaccharide (LPS) or live bacteria and in experimental peritonitis. Vallet et al. (44) reported a decrease in ileal mucosal PO\(_2\) in endotoxic dogs, and Hasibeder et al. (18) noted a decrease in jejunal mucosal PO\(_2\) in endotoxic pigs. Others have made similar observations in various animal models (1, 48). Recently, Theisen et al. (41) reported alterations in microvascular blood flow to the gut mucosa in a porcine model of hyperdynamic endotoxic shock, despite mesenteric blood flow being maintained.

However, other studies have suggested normal or elevated blood flow or tissue PO\(_2\) in endotoxic or experimental sepsis. VanderMeer et al. (45) reported ileal mucosal acidosis despite maintained mucosal perfusion and oxygenation in endotoxic pigs in association with normal or even higher mucosal PO\(_2\) levels, as measured with multiwired PO\(_2\) electrodes. Using colored microspheres, Revelly et al. (32) showed an increased mucosal flow to the gut in endotoxic pigs, and Crouser et al. (6) demonstrated that mucosal gut flow was maintained despite mucosal acidosis and alterations in gut oxygen metabolism in a feline ileal preparation. After cecal ligation and puncture in rats, there was a 42% increase in microvascular flow to the mid-jejunum in early sepsis shown by using laser-Doppler flow measurements and colloidal carbon infusion (46).

In addition to the possible effects of hypoperfusion on endotoxin-induced gut injury, LPS may induce alterations in gastrointestinal oxygen metabolism. Changes in ileal oxygen metabolism have been correlated with the severity of mitochondrial injury in feline ileum (7). Cellular alterations referred to as “cytopathic hypoxia” may be implicated in multiple organ failure, in which case production of ATP may be decreased despite normal PO\(_2\) values in the vicinity of mitochondria within.

GUT INJURY SEEN DURING SEPSIS has been associated with the development of organ dysfunction and multiple-organ failure (10, 27). However, the pathophysiological mechanisms underlying sepsis-induced gut injury are unclear, and whether it is caused by microcirculatory hypoxia or disturbances in cellular metabolic pathways associated with mitochondrial respiration remains controversial. Indeed, blood flow redistribution may result in regional ischemia in the gastrointestinal tract during sepsis despite a globally increased systemic oxygen delivery (D\(_{O2}\)). Gut alterations, including ileal mucosal acidosis (1, 12, 37, 45), increase in mucosal permeability (13, 37), and gut mucosal injury (6, 20, 30, 49), have often been reported after administration of lipopolysaccharide (LPS) or live bacteria and in experimental peritonitis. Vallet et al. (44) reported a decrease in ileal mucosal PO\(_2\) in endotoxic dogs, and Hasibeder et al. (18) noted a decrease in jejunal mucosal PO\(_2\) in endotoxic pigs. Others have made similar observations in various animal models (1, 48). Recently, Theisen et al. (41) reported alterations in microvascular blood flow to the gut mucosa in a porcine model of hyperdynamic endotoxic shock, despite mesenteric blood flow being maintained.

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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, including inactivation of pyruvate dehydrogenase, reversible inhibition of cytochrome oxidase by nitric oxide, including inactivation of pyruvate dehydrogenase, 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 water. Once in the maintained, a continuous intravenous infusion of lactated Ringer solution as fluid resuscitation throughout the experimental protocol and a 20-ml intravenous bolus of hydroxyethyl starch (6%, molecular weight 200,000; D0.5, 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 reopened 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 g for 20 min), and plasma was stored at −80°C.

Nitro group products of NO metabolism, nitrite (NO₂⁻), and nitrate (NO₃⁻) 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 stoichiometric 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). Ileal PCO₂ was corrected for incomplete equilibration time during the 30-min sampling periods by multiplying PCO₂ by 1.24 (5). Ileal mucosal-PaCO₂ gradient (PaCO₂ - PmCO₂) was calculated as the difference between ileal PCO₂ and PaCO₂.

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>8</td>
<td>265/234–296</td>
<td>68 (60–76)</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>8</td>
<td>261/234–289</td>
<td>67 (64–70)</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>8</td>
<td>265/250–273</td>
<td>67 (64–70)</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>8</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.
GUT DAMAGE DURING ENDOTOXIC SHOCK

38.5

peratures were maintained between means of 37.8 and the hypovolemic group (group 1) (Table 1). Body tempera-
ture decreased signiﬁcantly.

rank sum test. A logical damage was evaluated by using a Mann-Whitney

grade 1 toxilin and eosin. Mucosal histology was graded as previously

hyde saline followed by sectioning and staining with hemato-
xin, and the ileum was immediately removed. A segment

was taken for optical microscopy and ﬁxed in 10% formalde-
hyde saline followed by sectioning and staining with hemato-
xin 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. Bon-
ferroni adjustment was used for multiple comparisons. Lin-

ear regression was used to test the relation between gut

lactate release and \( P_{\text{aco}_2} \)-gap and \( Q_{\text{SMA}} \). The grade of histo-
lological damage was evaluated by using a Mann-Whitney

rank sum test. A \( P \) value of <0.05 was considered statisti-
cally signiﬁcant.

RESULTS

All groups received a similar amount of ﬂuids, except

the hypovolemic group (group IV) (Table 1). Body tem-

peratures were maintained between means of 37.8 and

38.5°C.

Hemodynamics. After LPS administration, mean ar-
teral 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 tran-
sient 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. \( P_{\text{aco}_2} \)-gap in-

creased 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). \( P_{\text{aco}_2} \) 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 lact-

cate 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 lactate 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

\( P_{\text{aco}_2} \) gap \( (r = 0.62, P = 0.0001) \) (Fig. 3).

Serum and gut concentrations of NO\textsubscript{2}/NO\textsubscript{3}. After

LPS administration, NO\textsubscript{2}/NO\textsubscript{3} (NOx) concentra-
tions 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;

\( *P < 0.05 \) vs. baseline.

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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 endotoxin 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 PCO$_2$ 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 PaCO$_2$ 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 aa$_3$ redox status did not depend on oxygen delivery (14). Finally,

**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 $Q_{\text{SMA}}$ (A) and PCO$_2$-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...
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 administration 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/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.

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