Estrogen increases sensitivity of hepatic Kupffer cells to endotoxin

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[1]*Laboratory of Hepatobiology and Toxicology, Department of Pharmacology,*
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Ikejima, Kenichi, Nobuyuki Enomoto, Yuji Iimuro, Ayako Ikejima, Dawn Fang, Juliana Xu, Donald T. Forman, David A. Brenner, and Ronald G. Thurman. Estrogen increases sensitivity of hepatic Kupffer cells to endotoxin. *Am. J. Physiol.* 274 (*Gastrointest. Liver Physiol.* 37): G669–G676, 1998.—The relationship among gender, lipopolysaccharide (LPS), and liver disease is complex. Accordingly, the effect of estrogen on activation of Kupffer cells by endotoxin was studied. All rats given estrogen intraperitoneally 24 h before an injection of a sublethal dose of LPS (5 mg/kg) died within 24 h, whereas none of the control rats died. Mortality was prevented totally by pretreatment with gadolinium chloride, a Kupffer cell toxicant. Peak serum tumor necrosis factor-α (TNF-α) values as well as TNF-α mRNA in the liver after LPS were twice as high in the estrogen-treated group as in the untreated controls. Plasma nitrite levels and inducible nitric oxide synthase in the liver were also elevated significantly in estrogen-treated rats 6 h after LPS. Furthermore, Kupffer cells isolated from estrogen-treated rats produced about twice as much TNF-α and nitrite as controls did in response to LPS. In addition, Kupffer cells from estrogen-treated rats required 15-fold lower amounts of LPS to increase intracellular Ca^{2+} than controls did, and Kupffer cells from estrogen-treated animals expressed more CD14, the receptor for LPS/LPS binding protein, than controls. Moreover, estrogen treatment increased LPS binding protein mRNA dramatically in liver in 6–24 h. It is concluded that estrogen treatment in vivo sensitizes Kupffer cells to LPS, leading to increased toxic mediator production by the liver.

lipopolysaccharide; tumor necrosis factor-α; nitric oxide; intracellular calcium; CD14

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 are also activated during the process (21) to produce chemical mediators [i.e., eicosanoids, interleukin-1 (IL-1), IL-6, tumor necrosis factor-α (TNF-α), superoxide, and nitric oxide (NO)]. Kupffer cells contain voltage-dependent Ca^{2+} channels (11), and intracellular Ca^{2+} is an important second messenger in the production and release of chemical mediators (5, 15). Indeed, Ca^{2+} channel blockers increased graft survival after transplantation (24) and reduced liver injury due to alcohol (12), presumably by preventing activation of Kupffer cells.

It is well known that sensitivity to endotoxin in vivo is increased during pregnancy, when estrogen levels are high. In 1935, Apitz (1) demonstrated that pregnant animals are more susceptible than nonpregnant animals to a generalized Shwartzman reaction induced by endotoxin. Furthermore, after a single injection of endotoxin, pregnant rats develop more severe inflammation and necrosis in the liver than nonpregnant rats (28). In addition, the syndrome of hemolysis, elevated liver enzymes, and low platelet count (HELLP syndrome), a serious complication of some preeclamptic and eclamptic patients (27), is mimicked by LPS treatment in pregnant animals (20). However, it is unclear how estrogen increases liver injury in pregnancy. One possibility is that female hormones alter susceptibility of the liver to endotoxin. The purpose of this study, therefore, was to evaluate the hypothesis that estrogen enhances the sensitivity of Kupffer cells to endotoxin.

MATERIALS AND METHODS

Estrogen treatment in vivo. Female Sprague-Dawley rats weighing between 200 and 250 g were used for all experiments. All animals were given humane care in compliance with institutional guidelines. Rats were given an intraperitoneal injection of estrogen (20 mg/kg estriol; Sigma Chemical, St. Louis, MO) 24 h before experiments. All control rats received saline vehicle without estrogen. A sublethal dose of LPS (5 mg/kg, *Escherichia coli* 0111:B4; Sigma Chemical) was injected intravenously via the tail vein, and survival was assessed after 24 h. Some rats were given gadolinium chloride (GdCl$_3$; 10 mg/kg in saline) intravenously 24 h before estrogen treatment. While rats were under pentobarbital anesthesia, serum and liver samples were collected at 1.5, 3, 6, 12, and 24 h after estrogen treatment and 1, 3, and 6 h after LPS injection and kept frozen at $-80°$C until assay.

Measurement of serum estrogen levels. Serum samples were collected 1.5 and 24 h after intraperitoneal injection of estriol and were stored frozen at $-20°$C until assay. Serum estriol levels were determined by RIA (31). The amount of ^{125}I-labeled estriol bound to antibody is inversely proportional to the concentration of the unlabeled estriol present. Separation of free and bound antigen is rapidly achieved using a double antibody system (19). An ultrasensitive unconjugated estriol procedure was employed (DSL-1400, Diagnostic Systems Laboratories, Webster, TX).

Blood sampling and measurement of TNF-α. Serial blood samples were collected for TNF-α determination as reported previously (14). Briefly, an intravenous catheter was placed into the femoral vein under methoxyflurane anesthesia (Metofane, Pittman-Moore, Mundelein, IL), and blood was drawn from a catheter before and 60, 150, 210, and 300 min after LPS injection (5 mg/kg). We collected 200 µl of whole blood

and then injected the same volume of lactated Ringer solution at each time point. Serum was mixed with the protease inhibitor aprotinin (Sigma Chemical) immediately, and samples were stored at $-80°C$ until assay. Serum TNF-α levels were measured using an ELISA kit (Genzyme, Cambridge, MA), and data were corrected for dilution.

Measurement of plasma nitrite levels. Some animals were killed before and 6 h after injection of LPS (5 mg/kg) to obtain plasma samples for the measurement of nitrite, which was determined colorimetrically using the Griess reaction (8). Briefly, 500 µl of plasma were mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthalene-ethylene-diamine dihydrochloride in 15% H_3PO_4) and incubated for 5 min at room temperature. The resulting product, *N*-(1-naphthyl)ethylenediamine, was quantitated spectrophotometrically at 550 nm. Nitrite levels were calculated using a standard curve generated with known concentrations of sodium nitrite.

Western blotting for inducible NO synthase and CD14. Total protein extracts of the liver or cultured Kupffer cells were obtained by homogenizing samples in a buffer containing 10 mM HEPES, pH 7.6, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 40 µg/ml Bestatin, 20 mM β-glycerophosphate, 10 mM 4-nitrophenyl phosphate, 0.5 mM Pefabloc, 0.7 µg/ml pepstatin A, 2 µg/ml aprotinin, 50 µM Na_3VO_4, and 0.5 µg/ml leupeptin. Protein concentration was determined using the Bradford assay kit (Bio-Rad Laboratories, Hercules, CA). Extracted protein was separated by 7.5% and 10% SDS-PAGE for inducible NO synthase (iNOS) and CD14, respectively, and transferred to polyvinylidene fluoride membranes. Membranes were blocked by Tris-buffered saline-Tween 20 containing 5% skim milk, probed with rabbit anti-mouse iNOS polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or mouse anti-rat ED9 monoclonal antibody (Serotec, Oxford, United Kingdom), followed by horseradish peroxidase-conjugated secondary antibody as appropriate. Membranes were incubated with a chemiluminescence substrate (ECL reagent, Amersham Life Science, Buckinghamshire, United Kingdom) and exposed to X-OMAT films.

RNA preparation, RT-PCR, and Northern blotting. Total liver RNA was prepared by guanidium-CsCl centrifugation as described previously (2). The integrity and concentration of RNA was determined by measuring absorbance at 260 nm followed by electrophoresis on agarose gels.

First-strand cDNA was transcribed from 1 µg RNA using Moloney murine leukemia virus RT (Life Technologies, Gaithersburg, MD) and an oligo(dT)$_{16}$ primer. PCR was performed using GeneAmp PCR system 9600 (Perkin Elmer, Foster City, CA). We amplified 1 µl of cDNA in a 50 µl reaction buffer containing 10 pmol of forward and reverse primers, 2.5 U *Taq* DNA polymerase, 250 mM 2′-deoxynucleoside 5′-triphosphates (dNTPs), and 1× PCR buffer (Perkin Elmer). The primer sets used in this study are shown in Table 1. The reaction mixture without enzyme and dNTPs was heated at 100°C for 4 min, and then a mixture of *Taq* polymerase and dNTPs was added at 80°C. Thereafter, 30 cycles of denaturing at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s followed by final extension at 72°C for 7 min were carried out. The size of PCR products was verified by electrophoresis in 1% agarose gel followed by ethidium bromide staining. Densitometrical analysis using NIH image software was performed for semiquantification of PCR products.

For Northern blotting, total RNA (10 µg) was separated in 1% agarose gel containing formaldehyde followed by capillary transfer to nylon membranes. Membranes were prehybrid-

Table 1. *Sequence of primer sets used for RT-PCR*

	Primers		Size of PCR Products, bp
iNOS	Forward:	5′-TAG AAA CAA CAG GAA CCT ACC A-3′	907
	Reverse:	5′-ACA GGG GTG ATG CTC CCG GAC A-3′	
TNF-α	Forward:	5′-CAC CAT GAG CAC GGA AAG CA-3′	692
	Reverse:	5′-GCA ATG ACT CCA AAG TAG ACC-3′	
IFN-γ	Forward:	5′-AAG ACA ACC AGG CCA TCA GCA-3′	547
	Reverse:	5′-AGC CAC AGT GTG AGT TCA GTC-3′	
IL-12	Forward:	5′-ATG GCC ATG TGG GAG CTG GA-3′	335
	Reverse:	5′-TTT GGT GCT TCA CAC TTC AG-3′	
β-Actin	Forward:	5′-ACC ACA GCT GAG AGG GAA ATC G-3′	281
	Reverse:	5′-AGA GGT CTT TAC GGA TGT CAA CG-3′	

iNOS, inducible nitric oxide synthase. TNF-α, tumor necrosis factor-α. IFN-γ, interferon-γ. IL-12, interleukin-12.

ized in a buffer containing 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), 100 µg/ml salmon sperm DNA, and 1× Denhardt's solution and hybridized in the same buffer with 10×10^6 cpm of random prime-labeled cDNA for LPS binding protein (LBP) overnight. Membranes were then washed with 2× SSC and 0.1% SDS at 50°C for 30 min and 0.1× SSC and 0.1% SDS at 55°C for 30 min and subjected to autoradiography. Subsequently, membranes were stripped and reprobed using cDNA for glyceraldehyde-3-phosphate dehydrogenase.

Kupffer cell isolation and culture. Kupffer cells from estrogen or vehicle-treated rats were isolated by collagenase digestion and differential centrifugation, using density gradients of Percoll (Pharmacia, Uppsala, Sweden) as described previously with slight modifications (23). Briefly, the liver was perfused in situ through the portal vein with Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (HBSS) containing 0.5 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA) at 37°C for 5 min at a flow rate of 26 ml/min. Subsequently, perfusion was with HBSS 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, and the filtrate was centrifuged twice at 50 *g* at 4°C for 3 min to remove parenchymal cells. The nonparenchymal cell fraction was washed with buffer and centrifuged on a density cushion of Percoll at 1,000 *g* for 15 min to obtain the Kupffer cell fraction, followed by washing with buffer again. The viability of isolated Kupffer cells was determined by trypan blue exclusion and routinely exceeded 90%. Cells were seeded onto 24-well culture plates (Corning, Corning, NY) or 25-mm glass coverslips at a concentration of 5×10^5 and cultured in DMEM (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U/ml of penicillin G and 100 µg/ml of streptomycin sulfate) at 37°C with 5% CO_2. Nonadherent cells were removed after 1 h by replacing the culture medium. All adherent cells phagocytosed latex beads, indicating that they were

Kupffer cells. Cells were cultured for 24 h before experiments. Cells seeded onto 24-well culture plates were incubated with fresh medium containing LPS (100 ng/ml, supplemented with 5% rat serum) for an additional 4 or 24 h, and samples were collected for TNF-α and nitrite measurements, respectively. Samples were kept at −80°C until assay. TNF-α and nitrite in the culture medium were determined by ELISA and the Griess reaction, respectively, as described above.

Culture of RAW 264.7 cells. RAW 264.7 cells, a mouse macrophage cell line, were cultured in DMEM (Gibco) containing 10% FBS and antibiotics at 37°C in 5% CO_2. Total RNA from RAW 264.7 cells was prepared using Trizol reagent (Life Technologies) according to the manufacturer's suggested protocol.

Measurement of intracellular Ca^{2+}. Intracellular Ca^{2+} in individual Kupffer cells was measured fluorometrically using the fluorescent Ca^{2+} indicator dye fura 2 and a microspectrofluorometer (Photon Technology International, South Brunswick, NJ) interfaced with an inverted microscope (Diaphot, Nikon). Kupffer cells cultured on coverslips were incubated in modified Hanks' buffer (115 mM NaCl, 5 mM KCl, 0.3 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 5.6 mM glucose, 0.8 mM $MgSO_4$, 1.26 mM $CaCl_2$, 15 mM HEPES, pH 7.4) containing 5 µM fura 2-AM (Molecular Probes, Eugene, OR) and 0.03% Pluronic F127 (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 at 510 nm were monitored in individual Kupffer cells. Each value was corrected by subtracting the system dark noise and autofluorescence, assessed by quenching fura 2 fluorescence with Mn^{2+} as described previously (11). Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was determined from the equation $[Ca^{2+}]_i = K_d[(R - R_{min})/(R_{max} - R)](F_0/F_s)$ where F_0/F_s is the ratio of fluorescence intensities evoked by 380 nm light from fura 2-pentapotassium salt loaded in cells using a buffer containing 3 mM EGTA and 1 µM ionomycin ($[Ca^{2+}]_{min}$) or 10 mM Ca^{2+} and 1 µM ionomycin ($[Ca^{2+}]_{max}$). R is the ratio of fluorescence intensities at excitation wavelengths of 340 and 380 nm, and R_{max} and R_{min} are values of R at $[Ca^{2+}]_{max}$ and $[Ca^{2+}]_{min}$, respectively. The values of these constants were determined at the end of each experiment, and a dissociation constant (K_d) of 135 nM was used (9).

Statistical analysis. All results except mortality data were expressed as means ± SE. Mortality was assessed using Fisher's test. Statistical differences between means were determined using analysis of variance (ANOVA) or ANOVA on ranks as appropriate. $P < 0.05$ was selected before the study to reflect significance.

RESULTS

Effect of estrogen on mortality after LPS injection. To assess the effect of estrogen on endotoxin shock, rats were given an intraperitoneal injection of estriol 24 h before intravenous injection of a sublethal dose of LPS via the tail vein. Serum estriol levels 1.5 and 24 h after estriol injection were 27 ± 9 and 6 ± 2 nM, respectively. Estriol in serum from controls was below detection limits. Figure 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, 100% mortality was observed in rats given estriol 24 h previously (20 mg/kg). Interestingly, mortality due to LPS in estrogen-treated rats was prevented totally by
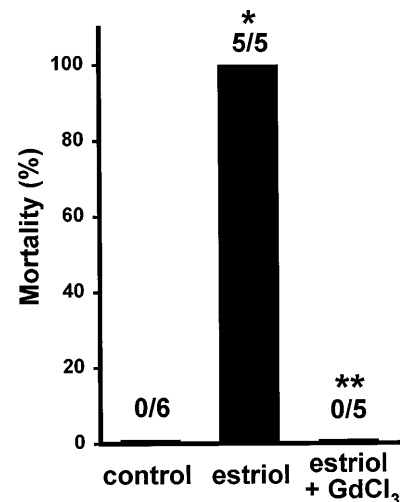

Fig. 1. Effect of estrogen on mortality due to lipopolysaccharide (LPS). Rats were given an intraperitoneal injection of estrogen (20 mg/kg estriol) 24 h before intravenous injection of a sublethal dose of LPS (5 mg/kg) via the tail vein. Some rats were pretreated with $GdCl_3$ (10 mg/kg, iv), a Kupffer cell toxicant, before estrogen treatment. Data represent 24-h mortality rates. * $P < 0.05$ vs. control. ** $P < 0.05$ vs. estriol, Fisher's test. Nos. above bars represent no. of dead animals per total no. of animals.

pretreatment with $GdCl_3$, a Kupffer cell toxicant, indicating that Kupffer cells are involved in this phenomenon.

Effect of estrogen on TNF-α production after LPS injection. Because TNF-α is a pivotal cytokine involved in the development of endotoxin shock, serum TNF-α levels were measured in estrogen-treated rats after LPS injection (Fig. 2A). As expected, serum TNF-α increased dramatically 150 min after injection of LPS (5 mg/kg) with a subsequent gradual decrease. Peak levels of TNF-α were twice as high in the estrogen-treated group as in the controls. Furthermore, mRNA for TNF-α in the liver was detected by RT-PCR (Fig. 2B). As expected, TNF-α mRNA was increased 1 h after LPS injection in control livers; however, values were about threefold higher at the same time in the estrogen-treated group. $GdCl_3$ pretreatment prevented the induction of TNF-α mRNA in the liver from estrogen-treated animals almost completely.

Effect of estrogen on NO production after LPS injection. NO produced from macrophages causes lethal hypotension during endotoxin shock. Therefore, plasma levels of nitrite, which reflect NO production in vivo, were determined. Figure 3A depicts the effect of estrogen on plasma nitrite levels after LPS injection. As expected, plasma nitrite increased markedly 6 h after LPS injection (5 mg/kg) in the control group. However, nitrite levels in estriol-treated rats reached 85 µM, a value 2.5 times higher than in controls, indicating that estrogen treatment in vivo increases NO production due to LPS. To determine if induction of NOS in the liver is responsible for this increase, iNOS was detected by Western blotting (Fig. 3B). iNOS was increased in the liver 6 h after LPS injection to levels about fivefold higher in livers from estrogen-treated rats than controls. Furthermore, mRNA for iNOS was about twofold
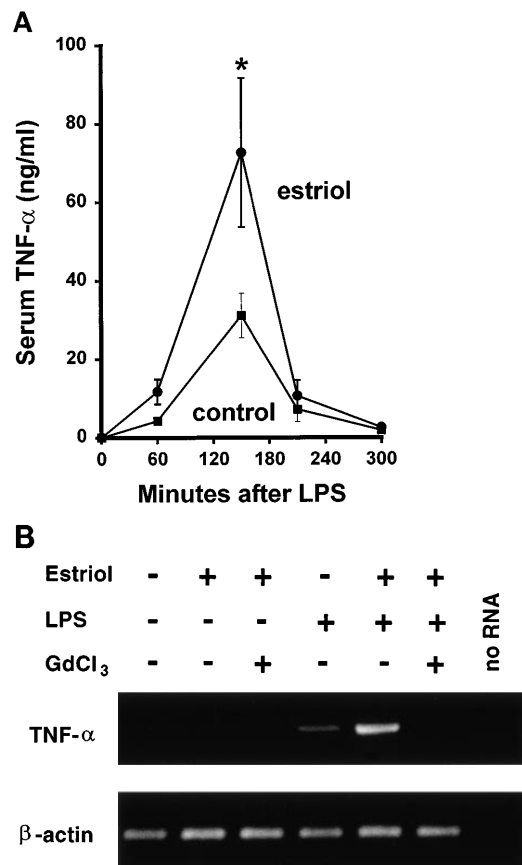
A

from estriol-treated animals produced about twice as much cytokine. Interestingly, addition of estriol (100 nM) to the culture medium for 24 h before LPS did not alter TNF-α production due to LPS (100 ng/ml) by isolated Kupffer cells (260 ± 27 and 312 ± 16 pg·10^6 cells^{-1}· 4 h^{-1}, control and estrogen-treated groups, respectively). Furthermore, Kupffer cells isolated from con-

A

B

B

C

Fig. 2. Effect of estrogen on tumor necrosis factor-α (TNF-α) production after LPS. A: blood samples were collected before and at 4 time points after an injection of LPS (5 mg/kg), and TNF-α was measured by ELISA. ELISA data represent means ± SE from 4 individual preparations. * $P < 0.05$ with Mann-Whitney's rank sum test. B: total RNA from the liver before and 1 h after LPS injection (5 mg/kg) was used for detection of TNF-α mRNA as described in MATERIALS AND METHODS. β-Actin was detected as a housekeeping gene, and ϕX174/*Hae* III was used to determine size of PCR products. Data for TNF-α mRNA are a representative picture of 3 individual experiments.

higher in estrogen-treated animals than in controls 6 h after LPS injection (Fig. 3C). Because it is known that interferon-γ (IFN-γ), which is produced by T lymphocytes and natural killer cells, is necessary for induction of NOS (4, 30), RT-PCR for IFN-γ mRNA was performed (Fig. 3C). mRNA for IFN-γ was increased 3 h after LPS injection in controls; however, estrogen pretreatment potentiated this increase about threefold. Moreover, since IL-12, which is produced by macrophages, is known to increase production of IFN-γ (18), we also studied IL-12 mRNA in the liver (Fig. 3C). IL-12 mRNA was induced 3 h after LPS injection, and levels were about sevenfold higher in estrogen-treated animals than in controls.

Effect of estrogen treatment in vivo on LPS-induced production of TNF-α and nitrite in isolated Kupffer cells. To confirm that Kupffer cells were the source of increased TNF-α in estrogen-treated animals, TNF-α production by isolated Kupffer cells was measured (Fig. 4A). Kupffer cells from control rats produced TNF-α in response to LPS (100 ng/ml). However, isolated cells
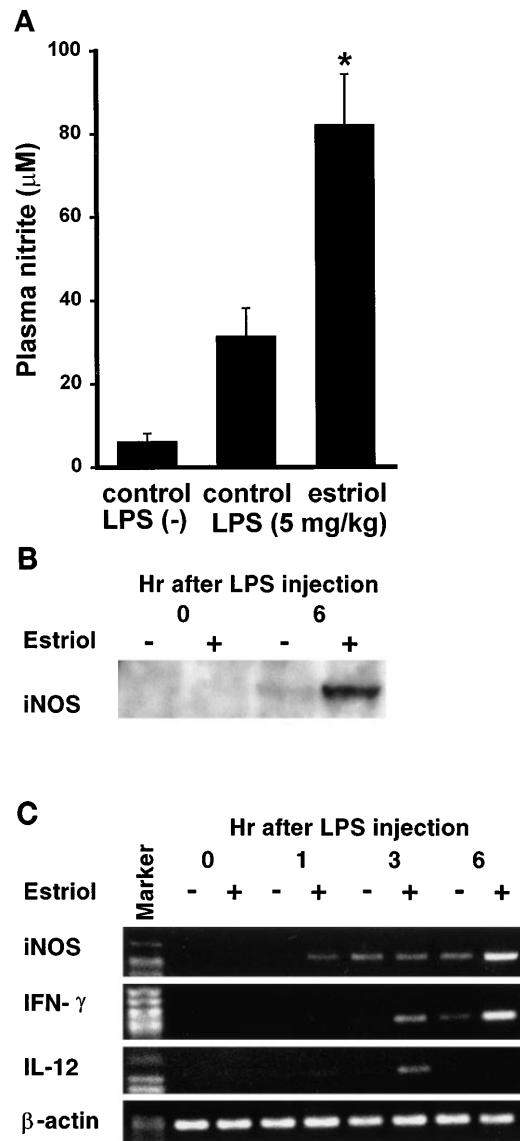
Fig. 3. Effect of estrogen on nitric oxide (NO) production due to LPS. A: estriol-treated rats were injected with LPS intravenously (5 mg/kg), and plasma samples were collected before and 6 h later. Plasma nitrite levels were determined colorimetrically. Nitrite data are means ± SE of 5 individual samples. * $P < 0.05$ vs. control given LPS, Mann-Whitney's rank sum test. B: protein extracts from whole liver before and 6 h after LPS injection were separated by 7.5% SDS-PAGE and immunoblotted using rabbit anti-mouse inducible NO synthase (iNOS) polyclonal antibody. Specific bands for iNOS (130 kDa) are shown. C: total liver RNA before and 1, 3, and 6 h after LPS injection (5 mg/ml) was used to detect iNOS, interferon-γ (IFN-γ), and interleukin-12 (IL-12) mRNA. β-Actin was also detected as a housekeeping gene, and ϕX174/*Hae* III was used to determine size of PCR products. RT-PCR data are a representative picture of 3 individual experiments.
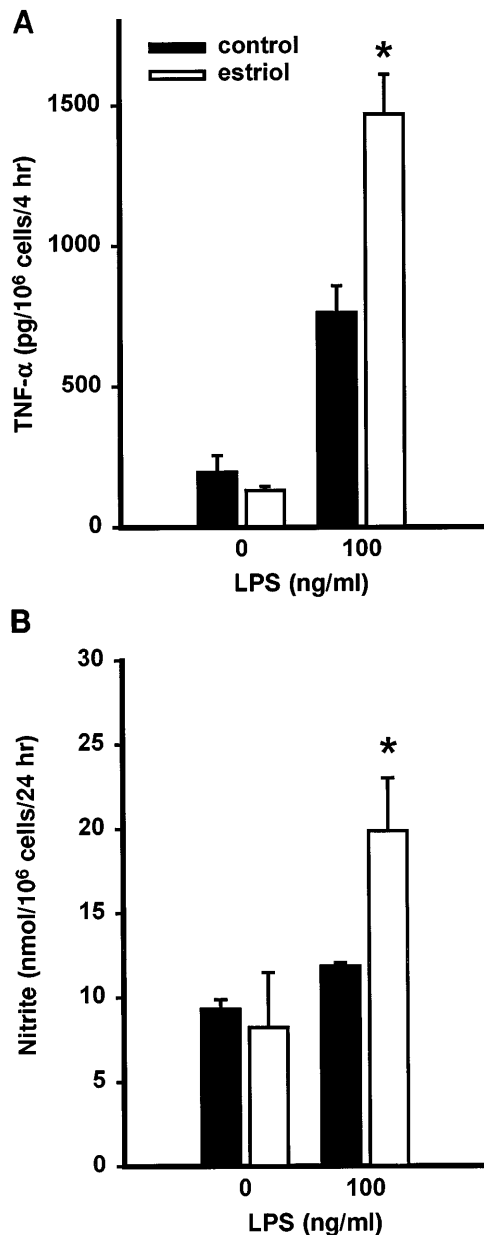
Fig. 4. Effect of estrogen on LPS-induced TNF-α and NO production by isolated Kupffer cells. Kupffer cells were isolated and cultured in 24-well plates with DMEM + 10% fetal bovine serum for 24 h (see MATERIALS AND METHODS). Culture medium was exchanged at 24 h with new medium with or without LPS (100 ng/ml, supplemented with 5% rat serum) and incubated for 4 or 24 h for TNF-α or nitrite measurements, respectively. TNF-α in the culture medium was measured by ELISA while nitrite was measured colorimetrically using the Griess reaction as detailed in MATERIALS AND METHODS. TNF-α (A) and nitrite (B) production in isolated Kupffer cells are depicted. Data represent means ± SE of experiments using cells from 4 individual rats. *$P < 0.05$ with 2-way analysis of variance (ANOVA).

trol rats produced small amounts of nitrite in the presence of LPS (100 ng/ml) (Fig. 4B). However, nitrite levels in the culture medium of Kupffer cells from estriol-treated rats were about twofold higher. Interestingly, addition of estriol (1 µM) to the culture medium for 24 h before LPS also did not alter nitrite production

due to LPS (100 ng/ml) (9.5 ± 1.3 and 9.9 ± 0.5 pmol·10^6 cells^{-1}· 24 h^{-1}, control and estrogen-treated groups, respectively).

Effect of estrogen on LPS-induced increases in intracellular Ca^{2+} in Kupffer cells. As reported previously (15), LPS increases intracellular Ca^{2+} transiently in isolated Kupffer cells. Here, Kupffer cells from control rats showed small increases in intracellular Ca^{2+} with 100 ng/ml of LPS (Fig. 5A) and maximal increases with two orders of magnitude higher concentrations (Fig. 5B). Surprisingly, Kupffer cells from estrogen-treated rats showed near-maximal increases in intracellular Ca^{2+} with only 100 ng/ml LPS (Fig. 5C). LPS increased intracellular Ca^{2+} in a dose-dependent manner in cells from control rats (Fig. 6). Kupffer cells isolated from estriol-treated rats, however, showed a significant, 15-fold shift of the dose-response curve to the left. On the other hand, increases in intracellular Ca^{2+} due to 100 ng/ml LPS in Kupffer cells from control rats incubated in vitro with estriol (1 µM) for 24 h were as small as values observed in cells from control rats (Fig. 5D), indicating that estrogen in vitro also has no effect on intracellular Ca^{2+} in Kupffer cells.

Effect of estrogen on CD14 expression in Kupffer cells and production of LBP in the liver. To determine whether the LPS/LBP receptor (CD14) was altered in Kupffer cells by estrogen treatment in vivo, Western blotting using ED9 antibody, which recognizes rat CD14, was performed (Fig. 7A). As expected, Kupffer cells from control rats expressed the 58-kDa CD14; however, the band was about twofold more intense in Kupffer cells from estrogen-treated rats. Furthermore, CD14 protein levels were not increased by estriol treatment (100 nM) in vitro for 24 h (data not shown).

Because LBP is necessary for CD14 to recognize LPS, mRNA for LBP in the liver after estrogen treatment was measured by Northern blotting (Fig. 7B). Interestingly, LBP mRNA was increased dramatically 6 h after estriol injection followed by a gradual decline. The peak levels of LBP mRNA reached after estriol injection were about 20-fold higher than controls. Furthermore, to confirm whether serum from estriol-treated rats contains functional LBP, RAW 264.7 cells, a mouse macrophage cell line, were incubated with LPS (1 ng/ml) and 1% serum from estriol-treated rats for 1 h. Subsequently, total RNA was prepared, and Northern blotting for TNF-α was performed (Fig. 7C). Indeed, rat serum increased TNF-α mRNA levels after LPS, indicating that RAW 264.7 cells recognize LBP in rat serum. This system was then used as an assay for bioactive LBP. LPS increased TNF-α mRNA in serum from rats treated with estriol for 6–24 h, indicating that LBP was increased in the serum after estrogen treatment. The LPS (1 ng/ml)-induced increase in TNF-α mRNA in RAW 264.7 cells was potentiated by serum from rats 6–24 h after estriol injection about threefold more than serum from controls. Serum from estrogen-treated animals did not increase TNF-α mRNA in the absence of LPS, confirming that serum itself did not contain any other
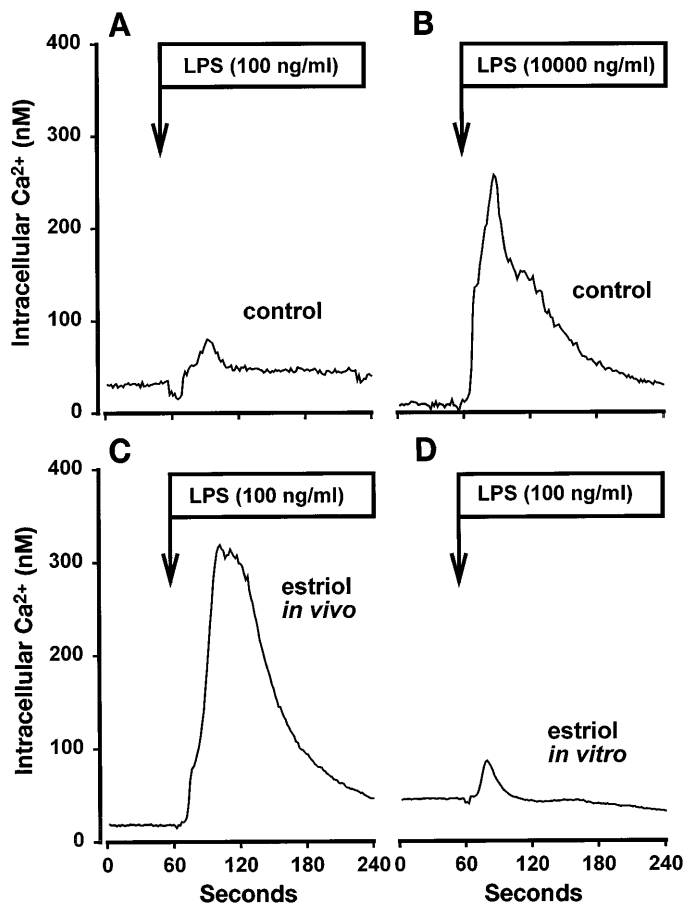
Fig. 5. Effect of estrogen on LPS-induced increases in intracellular Ca²⁺ in Kupffer cells. Intracellular Ca²⁺ in isolated Kupffer cells was measured fluorometrically using fura 2 as described in MATERIALS AND METHODS. Changes in intracellular Ca²⁺ after addition of LPS (supplemented with 5% rat serum) are plotted. *A*: LPS (100 ng/ml) was added to Kupffer cells from control rats. *B*: LPS (10,000 ng/ml) was added to Kupffer cells from control rats. *C*: LPS (100 ng/ml) was added to Kupffer cells from estriol-treated rats. *D*: LPS (100 ng/ml) was added to Kupffer cells from control rats 24 h after incubation with estriol (1 µM). Data are representative traces of experiments repeated 5 times.

stimulants of TNF-α mRNA in RAW 264.7 cells except LBP.

DISCUSSION

Estrogen increases sensitivity to endotoxin in vivo. In the present study, it was demonstrated that pharmacological doses of estrogen similar to levels encountered in late pregnancy increased mortality due to LPS (Fig. 1), confirming experiments by Nolan and Ali (22). This effect of estrogen was very impressive; however, precise mechanisms remained unclear. It is well known that macrophages, including Kupffer cells, contribute to the pathophysiology of endotoxin shock. Indeed, GdCl₃, a Kupffer cell toxicant, totally prevented mortality due to estrogen plus LPS (Fig. 1), indicating that Kupffer cells are necessary for this phenomenon.

Kupffer cells produce inflammatory cytokines (IL-1, IL-6, and TNF-α), eicosanoids, and free radicals, including NO, which causes organ damage (5). Increased
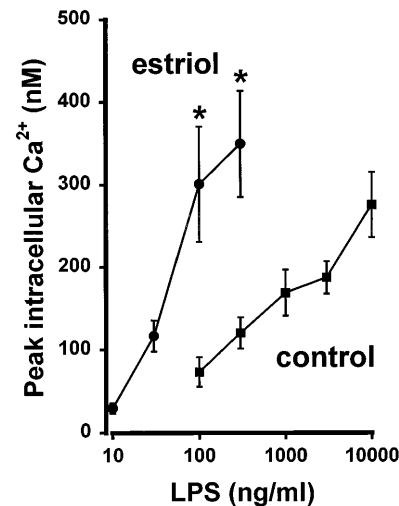

Fig. 6. Effect of estrogen on dose response of LPS on intracellular Ca²⁺ in Kupffer cells. Experimental design was as described in Fig. 5 legend, except that various doses of LPS were used. Means ± SE of peak intracellular Ca²⁺ levels from experiments using cells from 5 different rats were plotted. *$P < 0.05$ with 2-way ANOVA.

TNF-α during endotoxemia plays a pivotal role in endotoxin shock, because TNF-α mimics endotoxin shock and related multiple organ failure (25) and anti-TNF-antibody prevents it (26). As expected, peak serum TNF-α levels were about twofold higher in estrogen-treated animals (Fig. 2*A*), possibly explaining
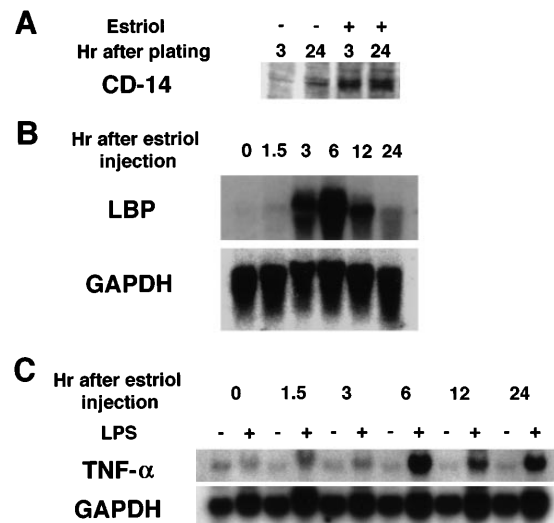

Fig. 7. Effect of estrogen on CD14 expression in Kupffer cells and production of LPS-binding protein (LBP) by the liver. *A*: protein extracts from cultured Kupffer cells 3 and 24 h after plating were analyzed by Western blotting using mouse anti-rat ED9 antibody. Specific bands for CD14 (58 kD) are shown. *B*: total RNA (10 µg) from livers before and 1.5, 3, 6, 12, and 24 h after estriol injection (20 mg/kg ip) was analyzed by Northern blotting for LBP. *C*: serum samples before and 1.5, 3, 6, 12, and 24 h after estriol injection were added to serum free RPMI 1640 medium (serum final concn, 1%) with 1 ng/ml LPS. Mouse macrophages (RAW 264.7) were incubated in this medium for 1 h, and total RNA was prepared. We analyzed 10 µg of total RNA by Northern blotting for TNF-α. Data shown are representative of 3 individual experiments. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

more severe shock and organ failure. It was reported (32) that in vivo treatment with estradiol augmented increases in serum TNF-α levels after LPS injection in the mouse, effects similar to those described here. Furthermore, estriol increased TNF-α mRNA in the liver 1 h after injection of LPS, whereas $GdCl_3$ prevented this increase (Fig. 2B), indicating that Kupffer cells are involved in the production of TNF-α in estriol-treated animals.

Moreover, NO produced by macrophages is a key factor in lethal hypotension during endotoxin shock (16, 17, 29). In this study, plasma nitrite levels after LPS were significantly higher in estrogen-treated rats than in controls (Fig. 3A), which most likely explains the higher mortality rate observed (Fig. 1). Western blotting and RT-PCR (Fig. 3, B and C) confirmed that increased levels of plasma nitrite in estrogen-treated animals are most likely due to elevated iNOS. TNF-α induces NO synthesis in macrophages (6, 7), so elevated levels of serum TNF-α in estrogen-treated animals are most likely responsible for increased NO production. It is also known that IFN-γ produced by T lymphocytes and natural killer cells (pit cells) can induce iNOS (4, 30) and the production of IFN-γ is positively regulated by IL-12 produced by macrophages (18). In this model, both IFN-γ and IL-12 RNA were increased by estrogen treatment (Fig. 3C). One possible sequence of events is that estrogen increases production of IL-12 by Kupffer cells in response to LPS, which increases production of IFN-γ by pit cells, which in turn induces NOS in Kupffer cells. Taken together, it is concluded that estrogen treatment increases production of toxic mediators from Kupffer cells, which is responsible for increased mortality after LPS.

As stated above, the effect of estrogen in vivo on endotoxin shock can most likely be explained by increased production of toxic mediators. Indeed, isolated Kupffer cells from estrogen-treated rats produced more toxic chemical mediators (e.g., TNF-α and nitrites) in response to LPS than cells from controls (Fig. 4). Surprisingly, estrogen treatment in vivo dramatically increased intracellular Ca^{2+} due to LPS, with a shift of the dose-response curve far to the left (Fig. 6). Although it has been reported that some macrophages contain estrogen receptors (3, 10), they have not been identified on Kupffer cells. Interestingly, addition of estrogen in vitro failed to change LPS-induced TNF-α and nitrite production and intracellular Ca^{2+}, supporting the idea that the effect of estrogen in vivo is indirect.

Estrogen increases both CD14 expression in Kupffer cells and LBP production by the liver. CD14 is a functional LPS/LBP receptor on Kupffer cells. Here, CD14 was increased by estrogen treatment in vivo (Fig. 7A), explaining why LPS increased intracellular Ca^{2+}, TNF-α, and nitrite in Kupffer cells isolated from estrogen-treated animals. Interestingly, LBP mRNA was also increased dramatically 6 h after injection of estrogen (Fig. 7B), suggesting that it is increased in the serum by estrogen. Indeed, serum from estrogen-treated animals potentiated the increase of TNF-α

mRNA by LPS in RAW 264.7 cells (Fig. 7C), confirming that functional LBP is increased in the serum by estrogen treatment. It is concluded that both LBP production by the liver and CD14 expression on Kupffer cells are increased by estrogen, thereby increasing toxic mediator production by Kupffer cells. It is possible that these changes in surface receptors and circulating binding protein could be involved in the pathology of the HELLP syndrome in pregnancy, since estriol levels in this study were in the range of those observed in late pregnancy.

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