Mechanisms of Hepatic Toxicity
II. Alcoholic liver injury involves activation of Kupffer cells by endotoxin

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Thurman, R. G. Mechanisms of Hepatic Toxicity. II. Alcoholic liver injury involves activation of Kupffer cells by endotoxin. Am. J. Physiol. 275 (Gastrointest. Liver Physiol. 38): G605–G611, 1998.—It is well known that females show a greater susceptibility to alcohol-induced liver injury than males. Additionally, females who consume alcohol regularly and have been obese for 10 years or more are at greater risk for both hepatitis and cirrhosis. Female rats on an enteral alcohol protocol exhibit injury more quickly than males, with widespread fatty changes over a larger portion of the liver lobule. Levels of plasma endotoxin, intercellular adhesion molecule-1, free radical adducts, infiltrating neutrophils, and nuclear factor-κB are increased about twofold more in livers from female than male rats after enteral alcohol treatment. Estrogen treatment in vivo increases the sensitivity of Kupffer cells to endotoxin. Evidence has been presented that Kupffer cells are pivotal in the development of alcohol-induced liver injury. Destruction of Kupffer cells with gadolinium chloride (GdCl3) or reduction of bacterial endotoxin by sterilization of the gut with antibiotics blocks early inflammation due to alcohol. Similar results have been obtained with anti-tumor necrosis factor-α antibody. These findings led to the hypothesis that alcohol-induced liver injury involves increases in circulating endotoxin, leading to activation of Kupffer cells, which causes a hypoxia-reoxygenation injury. This idea has been tested using pimonidazole, a nitroimidazole marker, to quantify hypoxia in downstream pericentral regions of the liver lobule. After chronic enteral alcohol, pimonidazole binding increases twofold. Enteral alcohol also increases free radicals detected with electron spin resonance. Importantly, hepatic hypoxia and radical production detected in bile are decreased by destruction of Kupffer cells with GdCl3. These data are consistent with the hypothesis that Kupffer cells participate in important gender differences in liver injury caused by alcohol.

endotoxin; hepatic injury; hypoxia-reoxygenation; gender

THE TOXIC EFFECTS of alcohol on the liver have been described elsewhere; however, mechanisms responsible for its hepatotoxicity have not been fully characterized. It is known that chronic alcohol ingestion stimulates hepatic oxygen consumption and causes fatty liver, hepatomegaly, inflammation, fibrosis, and cirrhosis. New evidence has now emerged implicating Kupffer cells in several aspects of this pathophysiology.

Because alcoholics are prone to infection, interest in the effect of alcohol on the reticuloendothelial system (RES) has increased (3). After alcohol consumption, significant alterations in host defense mechanisms occur, including changes in reticuloendothelial function as well as modified immune, lymphocyte, granulocyte, and platelet function (48). Recently, attention has turned toward the effect of alcohol on Kupffer cells (14). These cells are activated by gut endotoxin [lipopolysaccharide (LPS)] and have been shown to be involved in alcohol-induced liver damage (41). Conversely, the hepatocyte has been the chief focus of most studies on the effects of alcohol on liver function. The ability of Kupffer cells to eliminate and detoxify various exogenous and endogenous substances (e.g., endotoxin) is an important physiological regulatory function. Recent experiments have shown that Kupffer cells produce mediators that stimulate alcohol metabolism (11).

The lack of appropriate experimental models hampered studies on the mechanism of ethanol-induced liver injury in laboratory animals until Tsukamoto et al. (51) 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 exhibits several characteristics similar, although not exactly identical, to human alcoholic liver disease, including inflammation, pericentral necrosis, and ultimately fibrosis.

It has been suggested that the cascade of events leading to alcohol-induced hepatotoxicity is initiated by an increase in the delivery of endotoxin. We hypothesize that endotoxin stimulates Kupffer cells initially to produce mediators, a critical step in producing a hepatic hypermetabolic state [e.g., the swift increase in alcohol metabolism (SIAM)] in parenchymal cells. Subsequently, hypoxia develops in pericentral regions of the liver lobule, where toxic free radicals are formed on reintroduction of oxygen, resulting in cell death (Fig. 1).

In this study, we examine new evidence in support of the proposal that Kupffer cells play a pivotal role in hepatotoxicity after alcohol exposure, which may also explain gender differences. We focus predominantly on
new information obtained with an enteral alcohol delivery system.

**GUT INVOLVEMENT IN ALCOHOL-INDUCED LIVER DAMAGE**

Considerable recent evidence supports the hypothesis that the gut and endotoxin participate in alcoholic liver injury. Endotoxin (LPS), one of the components of the outer wall of gram-negative bacteria, has been implicated in sepsis, organ failure, and lethal shock. Elevated levels of circulating endotoxin delivered to the liver via portal blood can cause hepatic tissue injury. Figure 1 depicts our hypothesis that ethanol alters gut bacteria and causes an overgrowth of gram-negative flora. Endotoxin is increased in blood and activates Kupffer cells to produce receptors (CD-14) and mediators such as macrophage inflammatory protein-2 (MIP-2) or intercellular adhesion molecule-1 (ICAM-1), which bind polymorphonuclear neutrophils (PMN). Kupffer cells produce other mediators such as prostaglandin E2 (PGE2) or tumor necrosis factor-α (TNF-α) and α-hydroxyethyl radicals causing parenchymal cell (PC) injury. Blockage of Ca²⁺ channels with nimodipine, destruction of endotoxin with antibiotics, or treatment with gadolinium chloride (GdCl₃), a Kupffer cell toxicant, blocked the pathophysiological effects of ethanol in vivo.

**DIETARY EFFECTS**

Because malnutrition is frequently a complication of alcoholism, the effect of diet and ethanol on the gut flora is an important consideration (18). French (18), using the enteral ethanol model, has demonstrated that a diet rich in unsaturated fatty acids (i.e., linoleic or linolenic) is an absolute requirement for ethanol-induced liver damage. Rats fed a beef tallow diet show minimal hepatic injury after chronic ethanol exposure (18) and have normal gut bacterial flora (22). On the other hand, gram-negative bacteria increase in the jejunal microflora in alcoholics (9). Furthermore, ethanol-induced liver injury using enteral ethanol feeding is diminished when gut microflora are reduced after treatment with lactobacillus (38) or antibiotics (2). Lactobacillus can suppress the growth of a broad range of gram-negative bacteria due to the production of low-molecular-weight substances, whereas antibiotics destroy bacteria. Taken together, these studies are consistent with the hypothesis that the gut microflora can become more virulent after exposure to ethanol.

**ENDOTOXIN PARTICIPATES IN ETHANOL-INDUCED INJURY**

Under normal conditions, the gut mucosal layer is an imperfect barrier, allowing small amounts of antigens and other macromolecules (10) to pass through the intestinal wall into the blood. Both acute and chronic treatments with ethanol increase gut permeability to hemoglobin, horseradish peroxidase, and polyvinylpyrrolidone macromolecules. Furthermore, acute exposure to ethanol in vitro increases permeability of the isolated small intestine to labeled endotoxin in a dose-dependent manner (4). In alcoholic subjects, permeability of the small intestine to labeled EDTA is elevated about twofold (6). Physical chemical studies using electron spin resonance of interactions of lipids with membranes demonstrated that ethanol increases membrane fluidity due to alterations in the lipid and lipoprotein composition of the cell membrane. This modification of membrane fluidity due to ethanol may result in increased transport and absorption of macromolecules. Changes in membrane fluidity due to ethanol were observed almost 20 years before the significant experiments using the enteral ethanol model, which demonstrated that dietary requirements are involved in ethanol toxicity. However, it is still not clear whether 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 observations support the hypothesis that Kupffer cells are involved in hepatic injury caused by ethanol. First, Adachi et al. (1) have demonstrated that, when Kupffer cells in rats treated with enteral ethanol are inactivated by gadolinium chloride (GdCl₃), serum enzyme levels, fatty changes, inflammation, and necrosis are decreased significantly. Additionally, ethanol affects Kupffer cell functions such as phagocytosis, bactercidal activity, and cytokine production (Fig. 1) (52). Next, serum tumor necrosis factor-α (TNF-α) increases in alcoholics (47), supporting the idea that Kupffer cells are activated in patients with alcoholic...
liver disease. TNF-α is produced exclusively by the monocyte-macrophage lineage, and Kupffer cells are the major population of this lineage (14). Finally, Kupffer cells contain Ca²⁺ channels, and chronic ethanol treatment facilitates their opening. Intracellular Ca²⁺ is doubled in isolated Kupffer cells only 2 h after ethanol treatment in vivo. Moreover, Iimuro et al. (27) have recently demonstrated that nimodipine, a Ca²⁺ channel blocker, reduces ethanol-induced injury in the enteral ethanol model, suggesting that Ca²⁺ channels play a crucial role in Kupffer cell activation.

**TNF-α PLAYS A ROLE IN ALCOHOLIC INJURY**

A recent study has examined temporal changes due to acute ethanol to understand how ethanol causes both tolerance and sensitization to Kupffer cells (17). After ethanol administration, blood endotoxin is elevated maximally at about 1 h. Initially, ethanol causes tolerance by mechanisms that are still not clear. After 24 h of exposure to ethanol, however, CD-14 and TNF-α are elevated sixfold and threefold, respectively (Fig. 2). Furthermore, sterilization of the gut with antibiotics blocks both tolerance and sensitization to ethanol. Collectively, these observations support the hypothesis that both of these phenomena involve endotoxin and Kupffer cells (17).

It is hypothesized that activated Kupffer cells release mediators that are toxic to liver cells or serve as chemotactants for cytotoxic neutrophils that invade the liver (Fig. 1). Various mediators, including TNF-α, interleukins, prostaglandins, and oxygen radicals, are released from activated Kupffer cells (35). Monden et al. (36) have reported that TNF-α, superoxide, and interleukin-1 (IL-1) inhibit protein synthesis in cultured rat hepatocytes and that this effect can be observed in the supernatant of cultures of activated Kupffer cells. TNF-α and IL-1, which are directly cytotoxic to a variety of cell types, may be direct mediators of hepatocyte injury. A recent study has shown that rats administered ethanol enterally and injected with antibody to TNF-α are protected from ethanol-induced hepatic injury (26). Moreover, TNF-α and IL-1 stimulate neutrophil migration and activation and also induce protease and oxygen radical release (49). Cellular infiltration of activated neutrophils, which produce oxygen radicals and secrete other toxic mediators, may increase the inflammatory response, leading to cell injury and death. Indeed, inflammatory cell infiltration due to enteral ethanol is diminished by GdCl₃ treatment. Microcirculatory disturbances caused by vasoactive mediators released from Kupffer cells and neutrophils may enhance hypoxia and lead to a vicious cycle of pathophysiology.

Platelet-activating factor (PAF) is a very potent mediator generated in many cell types, including endothelial cells, neutrophils, macrophages, Kupffer cells, and platelets (13). PAF produces a wide range of physiological effects, which have been reviewed recently elsewhere (13) and include platelet aggregation, stimulation of macrophages, inflammation, ovulation, glycerogenesis, and endotoxin shock. Furthermore, arachidonic acid is released in response to PAF.

**ETHANOL ENHANCES THE EFFECT OF ENDOTOXIN**

Blood endotoxin is often elevated in alcoholics (8). In exciting experiments using the enteral ethanol model, it has been shown that levels of endotoxin in the blood begin to rise after ~2 wk of continuous intragastric administration of dietary ethanol (37). Endotoxin increases nearly fivefold in the systemic circulation, and levels of endotoxin in the portal circulation are most likely even higher. Interestingly, blood endotoxin is correlated (r = 0.84) with pathology (necrosis, steatosis, inflammation) (37). Bode et al. (8), Remmer (43), and our own group (2) have consistently been proponents of the theory that endotoxin plays a pivotal role in alcoholic liver injury.

It has been demonstrated that 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% (15). Furthermore, carbon uptake in the perfused liver is also elevated significantly, by about 50%, in rats treated with ethanol a few hours before perfusion. Similar results have been obtained in vivo (16).

Few data linking Kupffer cell function to chronic ethanol exposure have been reported. However, a recent study has reported that oxygen radical production by Kupffer cells is increased after chronic ethanol treatment (52). Others have reported that TNF release
and TNF mRNA expression are increased by ethanol, consistent with findings that TNF increases in alcoh-
olics (16). However, a number of studies have shown that ethanol paradoxically suppresses Kupffer cell function (40). Recently, ethanol has been shown to cause toler-
ance early (2 h), followed by sensitization in Kupffer cells 24 h later (Fig. 2). TNF production from Kupffer cells stimulated with LPS decreases about fourfold 2 h af-
after administration of ethanol and increases threefold at 24 h after ethanol (17). Both tolerance and sensitiza-
tion are blocked by antibiotics, implicating endotoxin and explaining an apparent paradox.

KUPFFER CELLS PLAY A ROLE IN HYPERMETABOLISM INDUCED BY ETHANOL

Israel and colleagues (30) were the first to describe a hypermetabolic state due to ethanol exposure. More-
over, Yuki and Thurman (54), using a perfused liver model, have shown that oxygen and ethanol uptake nearly double in just a few hours after treatment of rats with a single large dose of ethanol using a perfused liver model. They have also demonstrated that hormone-
mediated depletion of hepatic carbohydrate reserves plays a role in this process, which has been named the "swift increase in alcohol metabolism" (SIAM).

Elevation of ethanol metabolism occurs in concert with a reduction in both glycolysis and glycogen re-
erves during SIAM (54). Recently, studies have demon-
strated the involvement of Kupffer cells in carbohydrate metabolism (12). In these experiments, basal rates of oxygen and ethanol uptake are almost doubled after ethanol treatment and are blocked by GdCl3 (11). Thus increases in respiration and ethanol metabolism observed after ethanol treatment are blocked by inactiva-
tion of Kupffer cells. Specifically, Kupffer cells pro-
duce prostaglandins, primarily PGD2 and PGE2, which stimulate production of glucose from endogenous he-
patic glycogen by activating phosphorylase A (12). Importantly, conditioned medium from isolated Kupffer cells of ethanol-treated rats, which contained elevated levels of PGE2, stimulated parenchymal cell oxygen consumption (42). Thus regulation of SIAM clearly in-
volves a Kupffer cell component, which appears to be due to production of PGE2.

ETHANOL CAUSES HYPOXIA IN PARENCHYMAL CELLS

In addition to hypermetabolism, high doses of etha-
non alter hepatic microcirculation by stimulating endo-
thelein-1 production (23). Furthermore, enteral ethanol treatment causes hypoxia (19). Because ethanol also causes a compensatory increase in hepatic blood flow and thus could elevate hepatic oxygen levels, it has been proposed that this increase negates any effect of hypoxia due to hypermetabolism or microcirculatory disturbances (44). Therefore, pimonidazole, a 2-nitroim-
idazole hypoxia marker used in radiology to assess hypoxia in tumors, has been studied (5). Pimonidazole, which is reductively activated by nitroreductases, binds to thiol residues on proteins and macromolecules in the absence of oxygen, and adducts can be detected immu-
nochemically. Results of these experiments confirm that pericentral hypoxia occurs during SIAM and is blocked when Kupffer cells are destroyed with GdCl3 (11).

It has recently been demonstrated, using 2-nitroimid-
zoles and other techniques, that chronic ethanol treat-
ment using the enteral ethanol model also causes hypoxia (1). These data provide direct evidence that ethanol in vivo increases tissue hypoxia (5). By using the hypoxia marker pimonidazole, hypoxia can be quantitated in rats after 4 wk of ethanol feeding. Image analysis techniques have demonstrated that ethanol treatment for 1 or 4 wk increases pimonidazole binding in the liver from 18% (control) to 32–35% (ethanol) (5). Thus direct evidence has now been obtained demonstrat-
ing that hypoxia caused by ethanol treatment occurs in the clinically relevant enteral ethanol model or after acute ethanol treatment.

INVolVEMENT OF FREE RADICALS IN THE MECHanISM OF ETHANoL-INDUCED LIVER INJURY

Production of free radicals by ethanol has been sug-
spected as a factor in its hepatotoxicity. Although evi-
dence of lipid radical formation due to ethanol treat-
ment in vivo has been reported, only recently have free radicals from ethanol alone been detected in living animals (33). The application of the electron paramag-

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ICAM-1, intercellular adhesion molecule-1; LPS, lipopolysaccha-
ride.
namic resonance technique of spin trapping to the study of ethanol-treated alcohol dehydrogenase-deficient deer mice revealed 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 (33).

Free radical formation most likely participates in the progression of early events in alcoholic liver disease. Recently, Adachi et al. (1) detected a free radical in bile from rats exposed to ethanol on the Tsukamoto-French model. This free radical signal was diminished over 50% when Kupffer cells were destroyed after treatment with \( \text{GdCl}_3 \). Furthermore, bile from animals fed a control corn oil diet contained low concentrations of radical adducts. The free radical has been identified as \( \alpha \)-hydroxyethyl on the basis of the 12-line spectrum obtained when \([^{13}\text{C}]\text{ethanol}\) is used (32). Thus ethanol-derived free radicals are detected in the bile of enteral ethanol-fed rats after a high-fat, ethanol-containing diet. Although the precise pathways responsible for formation of free radicals remain unclear, a likely candidate is oxygen radical production by the NADPH oxidase system in Kupffer cells, since the electron paramagnetic resonance signal is reduced by \( \text{GdCl}_3 \) treatment. However, a reperfusion injury involving hypoxia and free radical formation via the xanthine-xanthine oxidase system cannot be excluded, especially since radicals in bile would be expected to originate from parenchymal cells.

**ROLE OF GENDER**

Evaluation of 1,600 alcoholic patients has identified three major independent risk factors for developing hepatitis and cirrhosis: consuming alcohol, being overweight for at least 10 years, and being female (39). Figure 3 depicts such a female. This painting is entitled “Malle Babbe” and was painted by Frans Hals and copied by Han van Meegeren, who successfully sold his forgeries to major art collectors and museums in the 1940s. “Malle Babbe” depicts an overweight, older woman who clearly enjoys a drink. Differences in sensitivity to ethanol in females are summarized in Table 1. In general, these studies demonstrate that alcohol consumption potentiates inflammatory responses, alcohol metabolism, hormone levels, hypoxia, free radicals, and endotoxin in females. In rats, a recent study using enteral ethanol feeding has established that ethanol causes greater hepatic injury in females (25). This study measures parameters including serum aspartate aminotransferase, pathological score, neutrophil infiltration, levels of circulating endotoxin, and expression of intercellular adhesion molecule-1. Interestingly, all parameters assessed are increased by ethanol about twofold more in females than in males. The most impressive histological change is the panlobular deposition of fat in livers from females after ethanol feeding compared with the well-known pericentral localization in males. Furthermore, significantly more hepatic infiltration of inflammatory cells is observed after ethanol administration in females. Ikejima et al. (29) have recently demonstrated that the endotoxin receptor CD-14 is elevated in Kupffer cells after treatment with estriol. Moreover, LPS is higher after ethanol in female rats (25). Thus, taken together, these data are consistent with the hypothesis that endotoxin and Kupffer cells are responsible for the increased susceptibility of females to ethanol.

More recently, tissue hypoxia has been quantitated using the hypoxia marker pimonidazole in male and female rats after enteral ethanol feeding (28). In this study, hypoxia marker binding is two to three times more intense in females than in males after 4 wk of ethanol treatment. Furthermore, nuclear factor-\( \kappa \text{B} \) (NF-\( \kappa \text{B} \)) is sensitive to oxidants and is elevated seven to eight times more in females than in males. Since NF-\( \kappa \text{B} \) also increases adhesion molecule synthesis and inflammatory cytokine production, these data may lead to the molecular mechanism of enhanced injury in females due to ethanol.

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

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