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MECHANISMS OF ALCOHOL-INDUCED HEPATOTOXICITY: STUDIES IN RATS

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1. ABSTRACT

Alcohol treatment results in increases in the release of endotoxin from gut bacteria and membrane permeability of the gut to endotoxin, or both. Females are more sensitive to these changes. Elevated levels of endotoxin activate Kupffer cells to release substances such as eicosanoids, TNF-alpha and free radicals. Prostaglandins increase oxygen uptake and most likely are responsible for the hypermetabolic state in the liver. The increase in oxygen demand leads to hypoxia in the liver, and on reperfusion, alpha-hyroxyethyl free radicals are formed which lead to tissue damage in oxygen-poor pericentral regions of the liver lobule.

2. INTRODUCTION

Alcohol-induced liver injury causes serious medical, financial and social problems. The disease progresses from fatty infiltration and follows a pernicious course of inflammation leading to irreversible damage; liver transplantation, at a cost of about $100,000 each, is the only known cure. The fourth leading cause of death in urban American males is alcoholic liver disease, and the number of female alcohol abusers has increased over the last 30 years. Although the hepatotoxic effects of alcohol have been described previously,(1) factors responsible have only been partially characterized.

Significant changes occur in host defense mechanisms after consumption of alcohol, including modified reticuloendothelial function as well as altered immune, lymphocyte, granulocyte and platelet functions (2) . Interest in the effect of alcohol on the reticuloendothelial system (RES) has mainly been in the context of the known predisposition of alcoholics to infection (3). Recently, however, attention has been directed toward the role of endotoxin and Kupffer cells (4). It is well known that chronic ethanol ingestion produces fatty liver, hepatomegaly, alcoholic hepatitis, fibrosis, and cirrhosis. It is now clear that Kupffer cells are key in at least the early aspects of this pathology.

It has been proposed that the cascade of events leading to alcohol-induced toxicity is initiated by endotoxin. We hypothesize that endotoxin initially activates the Kupffer cell, which is critical for producing a hypermetabolic state (i.e., the Swift Increase in Alcohol Metabolism; SIAM) in parenchymal cells. This leads to hypoxia in pericentral regions of the liver lobule where toxic free radicals are formed upon reintroduction of oxygen, causing cell death. This hypothesis was supported by the discovery that treatment with antibiotics and destruction of Kupffer cells with GdCl3 blocked alcohol-induced liver injury in the Tsukamoto-French model of continuous enteral alcohol administration *in vivo* (table 1) (5,6). The ability of Kupffer cells to remove and detoxify various exogenous and endogenous substances, such as endotoxin, is an important physiological process. Recent work has shown that Kupffer cells are required for alcohol-induced hypermetabolic state in experimental animals (7).

Several observations suggest that Kupffer cells are involved in liver injury caused by alcohol. First, alcohol has been reported to alter Kupffer cell functions such as phagocytosis, bactericidal activity and cytokine production (8). Second, the increase in serum TNF-alpha concentration in alcoholics (9) is consistent with the hypothesis that Kupffer cells of patients with alcoholic liver disease are activated; TNF alpha is produced exclusively by the monocyte-macrophage lineage, and the major cell type of this lineage is the hepatic Kupffer cell (10). Third, Kupffer cells, which are activated by calcium and contain Ca^{2+} channels, are opened by chronic ethanol (11). Reports that the calcium channel blocker nimodipine decreased alcohol-induced liver injury in the Tsukamoto-French model, suggests that Kupffer cell calcium channels play an important role in the mechanisms of alcoholic liver disease (12). Collectively, these observations are

Table 1. Effect of $GdCl_3$ and Antibiotic Treatment on Blood AST Levels in Tsukamoto-French Rats

Treatment	AST IU/L
Control	126 + 17
Ethanol	205 + 24*
Ethanol + $GdCl_3$	140 +12
Ethanol + Antibiotics	101 + 8

All rats were treated for 3 weeks with ethanol containing diet via gastric cannula as described by Tsukamoto and French. Rats treated with $GdCl_3$ received 10 mg/kg i.v. twice weekly and polymixin B and neomycin (150 and 450 mg/kg respectively) were added to the liquid diet. Mean $\pm$ SEM where n=5-10 and * $p < 0.01$ using ANOVA compared to control.

Table 2. Gender Differences in Plasma Endotoxin Levels and ICAM-1 Expression in Liver from Tsukamoto-French Rats

Gender	Endotoxin pg/ml	ICAM-1% of ICAM expressing tissue
Male		
❑ Control	8 +6	0.6 +0.3
❑ Ethanol	36 +10*	9.3 + 1.1*
Female		
❑ Control	15 +18	2.7 + 0.8
❑ Ethanol	88 +15*#	18.6+ 3.0*#

Male and female rats treated with high fat (control) or ethanol + high fat (ethanol) were maintained using the Tsukamoto-French model for 4 weeks. Plasma was collected for endotoxin from the portal vein into sterile syringes and analyzed using a LAL assay. ICAM-1 expression in liver was determined using a monoclonal antibody (1A29) and a Peroxidase linked anti-mouse second antibody with DAB to detect specific binding. Results are Mean $\pm$ SEM, n=4-7, * $p < .05$ versus control and # $p < 0.05$ comparing genders.

consistent with the hypothesis that chronic exposure to alcohol leads to activation of Kupffer cells.

3. GENDER STUDIES

Since it is known that women develop hepatic injury more rapidly and with exposure to less ethanol than men; we developed a new animal model to study female susceptibility to alcohol-induced liver injury. Female and male Wistar rats were given ethanol (11-12 g/kg/day) continuously for up to 4 weeks via intragastric feeding using the Tsukamoto-French model . Control rats were fed a high-fat diet without ethanol. There were no significant differences in body weight, mean ethanol concentrations (F: 230 ± 11 mg/dl; M: 228 ± 7 mg/dl), or the cyclic pattern of ethanol levels in urine (F: 5.2 ± 0.2 days; M: 5.5 ± 0.2 days). Furthermore, rates of ethanol elimination were similar between the genders.

Ethanol treatment elevated serum AST levels in male rats to 122 ± 10 IU/L after 4 weeks while in females, values increased more rapidly and reached higher levels (168 ± 18 IU/L, $p < 0.05$). Steatosis, inflammation, and necrosis assessed histologically developed more rapidly and were more severe in females than males. Indeed, steatosis due to ethanol exposure, which was localized in centrilobular areas in males, was panlobular in the female.

Table 2 summarizes gender differences after ethanol treatment. Plasma endotoxin levels were more than 2-fold higher in females than males following ethanol feedng and ICAM-1 expression in hepatic endothelial cells was also about 2-fold greater in females than males. Thus, a new model to study female susceptibility to alcoholic hepatitis has been developed (13). Initial work with this model fits the idea that endotoxin and Kupffer cells are responsible for the increased susceptibility of women to alcohol compared to men.

4. ROLE OF HYPOXIA AND FREE RADICALS

A possible mechanism of alcohol-induced liver injury involves hypoxia and free radical formation. Israel *et al.* demonstrated that chronic ethanol treatment increased hepatic oxygen uptake, (14) while Ji *et al*. reported that increased tissue respiration caused by chronic ethanol treatment made the intralobular oxygen gradient steeper (15). This stimulation in oxygen uptake is due to an alcohol-induced hypermetabolic state (16). Subsequent centrilobular hypoxia may be responsible for pericentral liver injury induced by ethanol. Destruction of Kupffer cells with $GdCl_3$ treatment prevented the elevation in oxygen uptake due to ethanol (7). In the Tsukamoto-French model, the rate of ethanol elimination, which is oxygen dependent, was elevated 2- to 3-fold in rats exposed to ethanol for 2 to 4 weeks; however, when Kupffer cells were destroyed by $GdCl_3$, this phenomenon was blocked. Indeed, centrilobular pathological changes are compatible with a mechanism involving hypoxia, and injury was increased when O_2 tension in the liver was decreased (17). Importantly, conditioned media from isolated Kupffer cells from ethanol-treated rats stimulated parenchymal cell oxygen consumption. Interestingly, this media contained elevated levels of prostaglandin E_2, which has been shown to elevate O_2 uptake (18). Kupffer cells likely participate in the alcohol-induced liver injury by stimulating oxygen uptake, thereby contributing to pericentral hypoxia.

4.1. Hypoxia

Recently, it was reported that high doses of ethanol impair hepatic microcirculation (19) by producing endothelin-1, (20) and we recently detected hypoxia directly in rats on the Tsukamoto-French protocol (21) and in the perfused liver after acute exposure to ethanol *in vivo* (22). These findings support the idea that hypoxia directly contributes to alcohol-induced liver injury. Since alcohol also causes a compensatory increase in hepatic blood flow resulting in an elevated oxygen, it has been argued that any effect of hypoxia due to hypermetabolism or microcirculatory disturbances would be blocked (23). However, use of the hypoxia marker pimonidazole confirmed that downstream hypoxia occurs after acute ethanol (SIAM) treatment (22). Indeed, when livers were perfused in the retrograde direction, hypoxia was switched from pericentral to periportal regions (24).

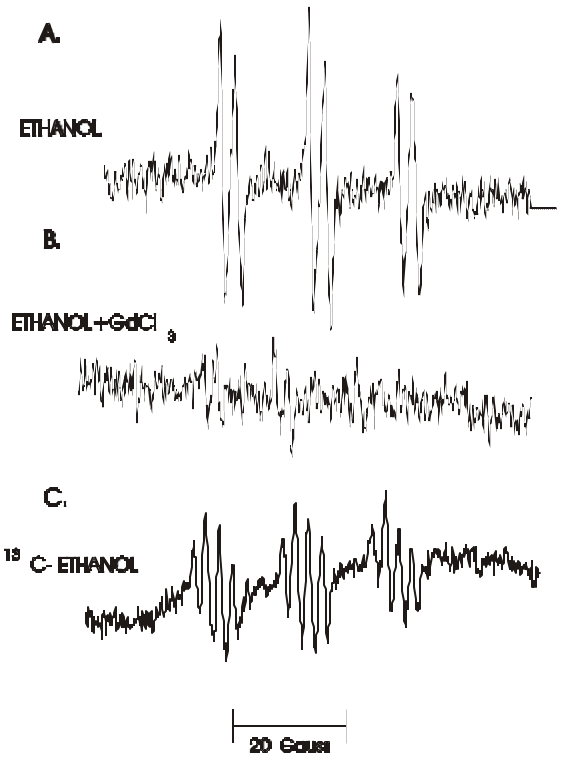

Figure 1. Alcohol produces free radical adducts in the liver. Representative ESR spectra of radical adducts in bile from rats treated for at least two weeks with continuous intragastric infusion of diet: (A) ethanol-containing, high-fat diet or (B) $GdCl_3$-treated rat given ethanol-containing diet or (C) ethanol treated rat followed by administration of ^{13} C-ethanol prior to bile collection. Bile ducts were cannulated under pentobarbital anesthesia and 100 mg/kg of the spin trap POBN was administered i.p. Bile samples were collected into vials containing 5 mM Desferal in order to prevent *ex vivo* free radical formation for 3-4 hours, frozen on dry ice, and analyzed for free radical adducts with ESR spectroscopy.

Studies using miniature oxygen electrodes and microfiber optics have also shown that oxygen uptake by the liver is dependent on local oxygen tension (25). Oxygen tension on the surface of livers from Tsukamoto-French rats was measured with miniature oxygen electrodes, utilizing the fact that the terminal portal venule stops about 200 microns from the liver surface. Lower values reflect relative hypoxia irrespective of mechanism (e.g., microcirculatory disturbances or hypermetabolism). Surface hepatic oxygen tension was decreased over 30% by alcohol treatment (6). Thus, direct evidence has now been obtained demonstrating that hypoxia begins after ethanol treatment in the Tsukamoto-French model and hypoxia was blocked when Kupffer cells were inactivated with $GdCl_3$.This was the first suggestion that an oxygen-sensing system was present in the liver. The importance of oxygen is underscored by the fact that rapid metabolic functions of the liver, such as urea and glucose synthesis, are dependent on the oxygen concentration gradient, whereas very slow metabolic processes, such as cytochrome P-450-dependent metabolism of drugs, predominate in pericentral areas irrespective of oxygen (26).

4.2. Free radicals

Free radical production has long been suggested to be a factor in hepatotoxicity due to ethanol. Although evidence of lipid radical formation due to ethanol treatment *in vivo* has been reported, free radicals from ethanol itself have only recently been detected in living animals (27). By applying the electron spin resonance (ESR) technique of spin trapping to the study of ethanol-treated alcohol dehydrogenase-deficient deermice (*Peromyscus maniculatus*), we detected the alpha-(4-pyridyl-1-oxide)-*N*-*t*-butylnitrone (POBN)/alpha-hydroxyethyl radical adduct in bile *in vivo* from animals given [1-^{13}C]ethanol and the spin trap POBN for the first time. Spin trapping is a technique in which a diamagnetic molecule reacts with a free radical to produce a more stable species, called a radical adduct, which is readily detectable by ESR. Radical adducts are substituted nitroxide free radicals, which tend to be relatively long-lived compared to free radicals which are very transient in biological systems. POBN radical adducts characteristically exhibit a six-line ESR spectrum (figure 1A), while ^{13}C substitution on the alpha-carbon of a spin-trapped species causes six additional lines due to the magnetic interaction of the ^{13}C with the unpaired electron. The subsequent production of a 12-line spectrum after administration of ^{13}C ethanol provides unequivocal physical evidence that the trapped radical arises from the labeled parent ethanol. Computer simulation of such an ESR spectrum gives spectral parameters called "hyperfine coupling constants" which can be compared with values of well-characterized radical adducts. The ^{13}C spectral effect and the hyperfine coupling constants identify the radical adduct as the POBN/α-hydroxyethyl radical adduct derived from ^{13}C labeled ethanol. The absorption of microwave energy at that particular magnetic field strength is rectified and detected by a microwave diode and recorded as the first derivative of the absorption peak.

Free radical formation (i.e., oxidative stress) likely participates in the progression of early events in alcoholic liver disease. Oxidative stress activates the transcription factor Nf kappa B and stimulates adhesion molecule synthesis leading to white cell sticking (28). Importantly, we have recently detected a free radical in the bile from rats exposed to ethanol on the Tsukamoto-French model (figure 1)(6). This free radical signal was reduced dramatically when Kupffer cells were eliminated with $GdCl_3$ (figure 1B). A six-line radical adduct spectrum was also detected from the bile of Tsukamoto-French rats treated with an ethanol-containing, high-fat diet (figure 1A) but not in bile from rats fed a chow diet (data not shown). Bile from animals fed the control corn oil diet also contained low concentrations of radical adducts. The free radical adduct was identified as α-hydroxyethyl by use of ^{13}C ethanol (figure 1C) (21). Thus, ethanol-derived free radical formation can be detected in the bile of Tsukamoto-French rats treated intragastrically with a high-fat and ethanol-containing diet. SOD/catalase-insensitive free

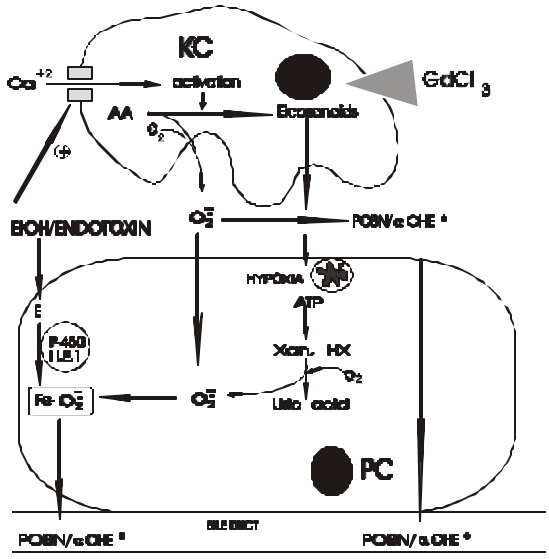

Figure 2. Scheme depicting working hypothesis for the involvement of endotoxin, Kupffer cells, hypoxia and free radicals in the mechanism of alcohol-induced liver injury. Alcohol consumption alters the membrane fluidity of the gut wall, making it more permeable to endotoxin. Blood endotoxin is elevated and enters the liver, where it is engulfed by Kupffer cells which become activated releasing TNF-alpha, PGE_2 and superoxide. Hepatocyte oxygen and ethanol metabolism is elevated, resulting in hypoxia in downstream regions of the liver lobule, which leads to generation of alpha-hydroxyethyl radicals. Blocking this cascade of events by sterilization of the gut with antibiotics or destruction of Kupffer cells with $GdCl_3$ prevents alcoholic liver injury.

radicals have also been obtained from livers of alcohol-treated rats after transplantation (29). Exact pathways responsible for formation of free radicals in alcohol-treated rats remain unclear; however, since the ESR signal was reduced with $GdCl_3$ treatment (figure 1 B), a likely candidate is oxygen radical production by the NADPH oxidase system in Kupffer cells and neutrophils (figure 2). On the other hand, a reperfusion injury involving hypoxia and free radical formation via the xanthine-xanthine oxidase system cannot be ruled out, especially since radicals in bile would be expected to arise from parenchymal cells.

5. ACKNOWLEDGMENTS

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