

Sex-related liver injury due to alcohol involves activation of Kupffer cells by endotoxin

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RG Thurman. Sex-related liver injury due to alcohol involves activation of Kupffer cells by endotoxin. Can J Gastroenterol 2000;14(Suppl D):129D-135D. Females have a greater susceptibility to ethanol-induced liver injury than males. Females who drink ethanol regularly and have been overweight for 10 years or more are at greater risk for both hepatitis and cirrhosis than males, and females develop ethanol-induced liver injury more rapidly and with less ethanol than males. Female rats on an enteral ethanol protocol exhibit injury more quickly than males and have widespread fatty changes over a larger portion of the liver lobule. Moreover, levels of plasma endotoxin, intracellular adhesion molecule-1, free radical adducts, infiltrating neutrophils and nuclear factor kappa B are doubled in female rat livers compared with male rat livers after enteral ethanol treatment. Additionally, estrogen treatment *in vivo* increases the sensitivity of hepatic macrophages or Kupffer cells to endotoxin. Evidence has been presented that Kupffer cells are pivotal in the development of ethanol-induced liver injury. Destroying Kupffer cells with gadolinium chloride or decreasing bacterial endotoxin by sterilizing the gut with antibiotics inhibits early inflammation due to ethanol. Similar results have been obtained with anti-tumour necrosis factor-alpha antibody. These data pointed to the hypothesis that ethanol-induced liver injury involves elevations in circulating endotoxin concentrations leading to activation of Kupffer cells, which causes a hypoxia-reoxygenation injury. This theory has been tested using pimonidazole, a 2-nitroimidazole marker, to quantify hypoxia in downstream, pericentral regions of the hepatic lobule. After chronic enteral ethanol treatment, pimonidazole binding doubles. Enteral ethanol also increases free radicals detected with electron spin resonance. Radical adducts, with coupling constants such as alpha-hydroxyethyl radical, have been shown to arise from ethanol. Importantly, hypoxia and radical production detected in bile are also decreased by the destruction of Kupffer cells with gadolinium chloride. These data support the hypothesis that Kupffer cells contribute to the vital sex differences in liver injury caused by ethanol.

Key Words: Endotoxin; Ethanol; Hepatic injury; Hypoxia-reoxygenation; Kupffer cells; Rats; Sex

Le degré d'atteinte hépatique d'origine éthylique selon le sexe dépend de l'activation des cellules de Kupffer par les endotoxines

RÉSUMÉ : Les femmes sont plus sujettes à l'atteinte hépatique d'origine éthylique que les hommes. En effet, celles qui boivent régulièrement et qui font de l'embonpoint depuis 10 ans et plus sont exposées à un plus grand risque d'hépatite et de cirrhose que les hommes, et chez elles, l'atteinte hépatique d'origine éthylique s'installe plus rapidement et après l'ingestion de quantités moindres d'alcool. Des rates soumises à un protocole éthylique entérique manifestent une atteinte hépatique plus rapidement que les mâles et présentent des anomalies adipeuses réparties sur une portion plus importante du lobule hépatique. De plus, les taux d'endotoxines plasmatiques, de molécule 1 de l'adhésion, d'adduits des radicaux libres, de neutrophiles infiltrants et de facteur kappa B nucléaire se trouvent multipliés par deux dans les foies des rates si on les compare aux rats soumis au même traitement. En outre, le traitement cestrogénique *in vivo* accroît la sensibilité des macrophages du foie (ou cellules de Kupffer) aux endotoxines. Selon les preuves accumulées, les cellules de Kupffer sont essentielles au développement de l'atteinte hépatique d'origine éthylique. La destruction des cellules de Kupffer au moyen de chlorure de gadolinium ou la réduction des taux d'endotoxines bactériennes obtenue par la stérilisation du tractus intestinal au moyen d'antibiotiques inhibe l'inflammation dès les premiers stades. Des résultats similaires ont été enregistrés avec l'anticorps dirigé contre le facteur de nécrose tumorale alpha. Ces données ont mené à l'hypothèse selon laquelle l'atteinte hépatique d'origine éthylique suppose des élévations des taux circulants d'endotoxines amenant une activation des cellules de Kupffer responsables des lésions d'hypoxie-reoxygénération. Cette théorie a été testée avec le pimonidazole, un marqueur du 2-nitro-imidazole, pour quantifier l'hypoxie dans des régions en aval et péricentrales du foie. Après le traitement à l'éthanol entérique, la fixation du pimonidazole double. L'éthanol fait aussi augmenter les taux de radicaux libres décelés par spectroscopie par résonance du spin électronique. Les adduits des radicaux, avec constantes de couplage, comme le radical de l'alpha-hydroxyéthyle, sont dérivés de l'éthanol. Fait à noter, l'hypoxie et la production de radicaux décelés dans la bile sont aussi atténuées par la destruction des cellules de Kupffer obtenue avec le chlorure de gadolinium. Ces données appuient l'hypothèse selon laquelle les cellules de Kupffer contribuent aux différences d'atteinte hépatique d'origine éthylique selon le sexe.

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Mechanisms responsible for the hepatotoxicity of ethanol have not been fully characterized despite years of research. Chronic ethanol ingestion stimulates hepatic oxygen consumption and causes fatty liver, hepatomegaly, inflammation, fibrosis and cirrhosis. Recently, exciting evidence has emerged implicating hepatic macrophages or Kupffer cells in several aspects of this pathophysiology.

Because alcoholics are susceptible to infection, interest in the effect of ethanol on the reticuloendothelial system has escalated (1). After consumption of ethanol, significant alterations in host defense mechanisms occur, including alterations in reticuloendothelial function as well as modified immune, lymphocyte, granulocyte and platelet function (2). Recently, attention has turned toward the effect of ethanol on Kupffer cells (3), which are activated by gut endotoxin or lipopolysaccharide (LPS), and have been shown to be involved in ethanol-induced liver damage (4). The hepatocyte has previously been the central focus of most studies on the effects of ethanol on liver function. The ability of Kupffer cells to eliminate and detoxify various exogenous and endogenous compounds (eg, LPS) is an important physiological regulatory function. Recent studies have demonstrated that Kupffer cells produce key mediators that stimulate ethanol metabolism (5).

Studies were hampered by the lack of an appropriate model for the examination of the mechanism of ethanol-induced liver injury in laboratory animals until Tsukamoto et al (6) introduced the *in vivo* rat model of continuous enteral ethanol administration. In addition to the steatosis present in other models of ethanol injury, this model exhibited several characteristics that were similar, but not exactly identical, to human alcoholic liver disease, including inflammation, pericentral necrosis and ultimately fibrosis.

It has been hypothesized that the cascade of events leading to hepatotoxicity by ethanol is initiated by an increase in circulating LPS. We hypothesized that LPS initially activates Kupffer cells to produce mediators – a necessary step in producing a hepatic hypermetabolic state (eg, the ‘swift increase in alcohol metabolism’ [SIAM]) in parenchymal cells. Subsequently, hypoxia occurs in pericentral regions of the liver lobule, where toxic free radicals are formed on re-introduction of oxygen, resulting in cell death (Figure 1). This article reviews new data in support of the proposal that Kupffer cells play a pivotal role in hepatotoxicity after ethanol exposure, examines sex differences and focuses predominantly on new information obtained with an enteral ethanol delivery system.

GUT FLORA AND ETHANOL-INDUCED LIVER DAMAGE

Considerable recent evidence supports the theory that the gut and LPS participate in alcoholic liver injury. LPS is one of the components of the outer wall of Gram-negative bacteria that is involved in sepsis, organ failure and lethal shock. Elevated levels of blood LPS circulating to the liver can cause hepatic tissue injury. Figure 1 illustrates our hypothesis that ethanol alters gut microflora, resulting in an increase in Gram-negative bacteria, which is the origin of LPS. Alterna-

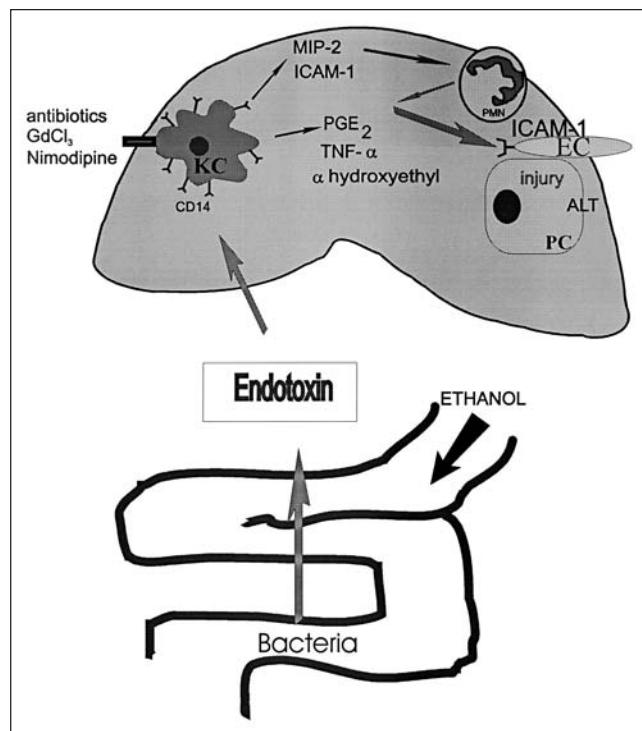


Figure 1) Working hypothesis depicting the mechanism by which ethanol elevates lipopolysaccharide (LPS) and stimulates Kupffer cells (KC). Ethanol alters gut bacteria, and an overgrowth of Gram-negative flora occurs. LPS is elevated in the blood and activates KC to produce receptors (CD14) and mediators such as macrophage inflammatory protein-2 (MIP-2) or intercellular adhesion molecule-1 (ICAM-1). Neutrophils (polymorphonuclear [PMN]) are activated and tumour necrosis factor-alpha (TNF-α) increases ICAM-1 expression on endothelial cells (EC). KC produce other chemokines such as prostaglandin E₂ (PGE₂) or alpha-hydroxyethyl radicals, which cause parenchymal cell (PC) injury. The inhibition of calcium channels with nimodipine, the destruction of LPS with antibiotics or the treatment with gadolinium chloride (GdCl₃), a KC toxicant, blocks the pathophysiological effects of ethanol *in vivo*

tively, ethanol may alter the permeability of the gut to macromolecules, thus increasing the release of LPS from the gut into the portal circulation.

DIETARY EFFECTS ON ETHANOL-INDUCED INJURY

Because undernourishment is frequently a complication of alcoholism, the effect of diet and ethanol on the gut flora is an important consideration (7). French and colleagues (7), using the enteral ethanol model, demonstrated that a diet high in unsaturated fatty acids (ie, linoleic or linolenic) is necessary for ethanol-induced liver injury. Rats fed a beef tallow diet show mild hepatic injury after chronic ethanol exposure and have normal gut bacterial flora (8). Alternatively, Gram-negative bacteria increase jejunal microflora in alcoholics (9). Furthermore, ethanol-induced liver injury using enteral ethanol feeding is decreased when gut microflora levels are diminished after treatment with lactobacillus (10) or antibiotics (11). Lactobacillus can suppress the development of a broad range of Gram-negative bacteria by produc-

ing low molecular weight substances, whereas antibiotics obliterate bacteria. Taken together, the results of these studies are consistent with the hypothesis that gut microflora can become more virulent after exposure to ethanol.

ROLE OF GUT-DERIVED LPS IN ETHANOL-INDUCED INJURY

Under normal conditions, the gut mucosal layer allows small amounts of antigens and other macromolecules to cross into the blood. Acute and chronic treatment with ethanol increases gut permeability to hemoglobin, horseradish peroxidase and polyvinylpyrrolidone macromolecules (12). Additionally, acute exposure to ethanol in vitro increases the permeability of the isolated small intestine to labelled LPS in a dose-dependent manner (13). In alcoholics, permeability of the small intestine to labelled ethylenediamine-tetra-acetic acid is doubled (14). Physical chemical studies of interactions of lipids with membranes using electron spin resonance demonstrated that ethanol increases membrane fluidity by altering the lipid and lipoprotein composition of the cell membrane. This alteration of membrane fluidity due to ethanol may result in enhanced transport and absorption of macromolecules. Changes in membrane fluidity by ethanol were observed almost 20 years before those significant experiments that used the enteral ethanol animal model, which demonstrated that dietary requirements are involved in ethanol toxicity. However, it is still not certain whether the dietary effects that prevent hepatic injury operate at the level of the gut mucosal barrier.

KUPFFER CELLS IN ETHANOL-INDUCED LIVER INJURY IN VIVO

Several findings support the hypothesis that Kupffer cells are involved in hepatic injury caused by ethanol. First, Adachi et al (15) demonstrated that, when Kupffer cells treated with enteral ethanol in rats are inactivated by gadolinium chloride in vivo, serum enzyme levels, fatty changes, inflammation and necrosis are significantly diminished. Additionally, ethanol affects Kupffer cell functions such as phagocytosis, bactericidal activity and cytokine production (Figure 1) (16). Serum tumour necrosis factor-alpha (TNF- α) levels are elevated in alcoholics (17), supporting the theory that Kupffer cells are activated in patients with alcoholic liver disease. In a recent study, plasma LPS, soluble TNF- α receptors and TNF- α were measured in patients who suffered from ethanol-induced cirrhosis (18). The data from this study were the first to report the strong correlation between plasma LPS and TNF- α soluble receptors in alcoholic cirrhosis. TNF- α is produced exclusively by the monocyte-macrophage lineage, and Kupffer cells are the primary population of this lineage (3). Lastly, Kupffer cells contain calcium ion channels, and chronic ethanol treatment promotes the opening of these channels. Intracellular calcium ion concentrations are increased twofold in isolated Kupffer cells only 2 h (early) after ethanol treatment in vivo. Moreover, Iimuro et al (19) recently reported that nifedipine, a calcium channel blocker, decreases ethanol-induced injury in the enteral ethanol model, suggesting that calcium channels have a crucial role in Kupffer cell activation.

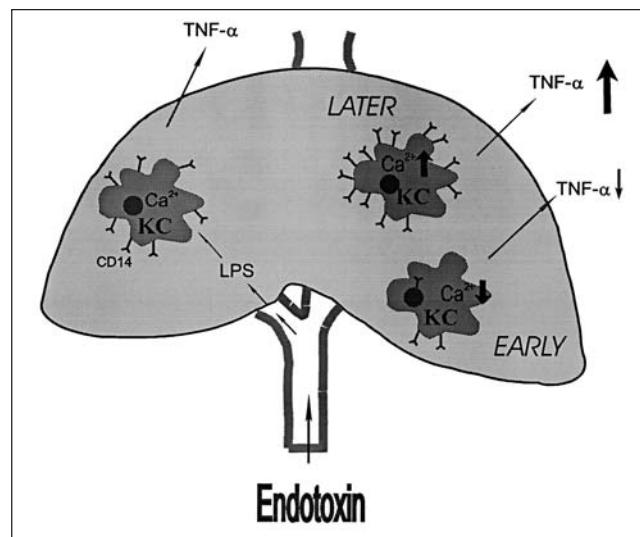


Figure 2) Hypothetical mechanism by which ethanol causes both the tolerance and sensitization of Kupffer cells (KC). As described in Figure 1, lipopolysaccharide (LPS) is elevated and removed by KC. After ethanol treatment, the tolerance of KC to LPS develops early (after approximately 2 h), but sensitization develops later (after 24 h). The endotoxin receptor CD-14 and the release of tumour necrosis factor-alpha (TNF- α) follows this pattern. CD-14 and TNF- α are blocked by antibiotics, thus pointing to LPS as the trigger

THE ROLE OF TNF- α IN ALCOHOLIC LIVER INJURY

A recent study examined temporal changes due to acute ethanol to evaluate the mechanism by which ethanol causes both the tolerance and sensitization of Kupffer cells (20). After ethanol administration, the level of blood LPS is elevated maximally at about 1 h (early). Initially, ethanol causes tolerance by mechanisms that are still not clear. After 24 h (late) of exposure to ethanol, however, CD14 and TNF- α are elevated sixfold and threefold, respectively (Figure 2). Additionally, sterilization of the gut with antibiotics blocks both the tolerance and the sensitization to ethanol. Taken together, these observations support the hypothesis that both of these phenomena involve LPS and Kupffer cells (20).

It is hypothesized that activated Kupffer cells release key mediators that are hepatotoxic or serve as chemoattractants for cytotoxic neutrophils that invade the liver (Figure 1). Various toxic mediators, including TNF- α , interleukins, prostaglandins (PGs) and oxygen radicals, are released from activated Kupffer cells (21). Monden et al (22) reported that TNF- α , superoxide and interleukin-1 inhibit protein synthesis in cultured rat hepatocytes, and that this effect can be observed in the media of cultures of activated Kupffer cells. TNF- α and interleukin-1, which are directly cytotoxic to a variety of cell types, may mediate hepatocyte injury. Moreover, a recent study demonstrated that rats administered ethanol enterally and injected with an antibody to TNF- α are protected from ethanol-induced hepatic injury (23). TNF- α and interleukin-1 stimulate neutrophil migration and activation, and also stimulate protease and oxygen radical release (24). Cellular infiltration of activated neutrophils, which produce

oxygen radicals and secrete other toxic mediators, may increase the inflammatory response, leading to hepatocellular injury and death. Indeed, inflammatory cell infiltration due to enteral ethanol is diminished by gadolinium chloride treatment. Disruption of the microcirculation caused by vasoactive mediators released from Kupffer cells and neutrophils may amplify hypoxia and lead to a vicious cycle of pathophysiology.

ETHANOL EXACERBATES THE EFFECT OF LPS

Previously, it has been reported that blood LPS concentrations are often elevated in alcoholics (25). In exciting experiments using the enteral ethanol model, levels of LPS in the blood begin to rise after about two weeks of enteral ethanol treatment (26). LPS concentrations increase nearly fivefold in the systemic circulation and are possibly higher in the portal circulation. Interestingly, blood LPS correlated ($r=0.84$) with pathology (necrosis, steatosis, inflammation, etc) (26). Bode et al (25), Remmer (27) and Adachi et al (11) have consistently been proponents of the theory that LPS plays a pivotal role in ethanol-induced liver injury.

Acute exposure to ethanol also activates Kupffer cells. During acute exposure to ethanol, carbon uptake by the perfused liver due to phagocytosis of particles by Kupffer cells increases about 25% (28). Carbon uptake is also increased significantly, about 35% in rat livers treated with ethanol a few hours before liver perfusion. Similar results have been obtained *in vivo* (29).

Few data linking Kupffer cell function to chronic ethanol exposure exist; however, a recent study has reported that oxygen radical production by Kupffer cells is elevated after chronic ethanol ingestion (16). Others have reported that TNF- α release and TNF- α mRNA expression are increased by ethanol, which is consistent with findings that TNF- α levels are increased in alcoholics (29). However, a number of studies have shown that ethanol paradoxically inhibits Kupffer cell function (30). Recently, ethanol treatment has been shown to cause tolerance after 2 h (early), followed by sensitization in Kupffer cells after 24 h (late) (Figure 2). TNF- α production from Kupffer cells stimulated with LPS decreases about fourfold 2 h after ethanol treatment but is elevated about threefold 24 h after ethanol treatment (20). Both tolerance and sensitization are blocked by antibiotics, implicating LPS and explaining this paradox.

KUPFFER CELLS PARTICIPATE IN ETHANOL-INDUCED HYPERMETABOLISM

Israel and colleagues (31) were the first to describe a hypermetabolic state due to ethanol exposure. Moreover, Yuki and Thurman (32) showed that oxygen and ethanol uptake increased twofold 2 to 3 h after a single large dose of ethanol using a perfused rat liver model. They also demonstrated that the hormone-mediated depletion of liver carbohydrate reserves participates in this process, a phenomenon that has been named 'SIAM'.

Ethanol metabolism increases with a reduction in both glycogenesis and glycogen reserves during SIAM (32). The involve-

ment of Kupffer cells in carbohydrate metabolism has been demonstrated (33). Moreover, oxygen and ethanol uptake were almost doubled after ethanol treatment – a phenomenon blocked by gadolinium chloride (5). Thus, increases in respiration and ethanol metabolism observed after ethanol treatment are blocked by the inactivation of Kupffer cells. Specifically, Kupffer cells produce PGs, primarily PGD₂ and PGE₂, which enhance the production of glucose from endogenous hepatic glycogen by activating phosphorylase A (33). Additionally, conditioned media from isolated Kupffer cells of ethanol-treated rats, which contain elevated levels of PGE₂, enhance parenchymal cell oxygen consumption (34). Therefore, regulation of SIAM clearly involves a Kupffer cell component, which appears to be due to production of PGE₂.

ETHANOL PRODUCES HYPOXIA IN PARENCHYMAL CELLS

In addition to hypermetabolism, high doses of ethanol change hepatic microcirculation by stimulating endothelin-1 production (35). Additionally, enteral ethanol administration causes hypoxia (36). Because ethanol also causes a compensatory increase in blood flow in the liver, which may elevate hepatic oxygen levels, it has been proposed that this increase negates any effect of hypoxia due to hypermetabolism or microcirculatory disturbances (37). Therefore, pimonidazole, a 2-nitroimidazole hypoxia marker used in radiobiology to assess local hypoxia in tumours, was studied (38). Pimonidazole, which is reductively activated by nitroreductases, binds to thiol residues on macromolecule proteins in the absence of oxygen, and adducts can be detected immunochemically. Pericentral hypoxia occurs during SIAM and is blocked when Kupffer cells are destroyed with gadolinium chloride (5).

It has been reported that chronic ethanol treatment using the enteral ethanol model also causes hypoxia by using 2-nitroimidazole markers and other techniques (15). These data provide direct evidence that ethanol increases tissue hypoxia *in vivo* (38). By employing the hypoxia marker pimonidazole, hypoxia can be quantified in rats after a month of ethanol feeding. Image analysis techniques have demonstrated that ethanol treatment for one or four weeks causes an augmentation of pimonidazole binding in the liver from 18% (control group) to 32% to 35% (ethanol-treated group) (38). Thus, direct evidence has been obtained demonstrating that hypoxia caused by ethanol treatment occurs in the clinically relevant enteral ethanol model or after acute ethanol treatment.

ROLE OF FREE RADICALS IN THE MECHANISM OF ETHANOL-INDUCED LIVER INJURY

Free radical production by ethanol has been implicated as a factor in its hepatotoxicity. Although evidence of lipid radical formation due to ethanol treatment *in vivo* has been reported, free radicals from ethanol alone been detected in living animals only recently (39). Ethanol-treated, alcohol dehydrogenase-deficient deer mice exhibited an alpha-(4-pyridyl-1-oxide)-*N*-*t*-butylnitrone (POBN)/alpha-hydroxyethyl radical adduct in bile after administration of ethanol and the spin

trap POBN using electron paramagnetic resonance technique and spin trapping (39).

Free radical formation most likely participates in the progression of primary events in alcoholic liver disease. Rats exposed to ethanol using the Tsukamoto et al (6) model of continuous enteral ethanol administration had a free radical in bile (15). This free radical signal decreased by over 50% when Kupffer cells were destroyed after treatment with gadolinium chloride. Furthermore, bile from rats fed a control corn oil diet contains low concentrations of radical adducts. The free radical has been identified as alpha-hydroxyethyl on the basis of the 12-line spectrum obtained when ^{13}C ethanol is used (40). Thus, ethanol-derived free radicals are detected in the bile of enteral ethanol-fed rats after a high fat, ethanol-containing diet. The precise pathways responsible for the formation of free radicals remain unclear. A possible candidate is oxygen radical production by the reduced nicotinamide adenine dinucleotide phosphate oxidase system in Kupffer cells, because the electron paramagnetic resonance signal is reduced by gadolinium chloride treatment. However, a reperfusion injury involving hypoxia and free radical formation via the xanthine-xanthine oxidase system and the cytochrome P450 2E1 system should not be excluded, especially because radicals in bile are expected to originate from parenchymal cells.

ROLE OF SEX

Three major independent risk factors for the development of hepatitis and cirrhosis have been identified after evaluation of 1600 alcoholic patients: consuming ethanol, being overweight for at least 10 years and being female. Sensitivity to ethanol in females is summarized in Table 1. In general, these studies demonstrate that ethanol consumption potentiates inflammatory responses, ethanol metabolism, hormone levels, hypoxia, free radicals and LPS in females. In rats, a study using enteral ethanol feeding established that ethanol causes more hepatic injury in females than in males (41). In that study, parameters including serum aspartate transaminase, pathological score, neutrophil infiltration, levels of circulating LPS and intracellular adhesion molecule-1 expression were evaluated. Interestingly, all parameters assessed were increased by ethanol treatment approximately two-fold in females compared with males. The most dramatic histological change is the panlobular deposition of fat in female livers after ethanol feeding, compared with the well known pericentral localization in males. Significantly more hepatic infiltration of inflammatory cells is observed after ethanol administration in the female. It has recently been demonstrated that the LPS receptor CD14 is elevated in Kupffer cells after treatment with estriol (42). Furthermore, LPS is higher after ethanol in female rats (41). Therefore, these data are consistent with the hypothesis that LPS and Kupffer cells are responsible for the increased susceptibility of females to ethanol.

Tissue hypoxia has been quantified using the hypoxia marker pimonidazole in male and female rats after enteral ethanol feeding (43). In this study, hypoxia marker binding is two to three times stronger in females than in males after a month of ethanol treatment. Furthermore, nuclear factor kappa B is

TABLE 1
Some factors for the increased susceptibility of females to ethanol-induced liver injury

Findings	Species studied	Reference
Higher levels of circulating LPS	Rat	41
Panlobular fat distribution in the liver	Rat	41
Greater liver injury with less ethanol	Rat	41
	Human	44
Elevated intercellular adhesion molecule-1 expression	Rat	41
More infiltrating neutrophils	Rat	41
Rapid ethanol metabolism	Rat	41
	Human	45,46
Increased mortality by estrogen due to LPS	Rat	42
Increased estrogen levels by ethanol	Human	47
Increased ethanol-induced injury by fat	Human	48
Decreased gastric alcohol dehydrogenase	Human	49
Elevated CD14 and lipid-binding protein with ethanol	Rat	20
Greater hypoxia and more free radicals	Rat	50
Greater fibrosis	Human	48
Injury due to ethanol blocked by ovariectomy, which is reversed by estrogen	Rat	51
Actin polymerization in Kupffer cells occurs only when ethanol and LPS are combined	Rat	52
Suppressed P450-mediated metabolism by LPS	Human	53
Lower cytokine-induced neutrophil chemoattractant after LPS	Rat	54
Decreased phagocytotic response to ethanol	Rat	54
Higher phospholipase A2 activity in lymphocytes and neutrophils	Human	55

LPS lipopolysaccharide

sensitive to oxidants and is increased seven to eight times more in females than in males. Because nuclear factor kappa B also increases adhesion molecule synthesis and inflammatory cytokine production, the above data may lead to the molecular mechanism of greater injury in females due to ethanol.

The use of the rat enteral feeding model will enable further mechanistic studies to provide insight into the pathophysiology of important sex differences due to alcohol. These data collectively demonstrate that females are more susceptible than males to ethanol-induced liver injury.

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