Gender differences in early alcohol-induced liver injury: role of CD14, NF-κB, and TNF-α

HIROSHI KONO,1 MICHAEL D. WHEELER,1 IVAN RUSYN,1 MIN LIN,2 VITOR SEABRA,1 CHANTAL A. RIVERA,1 BLAIR U. BRADFORD,1 DONALD T. FORMAN,3 AND RONALD G. THURMAN1

1Laboratory of Hepatobiology and Toxicology, Department of Pharmacology and 3Department of Pathology, University of North Carolina, Chapel Hill, North Carolina 27599–7365; and
2Department of Medicine, University of Southern California, Los Angeles, California 90033

The establishment of a continuous intragastric enteral feeding protocol in the rat by Tsukamoto and French (13, 41) represented a major development in alcohol research. With this model, not only is steatosis observed, which is characteristic of several animal models, but inflammation and necrosis also occur in ~4 wk, and fibrosis begins to develop within 12–16 wk in male rats. With this model, it has been reported that intestinal sterilization with antibiotics (2) or lactobacillus feeding (25) diminishes plasma endotoxin levels and liver injury. Furthermore, destruction of Kupffer cells with GdCl₃ prevents early alcohol-induced liver injury in males (1). These results are consistent with the hypothesis that Kupffer cells activated by gut-derived endotoxin play an important role in early alcohol-induced liver injury in males.

Ethanol increases plasma endotoxin levels and expression of the lipopolysaccharide receptor CD14 on Kupffer cells in male rats (15, 34). Moreover, Kupffer cells activated by endotoxin produce reactive oxygen species. These oxidants could activate transcription factors such as nuclear factor (NF)-κB, which regulates production of inflammatory cytokines and adhesion molecules, leading to liver injury (15). Tumor necrosis factor (TNF)-α, which plays a pivotal role in the inflammatory cytotoxic cascade, is involved in early alcohol-induced liver injury. Indeed, anti-TNF-α antibody reduces inflammation and necrosis in the Tsukamoto-French model (16). Furthermore, early alcohol-induced liver injury is diminished in TNF receptor-1 knockout mice given intragastric enteral ethanol (47).

Previous work from this laboratory has shown that endotoxin levels in plasma, intercellular adhesion molecule-1 (ICAM-1) expression in sinusoidal lining cells, the number of infiltrating neutrophils in the liver, and liver injury are significantly greater in females than in males (15). Accordingly, the purpose of this study was to determine whether CD14 expression on Kupffer cells, activation of NF-κB, and TNF-α mRNA in the liver are responsible for greater pathology in the female. In the present study, both genders received progressively increasing doses of ethanol by carefully challenging them as tolerance developed over a 4-wk period, since alcohol-induced liver injury is dependent on the dose of ethanol (9). This approach resulted in ethanol delivery in both genders that was ~50% greater than in previous...
studies (15, 27, 39). Under these conditions, the extent of early alcohol-induced liver injury was more severe in both genders, yet the injury in females developed earlier and was more extensive than in males.

MATERIALS AND METHODS

Animals and experimental protocol. Age-matched male and female Wistar rats were given high-fat control or ethanol-containing diets for 4 wk using the intragastric enteral protocol developed by Tsukamoto and French (13, 41). Daily caloric intake reached 230 kcal·kg\(^{-1}\)·day\(^{-1}\) Ethanol initially was delivered at 10 g·kg\(^{-1}\)·day\(^{-1}\) (35% of total calories) and was increased 0.6 g·kg\(^{-1}\)·2·days\(^{-1}\) until the end of the first week and then 0.6 g·kg\(^{-1}\)·4·days\(^{-1}\) until the end of the experiment (final alcohol delivered = 17·g·kg\(^{-1}\)·day\(^{-1}\); 40% of total calories). All animals received humane care in compliance with institutional guidelines, and alcohol intoxication was assessed carefully to evaluate development of tolerance to ethanol using a 0–3 point scoring system (0, normal; 1, sluggish movement; 2, loss of movement but still moving if stimulated; 3, loss of consciousness). Ethanol was increased progressively to challenge animals based on this assessment, allowing ~50% more ethanol to be delivered than in previous studies by this and other laboratories (15, 27, 39).

Diets. A liquid diet described by Thompson and Reitz (36), supplemented with lipotropes as described by Morimoto et al. (24), was used. It contained corn oil as fat (37% of total calories), protein (23%), carbohydrate (5%), minerals and vitamins, plus ethanol (35–40% of total calories) or isocaloric dextrose (control diet) as described elsewhere (40).

Urine collection and ethanol assay. Daily ethanol concentrations in urine, which are representative of blood alcohol levels (4), were measured. Rats were housed in metabolic cages that separated urine from feces, and urine was collected over 24 h in bottles containing mineral oil to prevent evaporation. Each day at 9 AM, urine collection bottles were changed and a 1-ml sample was stored at −20°C in a microtube for later analysis. The ethanol concentration was determined by measuring absorbance at 366 nm resulting from the reduction of NAD\(^+\) to NADH by alcohol dehydrogenase (7).

Clinical chemistry. Blood was collected via the aorta at 4 wk and centrifuged. Serum was stored at −20°C in a microtube until it was assayed. Aspartate aminotransferase (AST) was analyzed by standard enzymatic procedures (7). Estradiol was measured with no-extraction, solid-phase 125I-tube until it was assayed. Aspartate aminotransferase (AST) was measured after 4 wk by counting cells in three high-power fields (×400) per slide. Fat accumulation caused ballooning of hepatocytes and narrowing of the sinusoidal space. This could affect the number of hepatocytes and sinusoidal space in each field; therefore, the number of hepatocytes was also counted and the number of neutrophils was expressed per 100 hepatocytes. The mean value from three high-power fields was used for statistical analysis.

Kupffer cell preparation and Western blotting for CD14. Kupffer cells were isolated by collagenase digestion and differential centrifugation using Percoll (Pharmacal, Piscataway, NJ) as described elsewhere with slight modifications (29). Cells were plated on plastic culture dishes and cultured in RPMI 1640 media (GIBCO Life Technologies, Grand Island, NY) supplemented with 25 mM HEPES, 10% fetal bovine serum, and antibiotics (100 U/ml of penicillin G and 100 µg/ml of streptomycin sulfate). After 1 h of incubation, Kupffer cells were scraped from the side of cell culture plates and pelleted by centrifugation at 500 g for 7 min. Cells pellets were resuspended in 250 µl of suspension buffer with Triton X-100, agitated for 15 min at 4°C, and centrifuged at 12,000 g for 10 min at 4°C. The supernatant was removed, and the pellet was resuspended in suspension buffer. Protein was stored at −20°C for subsequent Western blotting.

Western blotting for CD14. Extracted proteins (10 µg) from each group were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride 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, Little Chalfont, UK) and exposed to X-Omat films (Eastman Kodak, Rochester, NY).

Neutrophil protein extraction and gel mobility shift assay. A gel mobility shift assay was used in this study to assess the amount of active protein involved in protein-DNA interactions. The limitations of this method are the amount of nuclear protein needed for assay, as well as the number of cells isolated from the rat liver. There are alternative approaches available, such as fluorescence in end-labeled oligonucleotides, to avoid use of radioactivity and transient transfection of cells with a NF-κB-dependent luciferase reporter gene.

Binding conditions for NF-κB were characterized and electrophoretic mobility shift assays were performed as described in detail elsewhere (48). Briefly, extracts (40 µg) from liver tissues were preincubated for 10 min on ice with 1 µg poly(dI·dC) and 20 µg BSA (both from Pharmacia Biotech, Piscataway, NJ) in a buffer that contained 1 mM HEPES (pH 7.6), 40 mM MgCl\(_2\), 0.1 M NaCl, 8% glycerol, 0.1 mM dithiothreitol, 0.05 mM EDTA, and 2 µl of a 32P-labeled DNA probe (10,000 cpm/µl; Cerenkov) that contained 0.4 ng of double-stranded oligonucleotide. Mixtures were incubated 20 min on ice and resolved on 5% polyacrylamide (29:1 cross-linking) and 0.4× Tris-borate-EDTA (TBE) gel. After electrophoresis, gels were dried and exposed to Kodak film. Specificity of NF-κB binding was verified by competition assays and the ability of specific antibodies to supershift protein-DNA complexes. In competition assays, 200-fold excess of the una-
beled oligonucleotide was added 10 min before addition of the labeled probe. In the supershift experiment, 1 µg of rabbit antisera against p50 protein (Santa Cruz Biotech, Santa Cruz, CA) was added to the reaction mixture after incubation with labeled probe, which was further incubated at 25°C for 3 min. Labeled and unlabeled oligonucleotides contained the consensus sequence for NF-κB (top strand, 5'-GCAGAGGG-GACCTTTCCGGA-3'; bottom strand, 5'-GTCGCCCAAGTGTCCTCTG-3') (5). Data were quantitated by scanning autoradiograms with GelScan XL (Pharmacia).

RNA isolation and RT-PCR amplification. Liver tissues were flash-frozen in liquid nitrogen and stored at −80°C until analysis. Samples (~50 µg) of liver tissue were collected, and total cellular RNA was extracted using the Qiagen Rneasy kit (Qiagen, Chatsworth, CA) according to the manufacturer’s instructions. For the synthesis of complementary DNA (cDNA), 1.0 µg of total RNA from each sample was resuspended in a 20-µl final volume of reaction buffer, which contained 25 mM Tris-HCl, pH 8.3, 37.5 mM KCl, 10 mM dithiothreitol, 1.5 mM MgCl2, 10 mM of each dNTP (Perkin Elmer Cetus, Norwalk, CT), and 0.5 mg oligo(dT)12–18 primer (GIBCO BRL). After the reaction mixture reached 42°C, 400 units of RT (GIBCO BRL) was added to each tube and the sample was incubated for 45 min at 42°C. Reverse transcription was stopped by denaturing the enzyme at 95°C. The reaction mixture was diluted with distilled water to a final volume of 50 µl. Commercially available PCR primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Clontech Laboratories (Palo Alto, CA). Primers for TNF-α contained the following sequences: TNF-α, sense (5'-ATGAGGACACAGAAGCATTG-3') and antisense (5'-TACACCTTTCTGACTGGAAT-3'); GAPDH, sense (5'-TGAAAGTGCCAGTGCAAGTTGGT-3') and antisense (5'-CATGGGGCATGGGTCCACAC-3'). The size of amplified PCR products was 276 bp for TNF-α and 983 bp for GAPDH.

Aliquots (5 µl) of synthesized cDNA were added to 45 µl of PCR mix containing 5 µl of 10× PCR buffer, 1 µl of each deoxynucleotide (1 mM each), 0.5 µl of sense and antisense primers (0.15 mM), and 0.25 µl of DNA polymerase (Gene Amp PCR kit; Perkin Elmer Cetus).

The reaction mixture was covered with mineral oil, and amplification was initiated by 1 min of denaturation at 94°C for 1 cycle, followed by multiple cycles (20-35) at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min using a GeneAmp PCR system 9800 DNA Thermal Cycler (Perkin Elmer Cetus). After the last cycle of amplification, samples were incubated for 7 min at 72°C. For each set of primers, dilutions of cDNA were amplified for 20, 23, 25, 28, 30, and 35 cycles to define optimal conditions for linearity to permit semiquantitative analysis of signal strength. When appropriate, the specificity of the PCR bands was confirmed by restriction site analysis of the amplified cDNA, which generates restriction fragments of the expected size (data not shown).

The amplified PCR products were subjected to electrophoresis at 75 V through 2% agarose gels (GIBCO BRL) for 1 h. The dX174 HaeIII digest was used for molecular markers (Sigma Chemical). Agarose gels were stained with 0.5 mg/ml ethidium bromide TBE buffer (ICN, Costa Mesa, CA) and photographed with type 55 Polaroid positive/negative film. The relative amount of mRNA transcript was determined using Eagle Eye II still video system (Stratagene, La Jolla, CA). Densitometric analysis of the captured image was performed on a Macintosh computer using NIH Image 1.62 analysis software. The area under the curve was normalized to GAPDH content.

Statistics. ANOVA or Student’s t-test was used for the determination of statistical significance as appropriate. For comparison of pathological scores, the Mann-Whitney rank sum test was used. A P value < 0.05 was selected before the study as the level of significance.

RESULTS

Body weight and ethanol concentrations in urine. Liquid diets were initiated 1 wk after surgery to allow for complete recovery (Fig. 1). All animals survived throughout the enteral feeding period. Since animals were age matched, males started the study heavier than females, as expected. Previous work has shown that age-matched or weight-matched animals respond similarly to ethanol (15). Weight gains during enteral feeding were similar in males and females and were similar to rats fed chow in both genders (data not shown). There were no differences in behavior scores between males and females during alcohol exposure.

Representative plots of daily urine alcohol concentrations in ethanol-treated male and female rats are depicted in Fig. 2. As reported previously by several groups (1, 27, 39), alcohol levels fluctuate in a cyclic fashion from 0 to >400 mg/dl for unknown reasons. Similar patterns were observed in both males and females, confirming previous work by limuro et al. (15). Mean urine alcohol concentrations were 145 ± 18 mg/dl in males and 157 ± 10 mg/dl in females at 2 wk but increased to 236 ± 6 mg/dl in males and 243 ± 11 mg/dl in females at 4 wk as a result of higher delivery rates of ethanol after tolerance developed. There were no significant differences in mean urine alcohol concentrations between males and females in this study.

Serum transaminase and estrogen levels. At 3 wk of ethanol treatment, serum AST levels reached 149 ± 33 IU/l in males and 224 ± 28 IU/l in females (data not shown). The mean urine estrogen levels were significantly higher in females than males, confirming the expected sex differences in estrogen levels (data not shown).

Fig. 1. Effect of ethanol on body weight of rats. Age-matched male (275–300 g) and female (200–225 g) Wistar rats were used in this study. Body weight was measured once per week. Values are means ± SE (n = 6). Male rats given control diet; ♂, male rats given ethanol-containing diet; ♀, female rats given control diet; □, female rats given ethanol-containing diet.
shown). These values were nearly identical to levels observed at 4 wk of ethanol treatment with the standard enteral protocol (15). After 4 wk, however, values reached 389 ± 647 IU/l in males and 727 ± 66 IU/l in females (Fig. 3). Thus transaminases were about twofold greater in females than in males. Here, AST levels were nearly fourfold higher than in a previous report from this laboratory in both genders (15). Serum AST levels increased dramatically after 4 wk in this study because rats received nearly 50% more ethanol.

In rats fed a high-fat control diet for 4 wk, serum estradiol levels were significantly greater in females than in males as expected (males, 5.0 ± 0.8 pg/ml; females, 42.7 ± 11.1 pg/ml). Furthermore, enteral ethanol did not affect serum estradiol levels (males 15.3 ± 5.0 pg/ml; females 50.0 ± 12.5 pg/ml).

Pathological evaluation. There were no pathological changes in male rats given the control diet for 4 wk, whereas slight steatosis was observed in females (Fig. 4). Dietary ethanol dramatically increased fatty infiltration after only 2 wk and caused mild inflammation and focal necrosis after 4 wk in ethanol-fed male rats. In contrast, the micro- and macrovesicular pattern of steatosis was more severe and panlobular in ethanol-fed female rats. The time course of changes in hepatic pathology is summarized in Fig. 5. After only 2 wk of ethanol treatment, the score for steatosis was 1.7-fold higher in females than in males. After 4 wk, inflammation and necrosis were also 1.4-fold higher in females than in males. The number of infiltrating neutrophils in the liver was minimal and similar between males and females in the absence of ethanol; however, significant increases were produced by 4 wk of ethanol treatment in both genders. Importantly, values were about twofold greater in females than in males (Fig. 6).

Plasma endotoxin levels. Basal endotoxin levels were near levels of detection (<10 pg/ml) and not different in both genders (data not shown). In controls, endotoxin levels were minimal over the 4-wk time course of this study (Fig. 7). Values were increased significantly by enteral ethanol in both genders after 4 wk. Moreover, after 4 wk of enteral feeding with higher delivery of ethanol in the final 2–4 wk period, endotoxin levels were 2.5-fold higher in this study than in a previous study with the standard protocol (15), but values in females were significantly higher than in males.

Effects of chronic enteral ethanol and gender on expression of CD14 on Kupffer cells. It was reported previously that CD14 expression was increased in the liver from rats fed a high-fat control diet without ethanol in the enteral feeding model (34). In cells from...
rats fed high-fat control diets in this study, CD14 expression on Kupffer cells was minimal and not different between males and females. However, enteral ethanol caused a 1.5-fold increase in expression of CD14 in both genders after 4 wk, and values in females were significantly greater than in males (Fig. 8).

Effects of chronic enteral ethanol and gender on NF-κB in the liver. The active form of the pleiotropic transcription factor NF-κB was minimal in livers from female and male rats fed a high-fat control diet for 4 wk (Fig. 9A). However, chronic enteral ethanol increased NF-κB activity significantly, nearly fivefold over control values after 4 wk. Moreover, this activity was nearly threefold greater in females than in males.

To confirm that protein binding in nuclear extracts to the labeled oligonucleotide probe was specific for the active form of NF-κB, gel shift assays were carried out either in the presence of excess unlabeled double-stranded oligonucleotide with a consensus sequence for NF-κB binding or with antibodies specific for the
NF-κB p50 subunit (Fig. 9B). In the absence of nuclear proteins, no protein-DNA complex was detected. Furthermore, unlabeled oligonucleotide that contained the NF-κB binding site could effectively compete for DNA binding with 32P-labeled probe. Moreover, addition of anti-p50 antiserum reduced the intensity of the complex and produced supershifted complexes with a higher molecular mass.

Effects of chronic enteral ethanol and gender on TNF-α mRNA expression in the liver. TNF-α mRNA expression in livers from rats fed a high-fat control diet for 4 wk was minimal in both genders (Fig. 10). However, ethanol treatment increased TNF-α mRNA expression significantly, nearly fivefold over control values after 4 wk. Moreover, expression was nearly threefold greater in females than in males.

DISCUSSION

Ethanol is a key factor in hepatic pathology in both genders. In a previous weight-matched study, liver injury assessed from transaminase release and pathology was greater in females than in males (15). However, inflammation and necrosis were mild, and fibrosis was not detected because the experiment was short term. Since the severity of clinical alcoholic hepatitis and cirrhosis is related to the amount of alcohol consumed (32, 38), here the enteral feeding model was modified to increase ethanol delivery. This goal was achieved by increasing alcohol levels in the diet based on behavioral assessment of development of tolerance to ethanol. Accordingly, animals given ethanol were observed frequently for signs of severe alcohol intoxication, i.e.,
sluggish movement, relaxation of legs, and loss of consciousness, using the 0–3 point scoring system detailed in MATERIALS AND METHODS. In this manner, alcohol delivery in Wistar rats in the 300- to 350-g weight range could be increased to 17–18 g·kg⁻¹·day⁻¹, yielding average blood alcohol levels in the 225- to 250-mg/dl range. Thus alcohol delivery and alcohol levels were increased, 50% over previous studies from this and other laboratories with adult rats in the same weight range (13, 15, 42). As a result, serum transaminases were about fourfold higher (Fig. 3) and pathology scores were elevated significantly in both genders (Fig. 5). Moreover, steatosis, inflammation, and necrosis were also greater in females than in males in this study.

Nutrition and dietary factors are important in the pathogenesis of alcoholic liver injury (12). Therefore, it is important that weight gain be demonstrated to rule out possible nutritional complications. Here, we followed a protocol recommended by Badger et al. (4) to allow complete recovery from surgery for 1 wk before alcohol administration is initiated. Steady weight gains were observed in both genders given ethanol over 4 wk in this study (Fig. 1). Furthermore, early alcohol-induced liver injury was present in wild-type mice but prevented in TNF receptor-1 knockout mice given enteral ethanol and similar diets (47). In this study, rats grew rapidly, making nutritional complications an unlikely explanation for these results.

Role of endotoxin, CD14, and NF-κB in gender differences in early alcohol-induced liver injury. Previous work has shown that intestinal sterilization with antibiotics (2) and suppression of endotoxin production with lactobacillus feeding (25) minimizes liver injury in the Tsukamoto-French model. Furthermore, destruction of Kupffer cells with GdCl₃ prevents hepatic pathology as well (1). In the present study, circulating endotoxin was significantly higher in females than in males after 4 wk of enteral ethanol (Fig. 7). Based on these data, it is concluded that Kupffer cells activated by gut-derived endotoxin are involved in early alcohol-induced liver injury (see Fig. 11) (37). Activated Kupffer cells produce reactive oxygen species (19). These oxidants could activate NF-κB and induce transcription of...
inflammatory cytokines, such as TNF-α, which is involved in early alcohol-induced liver injury. Indeed, TNF-α antibody reduces liver injury in the Tsukamoto-French model (8, 16). Furthermore, alcohol-induced liver injury is prevented in TNF receptor-1 knockout mice given enteral ethanol (20). Here, chronic enteral ethanol increased NF-κB activity much more in female than in male liver (Fig. 10). ICAM-1 expression on sinusoidal lining cells and the number of neutrophils in the liver were also significantly greater in females than in males (15) (Fig. 6). Furthermore, TNF-α mRNA expression in the liver was greater in females than males in this study (Fig. 10). NF-κB activation could be a result of higher endotoxin levels, leading to increased CD14 expression on Kupffer cells (Fig. 8). Increased inflammatory cytokine and adhesion molecule synthesis by enhanced activation of NF-κB could be one explanation for the greater liver injury observed in females (see Fig. 11). Alternatively, enhanced NF-κB activation in female rats given ethanol may reflect an antiapoptotic defensive response, since Rel A is known to be intimately involved in this process (33).

Possible role of estrogen in gender differences in early alcohol-induced liver injury. It is currently not clear why females have higher endotoxin levels in the blood. It is possible that gut permeability to endotoxin or endotoxin production by gut microflora is greater in females than in males, or that endotoxin clearance in females is compromised. Alternatively, estrogen might influence the gut microflora in females differently than in males, since some bacterial strains that have aryl steroid sulfatase activity for estrogen sulfates exist in rat and human intestine (43). Functional estrogen receptors exist in intestinal epithelial cells, suggesting that estrogen could affect the gut (35). Indeed, estrogen replacement increases gut permeability and endotoxin levels significantly in ovariectomized rats in the Tsukamoto-French model (46). During enteral feeding with or without ethanol, serum estrogen levels were higher in females than in males in this study, as expected (males 15.3 ± 5.0 pg/ml; females 50.0 ± 12.5 pg/ml). Thus
these data are consistent with the hypothesis that the effect of estrogen on the gut is involved in the mechanism of gender differences in early alcohol-induced liver injury. On the other hand, estradiol treatment increases the number of hepatic low-density lipoprotein receptors, leading to increased lipoprotein clearance and decreased serum lipoprotein levels (44). Since lipoprotein binds endotoxin, this could contribute to the higher endotoxin levels observed in females.

The endotoxin receptor CD14, which is on the surface of the plasma membrane of Kupffer cells, plays an important role in activation of Kupffer cells (10). Pharmacological doses of estrogen treatment in vivo increase CD14 expression on Kupffer cells, and this increase was blunted by intestinal sterilization with antibiotics (11, 17). Importantly, CD14 expression on Kupffer cells was significantly greater in females than in males after ethanol treatment in this study (Fig. 8). Moreover, estrogen increased CD14 mRNA expression in livers from ovariectomized rats given enteral ethanol (46). Together, these results are consistent with the hypothesis that estrogen is involved in upregulation of CD14 expression on Kupffer cells during ethanol exposure (see Fig. 11). Importantly, a previous study from this laboratory has demonstrated that estrogen treatment in vivo increases intracellular calcium and cytokine production due to lipopolysaccharide in isolated Kupffer cells, suggesting that estrogen also increases sensitivity of Kupffer cells to endotoxin (17). Alternatively, it was reported that chronic endotoxin administration did not increase hepatic CD14 mRNA expression in rats (18). Furthermore, endotoxin can activate NF-κB in Kupffer cells in a CD14-independent manner (6).

Clinical implications. In addition to amount of alcohol consumed, risk factors for alcoholic hepatitis and cirrhosis include gender and being overweight (28). The Tsukamoto-French model has long been shown to have a strong nutritional component, with injury dependent on unsaturated fat in the diet (26). Moreover, it is now clear that the gender differences seen in the clinic are reflected in this model (Fig. 4) (15). Although the advantages and disadvantages of animal models have been discussed (23), this model exhibits three important characteristics similar to those observed clinically. Namely, injury in the Tsukamoto-French model is dependent on fat, gender, and alcohol. Thus it represents an ideal animal model to study mechanisms of alcohol-induced liver injury.

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Address for reprint requests and other correspondence: B. U. Bradford, Laboratory of Hepatobiology and Toxicology, Dept. of Pharmacology, CB #3765, Mary Ellen Jones Bldg., Univ. of North Carolina at Chapel Hill, Chapel Hill, NC 27599–7365.

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