Estriol sensitizes rat Kupffer cells via gut-derived endotoxin

NOBUYUKI ENOMOTO,1 SHUNHEI YAMASHINA,1 PETER SCHEMMER,1 CHANTAL A. RIVERA,1 BLAIR U. BRADFORD,2 AYAKO ENOMOTO,2 DAVID A. BRENNER,2 AND RONALD G. THURMAN1

1Laboratory of Hepatobiology and Toxicology and Department of Pharmacology, and 2Division of Digestive Diseases and Nutrition and Department of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599

Enomoto, Nobuyuki, Shunhei Yamashina, Peter Schemmer, Chantal A. Rivera, Blair U. Bradford, Ayako Enomoto, David A. Brenner, and Ronald G. Thurman. Estriol sensitizes rat Kupffer cells via gut-derived endotoxin. Am. J. Physiol. 277 (Gastrointest. Liver Physiol. 40): G671–G677, 1999.—The relationship between gender and alcohol-induced liver disease is complex; however, endotoxin is most likely involved. Recently, it was reported that estriol activated Kupffer cells by upregulation of the endotoxin receptor CD14. Therefore, the purpose of this work was to study how estriol sensitizes Kupffer cells. Rats were given estriol (20 mg/kg ip), and Kupffer cells were isolated 24 h later. After addition of lipopolysaccharide (LPS), intracellular 

**ENDOTOXIN** (lipopolysaccharide [LPS]) is a component of the outer wall of gram-negative bacteria that causes many biological effects, including lethal shock and multiple organ failure. Kupffer cells, resident macrophages in the liver, not only remove gut-derived endotoxin but also are activated during the process to produce chemical mediators [i.e., eicosanoids, interleukin (IL)-1, IL-6, tumor necrosis factor-α (TNF-α), superoxide, and nitric oxide]. Kupffer cells contain voltage-dependent 

**lipopolysaccharide; tumor necrosis factor-α; CD14; intracellular calcium**
peroxidase, as described previously (5). Briefly, 8-cm segments of ileum were everted, filled with 1 ml of Tris buffer (125 mmol/l NaCl, 10 mmol/l fructose, 30 mmol/l Tris, pH 7.5), and ligated at both ends. The filled gut segments were incubated in Tris buffer containing 40 mg/100 ml of horseradish peroxidase (5). After 45 min, gut sacs were removed and blotted lightly to eliminate excess horseradish peroxidase, and the contents (750 µl) of each sac were collected carefully with a 1-ml syringe. Horseradish peroxidase activity in the contents of each sac was determined spectrophotometrically from the rate of oxidation of pyrogallol, as described elsewhere (5).

Kupffer cell preparation and culture. Kupffer cells were isolated by collagenase digestion and differential centrifugation with use of Percoll (Pharmacia, Uppsala, Sweden), as described elsewhere with slight modifications (22). Briefly, the liver was perfused through the portal vein with Ca2+ and Mg2+-free Hanks’ balanced salt solution at 37°C for 5 min at a flow rate of 26 ml/min. Subsequently, the liver was perfused with Hanks’ balanced salt solution containing 0.025% collagenase IV (Sigma Chemical) at 37°C for 5 min. After the liver was digested, it was excised and cut into small pieces in collagenase buffer. The suspension was filtered through nylon gauze mesh, and the filtrate was centrifuged at 450 g for 10 min at 4°C. Cell pellets were resuspended in buffer, parenchymal cells were removed by centrifugation at 50 g for 3 min, and the nonparenchymal cell fraction was washed twice with buffer. Cells were centrifuged on a density cushion of Percoll at 1,000 g for 15 min, and the Kupffer cell fraction was collected and washed with buffer again. Viability of cells determined by trypan blue exclusion was >90%. Cells were seeded onto 25-mm glass coverslips and cultured in DMEM (GIBCO Laboratories Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum and antibiotics (100 U/ml of penicillin G and 100 µg/ml of streptomycin sulfate) at 37°C with 5% CO2. Nonadherent cells were removed after 1 h by replacement of buffer, and cells were cultured for 24 h before experiments. Basically, Kupffer cells were incubated 24 h after seeding. For intracellular Ca2+ concentration ([Ca2+]i) measurements, Kupffer cells were prepared after seeding for 24 h on coverslips and loaded with fura 2 for 30 min. For TNF-α production, Kupffer cells were prepared after seeding for 24 h on 24-well plates, LPS containing medium was added, and samples were collected after 4 h for measurement by ELISA. In the case of CD14, Kupffer cells were prepared after seeding for 24 h on 6-cm

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Table 1. Effect of estriol on mortality due to LPS

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mortality, %</th>
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<tr>
<td>LPS 0 (0/4)</td>
<td></td>
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<tr>
<td>Estriol + LPS 50 (4/8)*</td>
<td></td>
</tr>
<tr>
<td>Antibiotics + estriol + LPS</td>
<td>0 (0/4)†</td>
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Rats were given estriol (20 mg/kg ip) 24 h before a sublethal dose of lipopolysaccharide (LPS, 5 mg/kg iv) via the tail vein. Some rats were treated with antibiotics. Data represent 24-h mortality rates; numbers in parentheses represent dead animals/total. *P < 0.05 vs. LPS. †P < 0.05 vs. estriol + LPS (Fisher’s test).

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Fig. 1. Photomicrographs (hematoxylin and eosin) of liver tissue. A: no treatment. B: 24 h after lipopolysaccharide (LPS, 5 mg/kg iv, Escherichia coli serotype 0111:B4, Sigma Chemical). C: 24 h of estriol exposure and 24 h of LPS. D: antibiotics for 4 days (150 mg/kg·day-1 of polymyxin B and 450 mg/kg·day-1 of neomycin), estriol for 24 h, and LPS for 24 h. Original magnification, ×100. Typical photomicrographs are shown.
culture dishes, and total protein extracts were obtained as described above. These times are optimal for each experiment. Time courses based on previous studies showed that cell seeding for 24 h is also optimal.

Measurement of $[\text{Ca}^2+]_i$. $[\text{Ca}^2+]_i$ was measured fluorometrically using the Ca$^{2+}$ indicator dye fura 2 and a microspectrofluorometer (PTI, South Brunswick, NJ) interfaced with an inverted microscope (Diaphot, Nikon, Japan). Kupffer cells were incubated in modified Hanks' buffer (115 mmol/l NaCl, 5 mmol/l KCl, 0.3 mmol/l Na$_2$HPO$_4$, 0.4 mmol/l KH$_2$PO$_4$, 5.6 mmol/l glucose, 0.8 mmol/l MgSO$_4$, 1.26 mmol/l CaCl$_2$, 15 mmol/l HEPES, pH 7.4) containing 5 µmol/l fura 2-AM (Molecular Probes, Eugene, OR) and 0.03% Pluronic F-127 (BASF Wyandotte, Wyandotte, MI) at room temperature for 60 min. Coverslips plated with Kupffer cells were rinsed and placed in chambers with buffer at room temperature. Changes in fluorescence intensity of fura 2 at excitation wavelengths of 340 and 380 nm and emission of 510 nm were monitored in individual Kupffer cells. Each value was corrected for dilution.

Western blotting for CD14. Kupffer cells were seeded onto 24-well plates and cultured in DMEM supplemented with 10% fetal bovine serum and antibiotics at 37°C in the presence of 5% CO$_2$. Cells were incubated with fresh media containing LPS (100 ng/ml supplemented with 5% rat serum) for an additional 4 h. Samples of media were collected and kept at −80°C until assay. TNF-α in the culture media was measured using an ELISA kit (Genzyme, Cambridge, MA), and data were corrected for dilution.

RESULTS

Effect of estriol on mortality due to endotoxin. To assess the effect of estriol on endotoxin shock, rats were given an intraperitoneal injection of estriol 24 h before an intravenous injection of a sublethal dose of endotoxin (LPS) via the tail vein. Table 1 depicts mortality 24 h after LPS. Obviously, all control rats survived for 24 h after a sublethal injection of LPS (5 mg/kg); however, 50% mortality was observed in rats given estriol 24 h earlier (20 mg/kg), confirming early work. Interestingly, mortality due to LPS in estriol-treated rats was prevented totally by gut sterilization with antibiotics, indicating that gut-derived endotoxin is involved in this phenomenon.

Effect of estriol and LPS on liver histology and serum transaminases. Liver specimens were collected for histology 24 h after administration of LPS (5 mg/kg). Histology was normal in control rats (Fig. 1A), whereas LPS caused focal necrosis and neutrophil infiltration in the liver, as expected (Fig. 1B). Twenty-four hours after estriol treatment, necrosis and neutrophil infiltration due to LPS were increased dramatically (Fig. 1C). These histological changes due to estriol were blunted by treatment with antibiotics (Fig. 1D).

Western blotting for CD14. Total protein extracts of cultured Kupffer cells were obtained by homogenizing samples in a buffer containing 10 mmol/l HEPES, pH 7.6, 25% glycerol, 420 mmol/l NaCl, 1.5 mmol/l MgCl$_2$, 0.2 mmol/l EDTA, 0.5 mmol/l dithiothreitol, 40 mg/ml bestatin, 20 mmol/l β-glycerophosphate, 10 mmol/l 4-nitrophenylphosphate, 0.5 mmol/l Pefabloc, 0.7 mg/ml pepstatin A, 2 mg/ml aprotinin, 50 mmol/l Na$_2$VO$_4$, and 0.5 mmg/ml leupeptin. Protein concentration was determined using the Bradford assay kit (Bio-Rad Laboratories, Hercules, CA). Extracted protein was separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were blocked by Tris-buffered saline-Tween 20 containing 5% skim milk and probed first with mouse anti-rat ED9 monoclonal antibody (Serotec, Oxford, UK), then with horseradish peroxidase-conjugated secondary antibody as appropriate. Membranes were incubated with a chemiluminescence substrate (enhanced chemiluminescence reagent, Amersham Life Science, Buckinghamshire, UK) and exposed to X-OMAT films (Eastman Kodak, Rochester, NY). Statistical analysis.Values are means ± SE. Statistical differences between means were determined using ANOVA or ANOVA on ranks as appropriate. P < 0.05 was selected before the study to reflect significance.
were collected 24 h later for serum AST and ALT measurements (Fig. 2). Mean values for AST and ALT in the control group were low, whereas values were increased slightly to 71 ± 3 and 64 ± 7 IU/l, respectively, with LPS treatment (5 mg/kg). In contrast, LPS increased transaminases dramatically to >700 IU/l in estriol-treated rats. This increase was also blunted significantly by antibiotics.

Effect of estriol treatment on gut permeability and portal endotoxin levels. Two hours after estriol treatment, gut permeability was increased dramatically (Fig. 3A). Levels were about threefold higher than values from control rats; however, permeability was not affected by treatment with antibiotics. Levels of endotoxin in peripheral blood plasma from rats in this study were below the limits of detection. Interestingly, portal endotoxin levels of 6 ± 3 pg/ml in normal rats were increased by estriol administration to 61 ± 19 pg/ml (Fig. 3B), an effect blocked by antibiotics.

Effect of estriol on LPS-induced increases in [Ca^{2+}] in Kupffer cells. As reported previously, LPS increases [Ca^{2+}] transiently in isolated Kupffer cells. Here, Kupffer cells from control female rats exhibited small increases in [Ca^{2+}] with 100 ng/ml LPS to 83 ± 6 nmol/l (Figs. 4A and 5A). [Ca^{2+}] in Kupffer cells from estriol-treated female rats, however, was increased to levels about threefold higher, confirming previous work (16) (Figs. 4B and 5A). This phenomenon was also blocked by treatment with antibiotics (Figs. 4C and 5A), indicating that the effect of estriol on Kupffer cells is dependent on gut-derived endotoxin. In contrast, addition of estriol in vitro did not alter LPS-induced increases in [Ca^{2+}], indicating that the effect of estriol on Kupffer cells is not direct. Although Kupffer cells from male control rats exhibited similar increases in [Ca^{2+}] with 100 ng/ml LPS to 114 ± 33 nmol/l, estriol had no effect on this phenomenon (Table 2). In castrated female rats, control Kupffer cells also showed similar increases in [Ca^{2+}] with LPS.
Table 2. Effect of estriol on LPS-induced increases in \([\text{Ca}^{2+}]_i\) in isolated Kupffer cells from male and female rats

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<thead>
<tr>
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<th>Estriol</th>
<th>([\text{Ca}^{2+}]_i), nM</th>
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<tbody>
<tr>
<td>Male</td>
<td>–</td>
<td>104 ± 4</td>
</tr>
<tr>
<td>Male</td>
<td>+</td>
<td>294 ± 33†</td>
</tr>
<tr>
<td>Ovariectomized male</td>
<td>–</td>
<td>114 ± 9</td>
</tr>
<tr>
<td>Ovariectomized male</td>
<td>+</td>
<td>145 ± 10*</td>
</tr>
<tr>
<td>Female</td>
<td>–</td>
<td>93 ± 6</td>
</tr>
<tr>
<td>Female</td>
<td>+</td>
<td>83 ± 6</td>
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</tbody>
</table>

Values are means ± SE of 4 rats per group. Intracellular \([\text{Ca}^{2+}]_i\) concentration (\([\text{Ca}^{2+}]_i\)) in isolated Kupffer cells was measured fluorometrically using fura 2. *P < 0.05 vs. ovariectomized control. †P < 0.05 vs. female control by ANOVA with Bonferroni’s post hoc test.
(2). Indeed, hepatic triglycerides were increased about 3-fold in women but only 1.5-fold in men 6 h after a single dose of ethanol (18). Therefore, estriol may have an additive effect on alcohol-induced fat accumulation in the liver.

On the other hand, Iimuro et al. (14) showed that plasma endotoxin levels were significantly higher in women than in men after exposure to ethanol. In the present study, estriol increased portal endotoxin via mechanisms most likely dependent on gut permeability (Fig. 3A), consistent with the hypothesis that higher plasma endotoxin levels lead to more extensive Kupffer cell activation in women than in men.

Recently, it was shown that ethanol-induced sensitization of Kupffer cells was caused by gut-derived endotoxin and involved increases in CD14 (9). Farhat et al. (10) demonstrated that estrogen promoted vasodilatation and stimulated microvascular permeability (6). It is also possible that treatment with estriol increases gram-negative bacterial species, a major source of endotoxin in the gut microflora, in the portal vein via increased gut permeability. In this study it was demonstrated that estriol indeed increased permeability of the isolated small intestine (Fig. 3A). As expected, permeability was not affected by treatment with antibiotics. This led to increases in plasma endotoxin, a phenomenon that was prevented by treatment with antibiotics (Fig. 3B). Thus it is concluded that estriol increases portal endotoxin by increasing gut permeability.

Kupffer cells are involved in potentiation of LPS-induced liver injury by estriol. In the present study it was demonstrated that pharmacological doses of estriol similar to levels encountered in late pregnancy increased mortality due to LPS (Table 1), confirming experiments by Nolan and Ali (21) and Ikejima et al. (16). This effect of estriol was impressive; however, precise mechanisms remain unclear. It is well known that macrophages, including Kupffer cells, contribute to the pathophysiology of endotoxin shock. Indeed, gadolinium chloride, a Kupffer cell toxicant, totally prevented mortality due to estrogen plus LPS (16), indicating that Kupffer cells are necessary for this phenomenon. Furthermore, antibiotics prevented mortality due to estrogen plus LPS (16), indicating that gut-derived endotoxin is also necessary for this phenomenon (Fig. 3B).

Kupffer cells are activated by endotoxin, leading to rapid increases in \([\text{Ca}^{2+}]\) followed by release of inflammatory mediators (e.g., cytokines and lipid metabolites) as well as reactive oxygen intermediates (20, 26, 30). TNF-\(\alpha\) is produced predominantly by the monocyte-macrophage lineage, and the predominant cell type of this lineage is the hepatic Kupffer cell (8). Moreover, \([\text{Ca}^{2+}]\) is required for LPS-induced expression of TNF-\(\alpha\) by a macrophage cell line (31). Increased TNF-\(\alpha\) plays a pivotal role in endotoxin shock and related multiple organ failure (28), and anti-TNF-\(\alpha\) antibody prevents it (29). TNF-\(\alpha\) stimulates generation of toxic superoxide anion from mitochondrial complex III in parenchymal cells, expression of factors for neutrophil chemotaxis (IL-8/CINC, MIP, MIP-2), and expression of intracellular adhesion molecule-1, leading to microcirculatory disturbances (19). However, the effect of estriol on Kupffer cells has not been studied in much detail. To try to understand the mechanisms of Kupffer cell sensitivity, here LPS-induced [\(\text{Ca}^{2+}\)], TNF-\(\alpha\), and CD14 were monitored in Kupffer cells. LPS-induced production of TNF-\(\alpha\) was enhanced in Kupffer cells isolated from rats treated with estriol (Fig. 5B). Patterns of LPS-stimulated increases in [\(\text{Ca}^{2+}\)] (Fig. 5A) and CD14 (Fig. 6) were similar. Thus it is concluded that estriol sensitizes Kupffer cells to endotoxin.

Estriol increases expression of CD14 in Kupffer cells. CD14 is a functional LPS/LBP receptor on Kupffer cells. Recently, it was demonstrated that ethanol-induced sensitization of Kupffer cells was caused by gut-derived endotoxin via mechanisms dependent on CD14 (9). Here, CD14 was increased by estriol treatment in vivo (Fig. 6), an effect that was blocked by antibiotics (Fig. 6). This most likely explains why estriol treatment increased [\(\text{Ca}^{2+}\)] and TNF-\(\alpha\) due to LPS in isolated Kupffer cells (Figs. 4 and 5). It is concluded that CD14 expression on Kupffer cells is increased by estriol, thereby increasing toxic mediator production by mechanisms involving gut-derived endotoxin. Our working hypothesis is that estriol increases gut permeability, leading to elevated portal endotoxin. Fearns and colleagues (11) reported that CD14 is increased by exposure to LPS, and upregulation of CD14 sensitizes Kupffer cells to LPS (9).

In summary, Kupffer cells isolated from rats treated with estriol exhibited sensitization to LPS. This phenomenon involves increases in gut permeability, elevated endotoxin levels, and increased CD14 expression on Kupffer cells.

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Address for reprint requests and other correspondence: R. G. Thurman, Laboratory of Hepatobiology and Toxicology, Dept. of Pharmacology, CB 7365, Mary Ellen Jones Bldg., University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7365.

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