Role of endotoxin in the hypermetabolic state after acute ethanol exposure

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Rivera, Chantal A., Blair U. Bradford, Vitor Seabra, and Ronald G. Thurman. Role of endotoxin in the hypermetabolic state after acute ethanol exposure. Am. J. Physiol. 275 (Gastrointest. Liver Physiol. 38): G1252–G1258, 1998.—This study investigated the role of endotoxin in the hypermetabolic state or swift increase in alcohol metabolism (SIAM) due to acute ethanol exposure. Female Sprague-Dawley rats (100–120 g) were given ethanol (5 g/kg) by gavage. Endotoxin measured in plasma from portal blood was not detectable in saline-treated controls; however, 90 min after ethanol, endotoxin increased to 85 ± 14 pg/ml, and endotoxin clearance was diminished by ~50%. Oxygen uptake in perfused livers was increased 48% by ethanol, and production of PGE2 by isolated Kupffer cells was increased similarly. These effects were blunted by elimination of gram-negative bacteria and endotoxin with antibiotics before ethanol administration. To reproduce ethanol-induced endotoxemia, endotoxin was infused via the mesenteric vein at a rate of 2 ng·kg⁻¹·h⁻¹. Endotoxin mimicked the effect of ethanol on oxygen uptake. The specific Kupffer cell toxicant GdCl3 completely prevented increases in oxygen uptake due to endotoxin. These findings demonstrate that endotoxin plays a pivotal role in SIAM, most likely by stimulating eicosanoid release from Kupffer cells.

Kupffer cells; liver; antibiotics; eicosanoids; gadolinium chloride

ENDOTOXIN IS A cell wall component of gram-negative bacteria that is cleared from the systemic circulation largely by Kupffer cells, the resident macrophages of the liver. Kupffer cells are activated by endotoxin to release toxic free radicals and cytokines that may cause injury to the surrounding parenchyma (11). For example, findings using an experimental model of chronic ethanol exposure demonstrated that destruction of Kupffer cells diminished radical formation and blunted liver injury (1). It is well known that a positive correlation exists between the extent of hepatic injury due to chronic ethanol exposure and blood levels of endotoxin in rats and humans (16, 20). Moreover, treatment of rats with agents that minimize gram-negative bacteria and endotoxin abolished alcohol-induced liver injury (2). Taken together, these findings support the hypothesis that Kupffer cells mediate many of the toxic effects of alcohol on the liver and endotoxin is the likely stimulus for Kupffer cell activation.

Previous work has shown that acute ethanol exposure causes a swift increase in alcohol metabolism (SIAM) characterized by increases in oxygen uptake and ethanol metabolism along with decreased rates of glycolysis (32). Alterations of the hepatic metabolic state have been attributed to the release of mediators such as prostaglandins from activated Kupffer cells (8, 23). In support of this idea, prior destruction of Kupffer cells prevented the hypermetabolic state due to ethanol (5). However, whether endotoxin is a stimulus for Kupffer cell activation during acute ethanol exposure is not known. Therefore, the present study was conducted to test the hypothesis that acute ethanol exposure increases circulating endotoxin to levels sufficient to activate Kupffer cells and induce a hypermetabolic state in the liver.

METHODS

Animal treatment. Ethanol (5 g/kg) or an equal volume of saline was administered by gavage to female Sprague-Dawley rats (100–120 g). One experimental group was treated with the antibiotics polymixin B (150 mg·kg⁻¹·day⁻¹) and neomycin (450 mg·kg⁻¹·day⁻¹) by gavage and in the drinking water for 5 days before ethanol to eliminate gram-negative bacteria and endotoxin (2). Blood ethanol levels were determined from the concentration of ethanol in breath measured by gas chromatography at 15-min intervals (14). After 2.5 h, livers of saline- or ethanol-treated rats were perfused via the portal vein with Krebs-Henseleit buffer (pH 7.4, 37°C) saturated with 95% oxygen-5% carbon dioxide in a hemoglobin-free, nonrecirculating system (25). The superior hepatic vena cava was also cannulated, and oxygen uptake was measured in the effluent perfusate as it flowed past a Clark-type oxygen electrode. Ethanol was infused in the portal vein at a final concentration of 2 mM; ethanol metabolism was determined from the difference between the influent and effluent concentrations by standard enzymatic procedures (4). All rats used in this study received humane care in accordance with institutional guidelines.

Endotoxin measurement. To measure endotoxin, rats were anesthetized, and heparinized blood samples (50 U/ml blood) were collected from the portal vein 30, 60, 90, 120, or 150 min after administration of saline or ethanol (n = 5 rats/time point). Blood was centrifuged at 150 × g for 10 min, and platelet-rich plasma was stored at −80°C. Just before assay, plasma samples were diluted 1:10 and heated to 75°C for 10 min to denature endotoxin-binding proteins that interfere with the assay (12). Tubes used for sample collection, storage, and assay preparation were borosilicate glass heated to 200°C overnight to destroy endotoxin. Endotoxin was measured kinetically using a chromogenic test based on the limulus amebocyte lysate assay (BioWhittaker). Pyrogen-free water and pooled normal rat plasma were used as controls. The concentration of endotoxin in each sample was calculated from a standard curve prepared for each assay.

Endotoxin clearance. Two hours after treatment of rats with saline or ethanol, blood samples were drawn from the tail vein to establish basal endotoxin values. Endotoxin (3

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µg/kg lipopolysaccharide; Escherichia coli serotype 0111:B4; Sigma Chemical, St. Louis, MO) suspended in pyrogen-free saline was administered via the tail vein. Blood samples were collected from the inferior vena cava 2, 4, 7, and 10 min after injection of endotoxin, and clearance was measured in platelet-rich plasma as described above.

Endotoxin infusion. Endotoxin was infused via the portal vein according to the method of Arita et al. (3). Briefly, rats were anesthetized, and the mesenteric vein was cannulated with siliconized PE-10 tubing that was advanced to the level of the portal vein. The cannula was attached to an infusion pump via PE-60 tubing. Endotoxin or an equal volume of pyrogen-free saline (vehicle control) was infused at a rate of 2 ng·kg⁻¹·h⁻¹ for 90 min to approximate levels of endotoxin observed during acute ethanol exposure. After a 30-min recovery period, livers were perfused, and oxygen uptake was measured. In one experimental series, rats were treated with the specific Kupffer cell toxicant GdCl₃ (10 mg/kg) 24 h before the infusion of endotoxin. GdCl₃ was dissolved in acidic saline and administered via the tail vein.

Gut permeability. Gut permeability was measured in isolated intestinal segments using horseradish peroxidase (HRP; 40,000 molecular weight), as described previously (6). Briefly, 4-cm segments of ileum were everted, filled with 1 ml of Tris buffer (125 mM NaCl, 10 mM fructose, and 30 mM Tris; pH 7.5), and ligated at both ends. The filled gut segments were incubated in Tris buffer containing 40 mg/ml HRP. After 45 min, gut sacs were removed and blotted lightly to eliminate excess HRP, and the contents (~750 µl) of each sac were collected carefully using a 1-ml syringe. HRP activity in the contents of each sac was determined spectrophotometrically from the rate of oxidation of pyrogallol as described elsewhere (10).

Prostaglandin measurement. Ethanol (5 g/kg) or an equal volume of saline was administered by gavage to control (untreated) and antibiotic-treated rats (see Animal treatment). Two hours later, Kupffer cells were isolated by collagenase digestion and differential centrifugation as described previously (22). The nonparenchymal cell fraction was centrifuged through a Percoll gradient at 1,000 g for 15 min. Viability determined by trypan blue exclusion was >90%. Cells were seeded on 60-mm culture dishes, and culture medium was exchanged after 1 h to remove nonadherent cells. Kupffer cells were incubated in RPMI-1640 supplemented with 10% fetal bovine serum, 100 U/ml penicillin G, and 100 µg/ml streptomycin sulfate. After 0, 2, or 4 h, the supernatant was collected and stored at −80°C for later determination of PGE₂ by radioimmunoassay (29).

Statistical analysis. Results were analyzed using two-way ANOVA, one-way ANOVA, or Mann-Whitney's rank-sum test as appropriate. P < 0.05 was selected before the study to reflect significance.

RESULTS

Ethanol increases circulating endotoxin. After a bolus dose of ethanol, blood ethanol levels increased rapidly, reached a maximum value of 215 ± 12 mg/100 ml after 1 h, and declined steadily over the next 3 h as expected (Fig. 1A). Endotoxin was measured in blood samples taken from the portal vein using the limulus lysate assay as described in METHODS and was undetectable in rats treated with saline. However, there was a marked transient increase in portal blood endotoxin levels beginning ~30 min after ethanol administration. Endotoxin reached a peak value of 85 ± 14 pg/ml after 90 min and declined to 9 ± 9 pg/ml after ~3 h (Fig. 1B).

Endotoxin reached maximal levels just before the peak of oxygen uptake (Fig. 1C). Antibiotics prevent the hypermetabolic state induced by ethanol. To index the hypermetabolic state, oxygen uptake and ethanol metabolism were measured in livers isolated from rats 2.5 h after treatment with saline or ethanol. Representative traces of hepatic oxygen uptake are shown in Fig. 2. Oxygen uptake reached steady-state levels after ~10 min of perfusion and was 128 ± 10 µmol·g⁻¹·h⁻¹ in livers from saline-treated rats; antibiotics did not alter basal rates of oxygen uptake (130 ± 13 µmol·g⁻¹·h⁻¹). Ethanol increased the rate of hepatic oxygen uptake 190 ± 16 µmol·g⁻¹·h⁻¹ as expected (Fig. 3A). In rats treated with antibiotics for 5 days before given ethanol,however, oxygen uptake only reached 139 ± 6 µmol·g⁻¹·h⁻¹ (Fig. 3A). After the infusion of ethanol directly in the
perfused liver, the rate of ethanol elimination was 37 ± 5 and 47 ± 3 µmol·g⁻¹·h⁻¹ in saline- and antibiotic-treated rats, respectively (Fig. 3B). The rate of ethanol elimination was increased significantly in livers of ethanol-treated rats to 68 ± 5 µmol·g⁻¹·h⁻¹, an effect largely blocked by antibiotics (48 ± 3 µmol·g⁻¹·h⁻¹).

Effect of ethanol on PGE₂ production by isolated Kupffer cells. In a previous study, it was shown that the release of PGE₂, which stimulated hepatocyte oxygen uptake, was increased in Kupffer cells isolated from rats after chronic ethanol exposure (23). To determine if antibiotics block PGE₂ production due to acute ethanol, Kupffer cells were isolated from control or antibiotic-treated rats 2 h after treatment with saline or ethanol. The time course of PGE₂ production by isolated Kupffer cells is shown in Fig. 4. Kupffer cells from saline-treated rats produced 30 ± 6 and 47 ± 3 pg/ml after 2 or 4 h, respectively. Treatment of rats with ethanol 2 h before Kupffer cell isolation increased PGE₂ production significantly, whereas antibiotics blunted the ethanol-stimulated release of PGE₂ to control levels.

Treatment of rats with endotoxin in vivo stimulates hepatic oxygen uptake. To mimic mild endotoxemia caused by ethanol, endotoxin was infused in the portal vein at a rate of 2 ng·kg⁻¹·h⁻¹ for 90 min. The total volume administered over the 90-min infusion was negligible (130 µl). Samples of portal blood were collected immediately after infusion and were analyzed for endotoxin. Levels of endotoxin were 145 ± 91 pg/ml and were not significantly different from levels measured 90 min after ethanol (85 ± 14). Oxygen uptake in livers isolated from rats infused with pyrogen-free saline was 98 ± 4 µmol·g⁻¹·h⁻¹ (Fig. 5), whereas low-dose endotoxin infusion significantly increased oxygen uptake to 234 ± 32 µmol·g⁻¹·h⁻¹. To determine if increases in hepatic oxygen uptake due to endotoxin are dependent on Kupffer cells, one experimental group was treated with the specific Kupffer cell toxicant GdCl₃ 24 h before giving ethanol. Treatment of rats with GdCl₃ completely prevented the increase in oxygen uptake due to exogenous endotoxin infusion (112 ± 12 µmol·g⁻¹·h⁻¹).

Ethanol diminishes endotoxin clearance. In an attempt to determine the cause(s) of increases in circulating endotoxin, gut permeability to HRP was evaluated. In contrast to more commonly used small-molecular weight-markers, HRP approximates the movement of large molecules such as endotoxin and has been shown to penetrate the mucosal barrier via both transcellular and paracellular pathways (28). In the present study, the amount of HRP in gut sacs of saline-treated control

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**Fig. 2.** Representative traces of hepatic oxygen consumption by isolated perfused liver. Typical traces of oxygen uptake by perfused livers of rats treated with saline, ethanol, or ethanol + antibiotics are shown. Traces from livers of rats treated with saline + antibiotics were similar to saline controls (data not shown). Oxygen uptake was measured continuously in the effluent perfusate using a Clark-type oxygen electrode.

**Fig. 3.** Antibiotics blunt the stimulation in oxygen and ethanol uptake due to acute ethanol (EtOH) exposure. Rats were given polymixin B and neomycin as described in METHODS. Oxygen uptake (A) and ethanol metabolism (B) were measured 2.5 h after 5 g/kg ethanol as described in METHODS; n = 6 rats; *P < 0.01 compared with control; #P < 0.05 compared with ethanol. Data were analyzed using 2-way ANOVA with Bonferroni’s post hoc test.
rats after the 45-min incubation period was 1.7 ± 0.3 U/ml. There was no significant increase in movement of HRP across the intestinal mucosa after ethanol treatment (1.5 ± 0.3 U/ml). Because HRP is much smaller than endotoxin, it is unlikely that ethanol increases the absorption of endotoxin. Therefore, the effect of ethanol on the rate of clearance of an injected dose of endotoxin was evaluated. Because a high degree of variability in rates of clearance was observed when rats were injected with endotoxin at levels achieved after ethanol administration, slightly larger amounts were given to produce more consistent results. Peak blood endotoxin levels were 30 ± 2 ng/ml in saline-treated rats and declined to 14 ± 3 ng/ml within 10 min (Fig. 6A). Ethanol significantly increased peak endotoxin to 49 ± 4 ng/ml, and the rate of endotoxin clearance was blunted by ~50% (Fig. 6A and B). Accordingly, the area under the endotoxin elimination curve was increased approximately twofold by ethanol (Fig. 6C).

**DISCUSSION**

Acute ethanol induces mild endotoxemia. It is well known that endotoxemia is associated with Kupffer cell activation and liver pathology due to chronic ethanol exposure (2, 16, 20). The purpose of the present study was to investigate the role of endotoxin in SIAM after acute ethanol exposure. Induction of transient mild endotoxemia was observed after a single dose of ethanol (Fig. 1). Endotoxin concentrations in portal blood reached peak levels before maximal induction of SIAM, which occurs ~2.5 h after ethanol (Fig. 3, B and C). Elimination of gram-negative bacteria and endotoxin prevented the hypermetabolic state (Fig. 3). Moreover, the effect of ethanol on oxygen uptake was mimicked by administering endotoxin at blood levels of endotoxin achieved after ethanol treatment (Fig. 5). Although the participation of other mediators such as catecholamines cannot be ruled out, these findings provide evidence that endotoxemia plays a pivotal role in the hypermetabolic state caused by ethanol.

Endotoxin mimics the effects of acute ethanol exposure. It is well known that endotoxemia resulting from burns, trauma, and sepsis is associated with a hypermetabolic state in humans (9, 24). Arita et al. (3) reported that endotoxemia significantly increased the resting metabolic expenditure of guinea pigs. However, these findings do not reflect the effects of ethanol, since levels of endotoxin used were as much as 1,000-fold higher than would be expected after acute ethanol exposure (Fig. 1B). Here, a model of intravenous infusion was employed to create mild endotoxemia. Blood levels of endotoxin achieved as a result of infusion were comparable to levels observed after treatment of rats with ethanol for similar times (Fig. 1). Infusion of endotoxin in this manner stimulated a Kupffer cell-dependent hypermetabolic state (Fig. 5). These findings demonstrate that even very mild endotoxemia is sufficient to increase hepatic oxygen uptake.

**Fig. 4.** Effect of ethanol on PGE2 production by isolated Kupffer cells. Kupffer cells were isolated 2 h after saline or ethanol treatment and were cultured for up to 4 h. PGE2 was measured in the culture medium by radioimmunoassay; n = 5 rats; *P < 0.05 using 2-way ANOVA with Bonferroni’s post hoc test.

**Fig. 5.** Effect of in vivo endotoxin infusion on hepatic oxygen uptake by the isolated perfused liver. Endotoxin (2 ng·kg⁻¹·h⁻¹) or an equal volume of pyrogen-free saline was infused directly in the portal vein for 90 min. One experimental group was treated with GdCl3 before infusion as described in METHODS. Livers were perfused, and oxygen uptake was measured; n = 5 rats; *P < 0.05 compared with saline; #P < 0.05 compared with the endotoxin group using Kruskal-Wallis 1-way ANOVA.
Mechanism of ethanol-induced endotoxemia. Endotoxin resides in the lower gut where gram-negative bacteria predominate. Under pathological conditions that compromise gut mucosal integrity, endotoxin can escape into the portal blood system where it is cleared largely by Kupffer cells, which are strategically placed along the sinusoids (31); endothelial cells also clear endotoxin to a lesser extent (27). Removal and catabolism of endotoxin by macrophages are primarily achieved through phagocytosis via scavenger receptors as reviewed elsewhere (15). Phagocytosis by Kupffer cells in vitro is inhibited by the addition of ethanol directly to the culture medium (18). Moreover, acute tolerance to tumor necrosis factor-α production by isolated Kupffer cells caused by ethanol was blocked by antibiotics. Treatment with ethanol in vivo reduced the rate of endotoxin clearance of high levels of endotoxin in rats and septic mice (13, 21). Results presented here support the findings that acute ethanol blunts endotoxin clearance (Fig. 6). Because no alteration in ileal permeability to HRP was noted, data presented here support the idea that the observed accumulation of endotoxin in portal blood is most likely due to decreased phagocytosis by macrophages. However, changes in permeability of the lower gut where endotoxin predominates cannot be ruled out.

Kupffer cell participation in the hypermetabolic state is mediated by endotoxin. Destruction of Kupffer cells with GdCl₃ largely blocked SIAM in a previous study (5), demonstrating that the hypermetabolic state is Kupffer cell dependent. However, the mechanism of Kupffer cell activation during ethanol exposure has not been identified. As demonstrated in Fig. 5, SIAM can be

![Fig. 6. Effect of ethanol on endotoxin clearance. Two hours after saline or ethanol treatment, endotoxin (3 µg/kg) was administered to rats via the tail vein. Rats were anesthetized, and blood samples were collected from the vena cava after 2, 4, 7, and 10 min. Endotoxin was measured in plasma as described in METHODS. Data are presented as means ± SE of at least 4 observations. A: time course of endotoxin clearance. *P < 0.05 using 2-way repeated-measures ANOVA. B: linear regression of clearance data from A. Slopes of the linear regression of saline and ethanol data are 0.07 and 0.03, respectively. C: average area under the curve; n = 5 rats; *P < 0.05 using Mann-Whitney's rank-sum test.](http://ajpgi.physiology.org/)

![Fig. 7. Proposed mechanism of the effect of alcohol and endotoxin on Kupffer cell activity. Data demonstrate that endotoxin (Etx) clearance, probably via the scavenger pathway, is diminished by alcohol, leading to a transient increase in blood endotoxin levels. Ethanol also increases lipopolysaccharide-binding protein (LBP), an opsonin necessary for endotoxin binding to CD14 receptors. Enhanced Kupffer cell activation via endotoxin-CD14 complexes is a likely result of these phenomena. It is well known that endotoxin-LBP binding to CD14 causes an increase in intracellular calcium in Kupffer cells, an important second messenger in the production of mediators such as PGE₂. This occurs largely via voltage-dependent calcium channels (VDCC). Here, acute ethanol stimulated PGE₂ production by isolated Kupffer cells, an effect blocked by elimination of gram-negative bacteria and endotoxin with antibiotics. Importantly, findings reported here provide direct evidence that the hepatic hypermetabolic state (swift increase in alcohol metabolism) caused by ethanol exposure is mediated by endotoxin and involves the release of factors such as PGE₂, which is known to stimulate oxygen uptake in hepatocytes.](http://ajpgi.physiology.org/)
mimicked with low-dose endotoxin infusion; this phenomenon is also blocked by destruction of Kupffer cells. Because antibiotics also blunted SIAM, it is concluded that endotoxin is responsible for activating Kupffer cells during acute ethanol exposure.

Endotoxin binding to CD14 receptors on Kupffer cells is known to be a potent activator of these cells. For endotoxin to be recognized by CD14, it must first form a complex with lipopolysaccharide-binding protein (LBP; see Ref. 26). Recent studies demonstrated that ethanol exposure upregulated serum LBP levels after only 2 h; however, there was no change in Kupffer cell CD14 expression (17). Because acute ethanol decreases endotoxin clearance, endotoxin increases in blood and binds to LBP. Kupffer cell activation by the endotoxin-LBP complex binding to CD14 is the most likely outcome. The consequence of the interaction of endotoxin with Kupffer cells via CD14 is the release of free radicals, cytokines, and eicosanoids (30). Eicosanoids play an important role in stimulating hepatic metabolism during endotoxemia (7, 8, 24). One of the major eicosanoids produced is PGE$_2$, which is responsible for the stimulation of hepatic respiration after ethanol exposure (23). Eicosanoids are primarily synthesized by Kupffer cells (66%) but also by hepatic endothelial cells (22%) and to a lesser extent by parenchymal cells (16%); see Ref. 19). Elimination of gram-negative bacteria and endotoxin from the gut before ethanol exposure prevented the production of PGE$_2$ by isolated Kupffer cells (Fig. 4) and blunted the hypermetabolic state (Fig. 3), supporting the hypothesis that endotoxin activates Kupffer cells to release eicosanoids that trigger SIAM.

Working hypothesis. The proposed role of endotoxin and Kupffer cells in SIAM is summarized in Fig. 7. It is concluded that acute ethanol administration rapidly diminishes the phagocytic ability of Kupffer cells, probably by inhibiting scavenger receptor function. This results in higher circulating levels of endotoxin, which complex with LBP. Elevated endotoxin-LBP complexes then bind to CD14 receptors on Kupffer cells and trigger the release of mediators such as PGE$_2$, which stimulate hepatic oxygen consumption and ethanol metabolism. This study provides the first direct evidence that endotoxemia plays a critical role in the hypermetabolic state after acute ethanol exposure and supports the hypothesis that endotoxin mediates many of the hepatic effects of ethanol.

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