Estrogen Is Involved in Early Alcohol-Induced Liver Injury in a Rat Enteral Feeding Model

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The aim of this study was to investigate whether reduction in blood estrogen by removal of the ovaries would decrease the sensitivity of female rats to early alcohol-induced liver injury using an enteral ethanol feeding model, and if so, whether estrogen replacement would compensate. Livers from ovariectomized rats with or without estrogen replacement after 4 weeks of continuous ethanol exposure were compared with nonovariectomized rats in the presence or absence of ethanol. Ethanol increased serum alanine transaminase (ALT) levels from 30 ± 6 to 64 ± 7 U/L. This effect was blocked by ovariectomy (31 ± 7) and totally reversed by estrogen replacement (110 ± 23). Ethanol increased liver weight and fat accumulation, an effect that was minimized by ovariectomy and reversed partially by estrogen replacement. Infiltrating leukocytes were increased 6.7-fold by ethanol, an effect that was blunted significantly by ovariectomy and reversed by estrogen replacement. Likewise, a similar pattern of changes was observed in the number of necrotic hepatocytes. Blood endotoxin and hepatic levels of CD14 messenger RNA (mRNA) and protein were increased by ethanol. This effect was blocked in ovariectomized rats and elevated by estrogen replacement. Moreover, Kupffer cells isolated from ethanol-treated rats with estrogen replacement produced more tumor necrosis factor α (TNF-α) than those from control and ovariectomized rats. It is concluded, therefore, that the sensitivity of rat liver to alcohol-induced injury is directly related to estrogen, which increases endotoxin in the blood and CD14 expression in the liver, leading to increased TNF-α production. (HEPATOLOGY 2000;31:117-123.)

It is well known that excessive intake of alcohol over a relatively long period of time leads to liver injury,[1] a disease characterized by steatosis, inflammation, necrosis, and finally fibrosis and cirrhosis. In the United States, women 18 years of age or older represent one third of all alcohol abusers.[2] Moreover, women have a significantly higher relative risk of developing alcohol-related liver disease than men at any given level of alcohol consumption.[3] This increased sensitivity of women to alcohol suggests the possible involvement of sex steroids in the pathogenesis of this disease; however, whether this hypothesis is valid has not been tested. Kupffer cells, sinusoidal endothelial cells, and hepatocytes contain estrogen receptors, and cell function can be influenced by exogenous estrogen through changes in estrogen receptor levels and occupancy.[4]

With a continuous intragastric ethanol dietary feeding model in the rat, a procedure developed by Tsukamoto et al.,[5] it was shown that early ethanol-induced liver injury was prevented by treatment with gadolinium chloride, a selective Kupffer cell toxicant, indicating that Kupffer cells are involved in the pathogenesis of early alcohol-induced liver disease.[6] Alcohol-induced liver injury was also minimized by sterilizing the gut with antibiotics, a method used to block gut-derived endotoxin production.[7] These findings led to the hypothesis that ethanol increases blood endotoxin,[8,9] which activates Kupffer cells to release many potent effectors and cytokines,[10] leading to early alcohol-induced liver injury.[11,12] Interestingly, like in the human, female rats also exhibited greater susceptibility to early alcohol-induced liver injury than males with the enteral ethanol feeding model and had 2-fold higher blood endotoxin levels than males.[9] Further, in an *in vitro* study, estrogen increased the sensitivity of Kupffer cells to endotoxin and increased the endotoxin receptor CD14.[13] Accordingly, the purpose of this study was to assess the effect of ovariectomy and estrogen replacement in ovariectomized rats on early alcohol-induced liver injury using the clinically relevant intragastric ethanol feeding model. Preliminary accounts of this work have appeared elsewhere.[14]

MATERIALS AND METHODS

Animals and Experimental Groups. Female Wistar rats (280-300 g) were used in the study. Intragastric cannulas were implanted into the forestomach using the method developed by Tsukamoto et al.[5] Animals were allowed to recover from surgery during the first postoperative week, with free access to chow and water. Thereafter, rats were infused continuously with a high-fat liquid diet (control group) or an isocaloric high-fat liquid diet containing ethanol (ethanol group) via a cannula for up to 4 weeks. In some rats, ovariectomy was performed at 4 weeks of age. When body weights reached 290 to 300 g, one group of ovariectomized rats (ovariectomy group) received ethanol like the ethanol group. Another group of ovariectomized rats received estrogen replacement, where 17 β-estradiol (15 μg/kg; Sigma Chemical Co., Louis, MO) was

Abbreviations: ALT, alanine transaminase; RNase, ribonuclease; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor α; mRNA, messenger RNA.

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administered subcutaneously every 4 days during the 4 weeks of intragastric dietary ethanol exposure (estrogen group), starting at the beginning of ethanol feeding. This protocol of estrogen replacement was chosen because it provides sufficient stimulus to trigger a luteinizing hormone (LH) surge on at least 4 consecutive days in ovariectomized rats.[15] All animals received humane care according to the criteria outline in the "Guide for the Care and Use of Laboratory Animals" (NIH publication 86-23, revised 1985).

Diet. The basic liquid diet was prepared according to Thompson and Reitz[16] and supplemented with lipotropes as described by Morimoto et al.[17] The composition of the diet was 23% of calories as protein, 37% of calories as fat (corn oil), and 5% from carbohydrates, minerals, and vitamins, plus 35% of calories from ethanol or isocaloric maltose-dextrin. Ethanol levels in the diet were increased gradually to 9 to 10 g/kg/d during the first week and were maintained at 10 to 12 g/kg/d during weeks 2 to 4. The volume of liquid diet (200 mL/kg) given to each rat was based on its body weight.

Alcohol Metabolism In Vivo. Rats receiving continuous enteral ethanol feeding were forced to breath into a closed heated chamber (37°C) for 20 seconds, and 1 mL of breath was collected with a gas-tight syringe at hourly intervals for 4 to 5 hours.[18] Concentrations of ethanol in breath were determined by gas chromatography, and rates of alcohol metabolism were calculated from linear decreases in blood alcohol concentration per unit time as described previously.[18]

Urine Collection and Assay for Ethanol. Rats were housed in metabolic cages that separate urine from feces, and urine samples were collected over 24 hours for each rat. Ethanol levels in urine were determined daily by measuring absorbance at 366 nm resulting from the reduction of nicotinamide adenine dinucleotide to nicotinamide adenine dinucleotide by alcohol dehydrogenase.[19]

Measurement of Serum Estrogen and Alanine Transaminase Levels. Blood was collected via the inferior vena cava at necropsy 3 days after the last injection of estrogen and serum was stored at −20°C until assay. The estradiol measurement is a no-extraction, solid phase ^{125}I-radioimmunoassay (Coat-A-Count Estradiol; Diagnostics Products Corp., Los Angeles, CA) based on antibody-coated tube technology.[20,21] After incubation for 1 hour at 37°C, separation of bound from free estradiol is achieved by a decanting process. The remaining radioactivity is then counted in a gamma counter with counts inversely related to the amount of estradiol present in the sample. The quantity of estradiol was determined by using known standards and a calibration curve. Alanine transaminase (ALT) was analyzed by standard enzymatic procedures.[19]

Quantitation of Steatosis, Infiltrating Leukocytes, and Necrosis. An image acquisition and analysis system (Image-1/AT; Universal Image Corp, Chester, PA) incorporating an Axioskop 50 microscope (Carl Zeiss, Inc., Thornwood, NY) was used to capture and analyze tissue sections using a modification of previously published techniques.[22] The extent of fat accumulation in pericentral regions of the liver lobule was defined as the percent of the field area. Average measurements from each tissue section (5 fields per section) were pooled to determine means. The total number of infiltrating leukocytes (including neutrophils and mononuclear cells), hepatocytes, and necrotic hepatocytes was counted in a 100 mm^2 area with a magnification of 200×. Five areas per section were randomly selected and counted.

Endotoxin Assay. Blood collection and measurement of plasma endotoxin are described elsewhere.[9,23] In short, blood samples were handled under pyrogen-free conditions and centrifuged at 1,200 rpm for 10 minutes. Plasma was stored at −70°C until measurement of endotoxin with a Limulus Amebocyte Lysate test kit (Kinetic-QCL; BioWhittaker, Walkersville, MD).[24]

RNA Preparation and Ribonuclease Protection Assay for CD14. Total liver RNA was prepared from frozen tissues by the guanidium/CsTFA centrifugation method (QuickPrep total RNA extraction kit; Amersham Pharmacia Biotech, Piscataway, NJ). The quantity and purity of RNA was verified by measuring the absorbance at A260 and A280. Further, the integrity of RNA was confirmed by electrophoresis on a formaldehyde-denaturing agarose gel. To obtain a riboprobe for CD14, the full coding region of rat CD14 was amplified using reverse transcription polymerase chain reaction and subcloned into TOPO pCR II vector (Invitrogen, Carlsbad, CA). The sequence was identical with the reported sequence,[25] which was confirmed by direct sequencing using ABI PRISM 310 genetic analyzer (PE Applied Biosystems, Foster City, CA) and the dye terminator cycle sequencing FS ready reaction kit. The plasmid was linealized by restriction enzyme (*Bgl* II) digestion and served as a template for *in vitro* transcription. The radiolabeled riboprobe for rat CD14 was then generated using the MAXIscript *in vitro* transcription kit (Ambion Inc., Austin, TX) in the presence of a ^{32}P UTP and SP6 RNA polymerase. The riboprobe for rat cyclophilin and RNA ladder were also transcribed with T7 RNA polymerase using pTRI-cyclophilin-rat antisense control template (Ambion Inc.) and RNA template set (Wako Chemical Inc., Osaka, Japan). Ribonuclease (RNase) protection assay was performed using RPA III kit (Ambion Inc.) according to the manufacturer's instructions. Briefly, 20 mg of total RNA was hybridized with the radiolabeled probes (2×10^5 cpm each) in hybridization solution at 45°C overnight. The reaction was then incubated with RNase A/T1 at 37°C for 30 minutes and precipitated with RNase inactivation/precipitation solution. The reaction was separated on a denaturing 5% polyacrylamide/urea gel and exposed to films at −80°C.

Western Blotting for CD14. Extracted proteins (10 μg) from each group were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. Membranes were blocked by Tris-buffered saline-Tween 20 containing 5% skim milk, probed with mouse anti-rat ED9 monoclonal antibody (Serotec, Oxford, UK), followed by horseradish peroxidase–conjugated secondary antibody as appropriate. Membranes were incubated with a chemiluminescence substrate (ECL reagent; Amersham Life Science, Buckinghamshire, UK) and exposed to X-OMAT film (Eastman Kodak Company, Rochester, NY).

Kupffer Cell Isolation and Culture. Kupffer cells were prepared from rats treated for 4 weeks with enteral ethanol, using techniques described previously.[26] In brief, the liver was digested with collagenase, excised, and the Gilsson capsule broken by shaking in Hank's balanced salt solution buffer. The suspension was filtered through sterile nylon gauze, and the filtrate was centrifuged twice at 50*g* for 3 minutes to separate parenchymal from nonparenchymal cells. The supernatant was collected, and the nonparenchymal cell supernatant fraction was centrifuged at 500*g* for 7 minutes. The pellet was resuspended in buffer and layered gently on a density cushion of Percoll and centrifuged for 15 minutes at 1,500*g*. The Kupffer cell fraction was collected and washed with Hank's balanced salt solution. Cells were seeded onto 25-mm glass coverslips and incubated in Dulbecco's modified Eagle's medium (GIBCO Laboratories Life Technologies Inc., 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% CO_2. Nonadherent cells were removed after 1 hour by replacing the culture medium. All adherent cells phagocytosed latex beads, indicating that they were Kupffer cells. Cells were cultured for 24 hours before experiments.

Tumor Necrosis Factor α Production by Kupffer Cells. Isolated Kupffer cells were cultured in 24-well culture plates (Corning Inc., Corning, NY) at a density of 5×10^5 cells/well for 24 hours before experiments using media and conditions described previously. Lipopolysaccharide (LPS; Sigma Chemical Co.) was added to media (1 μg/mL) for 4 hours. Samples were stored at −80°C and tumor necrosis factor α (TNF-α) in the culture media was determined using an enzyme-linked immunosorbent assay kit (Genzyme Corp., Cambridge, MA).

Estrogen Supplementation in Normal Rats. Twelve female Wistar rats (weighing 280-300 g) were allocated to 2 experimental groups and received either vehicle (sesame oil) or estrogen (β-estradiol in sesame oil, see previous protocol). The animals had free access to chow and tap water. On days 7 and 24 after the first injection,

ethanol elimination rates were determined after an acute dose of ethanol (4.5 g/kg). Animals were killed on day 28. Livers were removed for histology and blood was collected for measurement of serum ALT.

Statistical Analysis. All results were expressed as mean ± SEM. Statistical differences between means were determined using analysis of variance (ANOVA) as appropriate. $P < .05$ was selected before the study to reflect significance.

RESULTS

Body/Liver Weights, Urine Ethanol, and Blood Estradiol Levels. When adult female Wistar rats reach 300 g, they no longer gain weight. As expected, no significant body weight gain was observed after 4 weeks of high-fat liquid diet feeding with or without ethanol, because these rats were in the 300-g weight range (Table 1). There were also no differences in body weights among the groups studied. Liver to body weight ratios were determined at necropsy (Table 1). This value was about 4.2% in rats receiving high-fat control diet (control) and was increased nearly 2-fold by 4 weeks of ethanol exposure (ethanol group). However, the ethanol-enhanced ratio was attenuated about one third by ovariectomy (7.6 vs. 5.3%). Moreover, this phenomenon tended to be reversed by estrogen replacement. During the continuous enteral feeding period, urine ethanol concentrations of the 3 ethanol-fed groups of animals fluctuated as previously observed,[9] with an average urine ethanol level of about 180 mg/dL. Further, there was no difference in rates of ethanol elimination between the groups studied (Table 1). Ethanol elimination rates were also not significantly different when expressed per gram liver (data not shown). Before ethanol exposure, the serum estradiol levels in the nonovariectomized rats (control and ethanol groups) were 38 ± 4 pg/mL, values that were reduced significantly by ovariectomy (13 ± 4 pg/mL). After 4 weeks of enteral feeding, blood estradiol levels in the control and ethanol groups were about 50 pg/mL (Table 1). In contrast, in the ovariectomy group, values were about 10-fold lower. As expected, estrogen replacement in ethanol-fed ovariectomized rats elevated estrogen levels 5-fold over controls and 55-fold over the ovariectomy group.

TABLE 1. Effects of Ovariectomy and Estrogen Replacement on Routine Parameters in Rats Treated With or Without Ethanol

	High-Fat Control	Ethanol	Ethanol + Ovariectomy	Ethanol + Ovariectomy + Estrogen
Preoperative body weight (g)	292 ± 3	293 ± 5	310 ± 8	312 ± 7
Body weight at necropsy (g)	295 ± 4	295 ± 5	315 ± 8	316 ± 9
Liver to body weight ratio (%)	4.2 ± 0.3	7.6 ± 0.3*	5.3 ± 0.4†	6.2 ± 0.5*
Average urine ethanol (mg/dL)	NA	181 ± 23	185 ± 31	178 ± 27
Ethanol elimination rates (mmol/kg/hr)	NA	5.8 ± 0.3	6.8 ± 1.1	6.8 ± 0.7
Serum estradiol at autopsy (pg/mL)	48 ± 12	54 ± 14	4 ± 1*†	224 ± 66*†‡
Serum ALT (U/L)	30 ± 6	64 ± 7*	31 ± 7	110 ± 23*†‡
Steatosis§	0	30 ± 1.9*	20 ± 0.6*†	24 ± 2.4*
Infiltrating leukocytes	1.0 ± 0.3	6.7 ± 0.7*	4.3 ± 0.2*†	6.8 ± 0.4*‡
Necrotic cells	1.0 ± 0.3	21 ± 2.2*	12 ± 1.0*†	18 ± 1.2*‡

NOTE. Ethanol elimination rates, urine concentrations of ethanol, serum estradiol and ALT levels, quantitation of infiltrating leukocytes, and necrotic cells were determined as described in Materials and Methods. There were 6 to 7 animals per group. Data are expressed as mean ± SEM.

Abbreviations: NA, not applicable.

*$P < .05$ compared with the control group.

†$P < .05$ compared with the ethanol group.

‡$P < .05$ compared with the ovariectomy group by one-way ANOVA using Tukey's post-hoc test.

§Image analysis was used to quantitate steatosis in livers from ethanol-treated rats; the extent of fat accumulation was defined as the percent of the field area.

Blood Transaminases. The effects of ovariectomy and estrogen replacement on serum levels of ALT after 4 weeks of ethanol exposure are also shown in Table 1. ALT levels in rats fed a high-fat control diet were about 30 U/L, values within the normal range. Four weeks of enteral ethanol feeding resulted in 2-fold increases in ALT, an effect that was prevented by ovariectomy ($P < .05$, compared with the ethanol group). Estrogen replacement, however, significantly increased ALT levels to levels about 2 times higher than values in the ethanol group ($P < .01$, compared with other groups).

Fatty Accumulation in Livers. The increased liver to body weight ratio by ethanol is most likely caused by enhanced fat accumulation depicted in representative photomicrographs of livers from the 4 experimental groups (Fig. 1). Steatosis was not observed in rats receiving control diet (Fig. 1A); however, severe and panlobular fatty accumulation was observed in livers from the ethanol group (Fig. 1B). Livers from ovariectomized rats given ethanol showed less extensive steatosis, mainly in periportal areas (Fig. 1C). On the other hand, ethanol-treated ovariectomized rats with estrogen replacement (Fig. 1D) exhibited a similar pattern of steatosis to that observed in nonovariectomized rats fed ethanol.

No steatosis in livers from rats fed the control diet was detected by image analysis (Table 1). In the nonovariectomized ethanol-treated rats (ethanol group), steatosis reached 30% ± 2% of the total hepatic area; however, the magnitude of this phenomenon was reduced significantly by ovariectomy (20% ± 0.6%, $P < .05$ compared with the ethanol group) and partially reversed by estrogen replacement.

Hepatic Inflammation and Necrosis. Effects of ovariectomy and estrogen replacement on leukocyte infiltration (including neutrophils and mononuclear cells) in alcohol-treated rats are shown in Table 1. Only a few leukocytes were observed in livers from animals fed the control diet. The number of infiltrating leukocytes was increased 7-fold in ethanol-fed animals. Ovariectomy, however, significantly diminished this number to about two thirds that of the ethanol group. This reduced infiltration by ovariectomy was totally reversed by estrogen replacement. Thus, rats with regular ovary function or estrogen replacement therapy have more severe ethanol-induced hepatic inflammation. Likewise, necrotic hepatocytes were nearly undetectable in rats fed the high-fat control diet (Table 1). The number of necrotic hepatocytes in the ethanol group was about 2-fold higher than in livers from ovariectomized rats fed ethanol ($P < .01$). When ovariectomized rats were given estrogen replacement, the number of necrotic cells was elevated to a level comparable with that of ethanol-treated nonovariectomized rats (ethanol group) and was significantly higher than values in the ovariectomy group ($P < .05$).

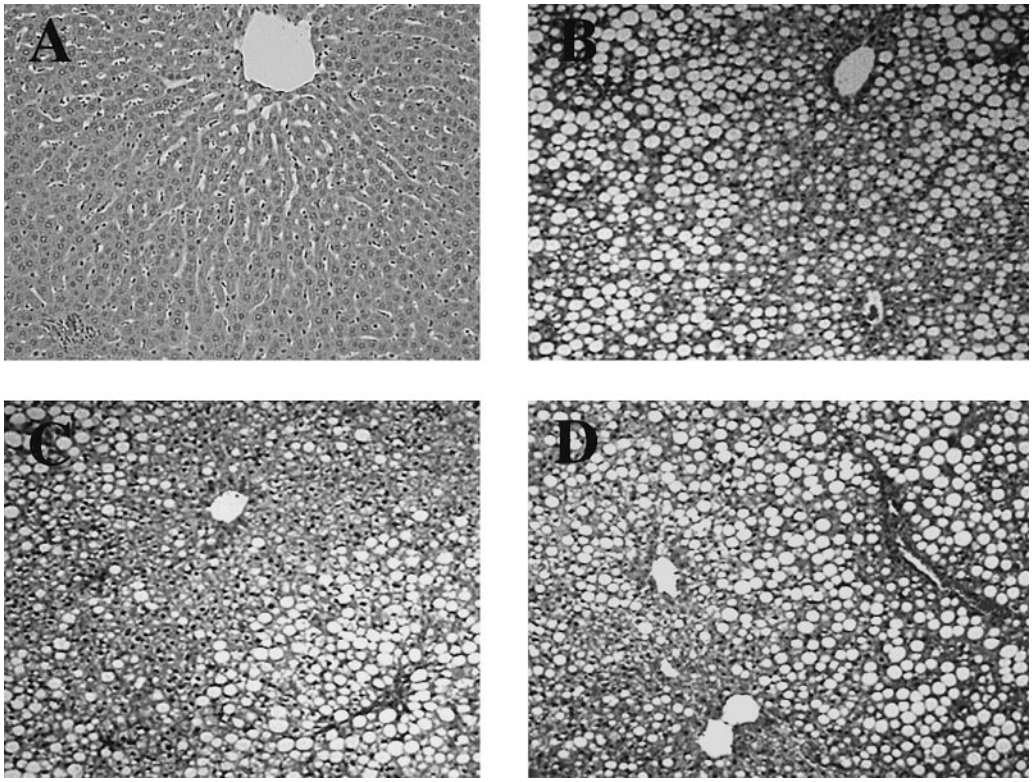

FIG. 1. Representative photomicrographs of livers after 4 weeks of ethanol exposure. Rats were treated as detailed in Materials and Methods. (A) Histology of liver of a female rat fed a high-fat control diet. (B) Liver from a female rat fed ethanol. (C) Liver from an ovariectomized rat fed ethanol. (D) Liver from an ethanol-fed ovariectomized rat with estrogen replacement. (Original magnification ×100.)

Blood Endotoxin. Plasma endotoxin levels in rats fed control diet were around 14 pg/mL (Fig. 2). After 4 weeks of continuous intragastric ethanol feeding, endotoxin in rats with normal ovary function (ethanol group) reached average levels of 50 pg/mL ($P < .05$ compared with controls). Ovariectomy significantly reduced blood endotoxin about 8-fold ($P < .05$). As expected, estrogen replacement elevated values about 10-fold.

Hepatic CD14 Expression. CD14 is an integral part of the endotoxin receptor.[27] RNase protection assay was used here to detect rat CD14 messenger RNA (mRNA) (Fig. 3A). CD14 mRNA was less intense in tissues from nonovariectomized rats fed the high-fat control diet than in rats fed the ethanol diet, indicating that ethanol enhanced transcription of CD14 mRNA. The band in ovariectomized rats given ethanol was much weaker; however, in ethanol-fed ovariectomized rats given estrogen replacement, it was as intense as nonovariectomized rats given ethanol. These differences in CD14 mRNA were also reflected in CD14 protein measured using Western blotting (Fig. 3B). There was significantly less CD14 protein in livers from rats fed the high-fat diet or ovariectomized rats fed ethanol than the ethanol-treated nonovariectomized rats or ovariectomized rats with estrogen replacement. Thus, CD14 synthesis and expression is influenced by estrogen.

TNF-α Production by Kupffer Cells. In the absence of enteral ethanol exposure, TNF-α production by isolated Kupffer cells was minimal (Fig. 4). However, after 4 weeks of ethanol, TNF-α production from cells from rats with normal ovaries and ovariectomized rats increased more than 2-fold over basal levels. On the other hand, in ethanol-fed ovariectomized rats with estrogen replacement, TNF-α production increased about 5-fold over basal levels and was significantly elevated over the ovariectomy group ($P < .05$).

Effects of Estrogen Administration in Normal Rats on Ethanol Elimination and Liver. Ethanol elimination rates in normal rats receiving vehicle were 6.6 ± 0.5 and 6.2 ± 0.7 mmol/kg/hr on days 7 and 24, which were not different compared with rats given β-estradiol (5.3 ± 0.8 and 5.5 ± 0.7 mmol/kg/hr). After 4 weeks, serum ALT levels were all within normal range (below 30 U/L) regardless of estrogen administration or not. Likewise, no liver damage was observed in normal rats.

DISCUSSION

Estrogen Exacerbates Early Alcohol-Induced Liver Injury. The increased incidence of alcohol-induced liver injury in women and in animal models has provided evidence for the involvement of sex steroids in the pathogenesis of this disease.[3,9] The major conclusion from this study is that female subjects are more susceptible to alcohol-induced liver injury, at least in part because of higher levels of estrogen in blood as shown in this clinically relevant animal model. In the present study, early alcohol-induced liver damage in rats with normal ovary function was much more severe than in ovariectomized rats with lower levels of blood estrogen after 4 weeks of continuous enteral ethanol feeding (Table 1, Fig. 1). When ovariectomized animals were given estrogen replacement, hepatic injury was as serious as in rats with normal ovary function, consistent with the hypothesis that estrogen replacement is linked to liver injury. Moreover, the extent of alcohol-induced liver injury correlated with circulating levels of estrogen, a situation that can also be seen in male patients.[1,28] However, the same dose of β-estradiol given to normal rats for the same period of time did not affect ethanol elimination or liver histology.

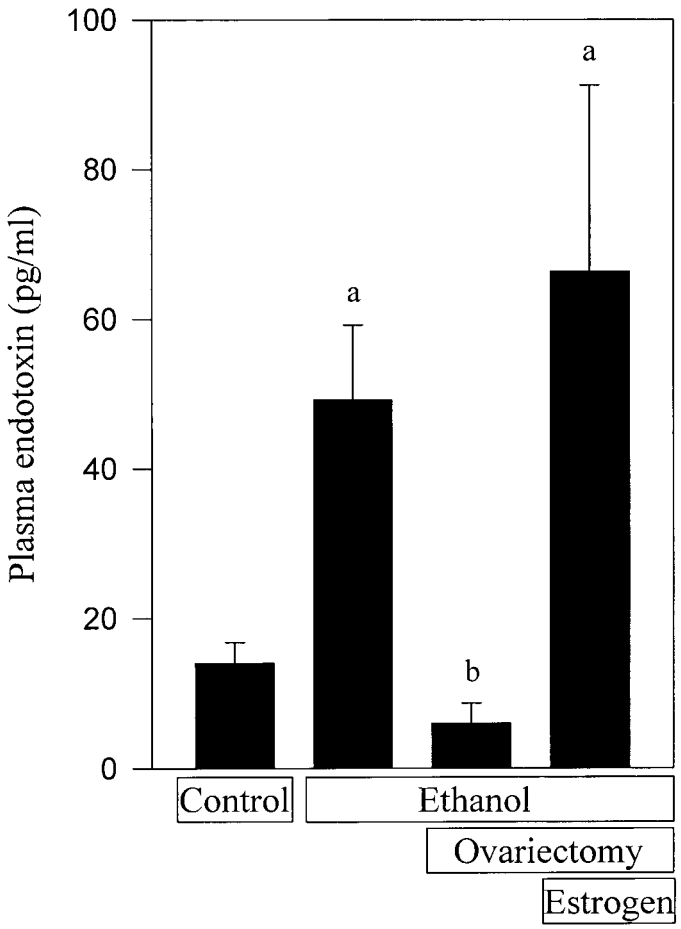

FIG. 2. Effects of ovariectomy and estrogen replacement on blood endotoxin levels 4 weeks after ethanol exposure. Plasma endotoxin levels in rats given a high-fat control diet and an ethanol diet were determined by the Limulus amebocyte lysate assay described in Materials and Methods. Data are presented as mean ± SEM. (a) $P < .05$ compared with controls. (b) $P < .05$ compared with nonovariectomized rats fed ethanol and ethanol-treated ovariectomized rats with estrogen replacement by one-way ANOVA with Tukey's post-hoc test.

Possible Mechanisms of Increased Early Alcohol-Induced Liver Injury by Estrogen. The mechanism responsible for estrogen-enhanced alcohol hepatotoxicity remains unclear.[29] However, evidence from the present study shows that ovariectomy and/or estrogen replacement did not influence the cyclic pattern of urine ethanol, average levels of urine ethanol, or the rate of ethanol elimination, suggesting that estrogen does not affect ethanol metabolism under these conditions. It is well known that Kupffer cells activated by gut-derived endotoxin (LPS) produce inflammatory cytokines (interleukin 1, TNF-α), eicosanoids, and free radicals, which cause liver injury.[10,11] Moreover, blood endotoxin levels are elevated in alcoholics[30] and in animals fed alcohol.[8,9,29] In this study, plasma endotoxin levels were significantly higher in rats with normal ovary function and in ovariectomized rats given estrogen replacement than in ovariectomized animals exposed chronically to ethanol (Fig. 2). Thus, estrogen may affect gut permeability to endotoxin or endotoxin production by gut microflora. Indeed, it has been reported that intestinal epithelial cells contain estrogen receptors.[31]

Previous studies from this laboratory showed that estrogen increased sensitivity of hepatic Kupffer cells to endotoxin.[13] It increased serum TNF-α as well as TNF-α mRNA in the liver after LPS stimulation about twice as much in the estrogen-treated group as in untreated controls.[13] In line with the previous finding, both CD14 mRNA and protein were reduced by ovariectomy (Fig. 3). Moreover, the present study also showed that Kupffer cells isolated from ovariectomized rats with estrogen replacement produced more TNF-α than ovariectomized animals after 4 weeks of ethanol feeding (Fig. 4). Taken together, these results are consistent with the

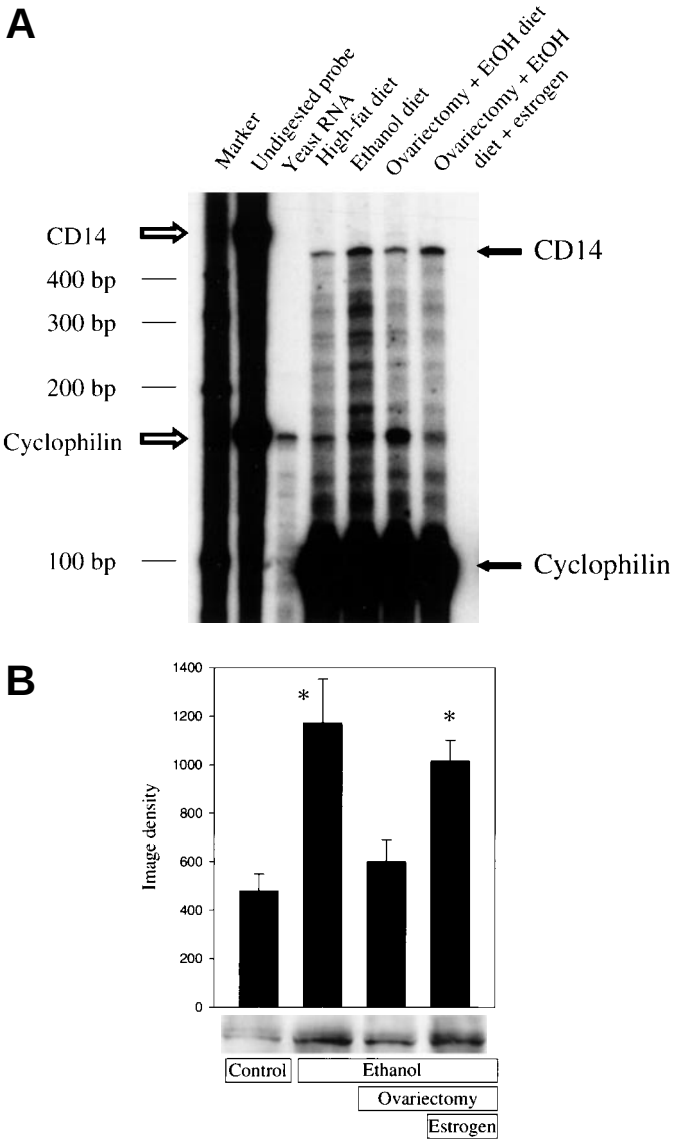

FIG. 3. Effects of ethanol, ovariectomy, and estrogen replacement on hepatic CD14 expression after 4 weeks of ethanol exposure. CD14 mRNA in the liver was measured by RNase protection assay (A), and CD14 protein in the liver was detected by Western blotting (B), as described in Materials and Methods. In the RNase protection assay (A), 20 μg of total RNA was analyzed by dual probe RNase protection assay using rat cyclophilin probe as a housekeeping gene. Yeast transfer RNA was used as a negative control. The same amount of cyclophilin could been clearly seen in all lanes with a shorter exposure (data not shown). A representative band from each group is shown. *Open arrows* indicate undigested probes for rat CD14 (597 bp) and rat cyclophilin (165 bp). *Solid arrows* indicate protected bands for CD14 (517 nt) and cyclophilin (103 nt). Data from Western blotting (B) are expressed as mean ± SEM. *$P < .05$ compared with nonovariectomized rats fed a high-fat control diet and ovariectomized rats fed ethanol by one-way ANOVA with Tukey's post-hoc test.

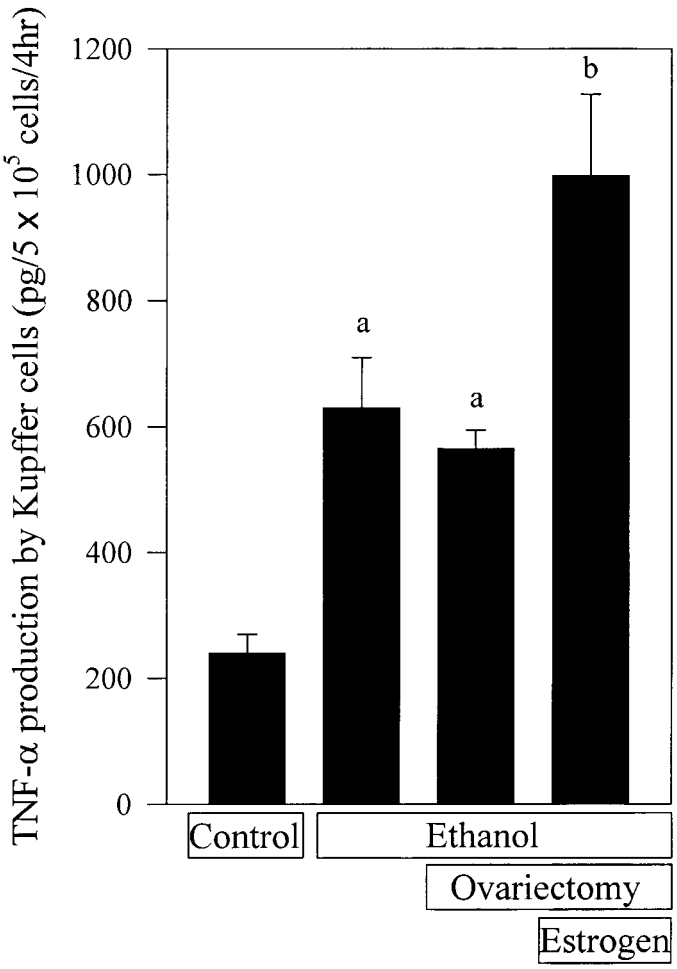

FIG. 4. Effects of ovariectomy and estrogen replacement on TNF-α production by Kupffer cells isolated 4 weeks after ethanol exposure. Kupffer cell isolation and quantitation of TNF-α are described in Materials and Methods. Cultured Kupffer cells were stimulated by LPS (1μg/mL) for 4 hours. Data are expressed as mean ± SEM. (a) $P < .05$ compared with controls. (b) $P < .05$ compared with all other 3 by one-way ANOVA using Tukey's post-hoc test.

hypothesis that during ethanol exposure, estrogen up-regulates CD14 expression in Kupffer cells. Sensitized Kupffer cells are activated by increased blood endotoxin because of estrogen-enhanced gut permeability, leading to elevated TNF-α production (Fig. 4) and ultimately to alcohol-induced tissue destruction. In addition, other factors might be involved in the development of alcohol-induced liver injury in this model, because no differences in TNF-α levels between ethanol-fed rats and ovariectomized ethanol-fed animals were observed.

In summary, rats with lower estrogen levels developed less alcohol-induced liver injury compared with animals with higher estrogen levels, because ovariectomy decreased serum ALT, liver weights and hepatic fatty accumulation, inflammation, and necrosis. Estrogen supplementation reversed the beneficial effects of ovariectomy on early alcohol-induced liver injury, clearly implicating the hormone in pathology. The increased sensitivity of female subjects to alcohol is partially because of estrogen-enhanced up-regulation of CD14 expression on Kupffer cells, thereby increasing sensitivity to gut-derived endotoxin, which is also elevated by estrogen.

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