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MOLECULAR PHARMACOLOGY, 47:1028–1034 (1995).

Free Radical Adducts in the Bile of Rats Treated Chronically with Intragastric Alcohol: Inhibition by Destruction of Kupffer Cells

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Received December 8, 1994; Accepted February 16, 1995

SUMMARY

Free radical products have previously been detected in rodents after chronic feeding with an ethanol-containing, high-fat diet. The significance of reactive free radical formation in ethanol-induced hepatotoxicity has been difficult to assess because most rodent models exhibit only fatty liver. However, serious hepatic damage resembling clinical alcoholic liver injury (e.g., steatosis, inflammation, and necrosis) occurs in rats after continuous intragastric administration of an ethanol-containing, high-fat diet developed by Tsukamoto and French. Accordingly, rats treated with ethanol for at least 2 weeks using this protocol were administered the spin trap α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone, and bile samples were collected. A six-line radical adduct spectrum was detected in the bile of ethanol-treated rats. A similar spectrum of lower intensity was detected with rats fed a high-fat diet without ethanol, but little or no radical adduct signal was detected with chow-fed animals. For both treatment groups, α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone and extra ethanol were given acutely. Destruction of Kupffer cells by chronic treatment with $GdCl_3$ decreased by about 50% the radical adduct formation in rats fed the ethanol-containing, high-fat diet. This radical species was largely ethanol derived, because addition of [^{13}C]ethanol produced a 12-line spectrum, indicating the formation of α-hydroxyethyl radical. Ethanol treatment also caused hypoxia (detected on the liver surface *in vivo* with oxygen electrodes), which was reflected in a dose-dependent decrease in oxygen tension with ethanol. The effect was blocked by $GdCl_3$. Hepatic damage detected by histology was prevalent in ethanol-treated rats but only mild fatty liver was observed in high-fat diet-fed controls. $GdCl_3$ treatment eliminated hepatic damage due to high-fat and ethanol diets, and when all groups were compared a significant correlation between liver injury and radical adduct signal was observed. Thus, free radical formation in ethanol-treated rats has been detected for the first time in a model that exhibits injury characteristic of human alcoholic injury, and signal intensity correlates with hepatotoxicity. Moreover, the decrease in both free radical formation and hepatic damage produced by $GdCl_3$ implicates Kupffer cells in the development of alcoholic liver injury. This important pathophysiological process may involve direct production of reactive oxygen species or indirect actions of mediators on parenchymal cells.

A number of studies have shown that ethanol administration *in vivo* is associated with the production of free radicals and oxidative stress. Lipid peroxidation has been measured (1), and hepatic antioxidants decrease after ethanol administration (2–4), but the most direct evidence for free radical activity comes from the detection of spin-trapped free radicals from living animals. Reinke *et al.* (5) detected lipid-derived radical adduct species in heart and liver of rats treated with high dietary fat (corn oil) and ethanol. With deer mice fed a corn oil- and ethanol-containing diet, both lipid-derived and ethanol-derived radical adduct species were detected in bile *in vivo* (6). Radical adduct signal intensity was dependent on prior feeding of both ethanol and high fat, and radical adduct signal was produced by the animal, rather than in the collected sample. An ethanol-derived radical adduct was later detected in Folch extracts of rat liver as well (7). The source of free radicals after ethanol treatment is not known, but free radicals from ethanol or lipid most likely arise as secondary radicals formed by reactive oxygen species (8).

Reactive oxygen species can be formed in the body from a number of different sources. The enzyme cytochrome P-450 II.E.1 is induced by ethanol treatment and is an excellent source of superoxide free radicals (9). Indeed, Knecht *et al.* (8) found that α-hydroxyethyl free radical formation by micro-

This work was supported, in part, by Grants AA03624 and AA09156 from the National Institute on Alcohol Abuse and Alcoholism.

ABBREVIATIONS: AST, aspartate aminotransferase; POBN, α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone.

somes was dependent on superoxide produced by cytochrome P-450 and on trace transition metals. However, whether such chemistry could be significant *in vivo* is not certain, considering the presence of high levels of the cytosolic enzyme superoxide dismutase and the near absence of free transition metal ions in living cells.

Reactive oxygen species are also produced by various white blood cells as part of their response to pathogens. Of particular interest is possible superoxide anion radical production by Kupffer cells, the resident macrophages of the liver. Superoxide production has been detected after stimulation of Kupffer cells by hypoxia/reperfusion or after priming and challenge with tumor necrosis factor and phorbol esters or opsonized zymosan (10, 11). Furthermore, ethanol has been shown to activate Kupffer cells, possibly by making their calcium channels easier to open (12, 13), and it was recently shown that inactivation of Kupffer cells with the selective toxicant $GdCl_3$ diminished reperfusion injury in perfused liver (14).

Although reactive free radical formation would seem to be destructive to the liver and thus causally related to human alcoholic liver injury, this relationship has been difficult to assess experimentally, because free radical metabolism has been detected only in rodents, whereas serious hepatic injury has been observed primarily in humans and nonhuman primates. However, hepatic damage in rats similar to that observed in humans (e.g., steatosis, inflammation, and necrosis) is characteristic of the Tsukamoto-French model (15), where rats are treated with continuous intragastric administration of an ethanol-containing, corn oil-based, high-fat diet. Thus, one purpose of this study was to determine whether free radical products could be detected in rats treated using this model. Also, because $GdCl_3$ treatment (to destroy Kupffer cells) of rats undergoing the Tsukamoto-French protocol has been reported to decrease ethanol-induced hepatotoxicity (16), another purpose of this study was to determine the effect of $GdCl_3$ treatment on free radical formation, to assess the possible contribution of free radicals from Kupffer cells to the pathophysiology of alcoholic liver disease.

Materials and Methods

Animals and sample collection. Male Wistar rats (300–320 g) were used in this study. Intragastric cannulae were inserted according to the method developed by Tsukamoto *et al.* (15), and animals were continuously infused with a high-fat liquid diet for at least 2 weeks. The diet contained corn oil as fat (37% of total calories), protein (23%), carbohydrate, minerals, and vitamins, plus ethanol (ethanol diet) or isocaloric dextrose (control diet). The percentage of ethanol in the diet was adjusted individually between 7 and 9% (by volume), depending on urinary alcohol concentrations and the observed degree of intoxication. For some animals, $GdCl_3$ (10 mg/kg) was administered intravenously twice weekly, to kill Kupffer cells (16).

Twenty-four-hour urine samples were collected for each rat, and the ethanol concentration was determined by enzymatic procedures (17). Concentrations of ethanol in breath, which closely correspond to blood alcohol measurements, were determined by gas chromatography (18). The ethanol-containing diet was removed from ethanol/high fat-treated animals several hours before bile cannulation, but significant ethanol remained, as shown by breath ethanol measurements (176 ± 42 mg/dl). Therefore, ethanol (2 g/kg) was administered intragastrically to high fat- and chow-fed animals before administration of the spin trap; this amount of ethanol was estimated to produce comparable levels of ethanol in these animals (i.e., 200 mg/dl ethanol). Nonetheless, levels of ethanol were still slightly higher in ethanol-fed rats. For some ethanol-fed animals, the diet was discontinued so that ethanol from the diet was negligible before additional [^{13}C]ethanol (2 g/kg) was administered.

Rats treated for at least 2 weeks, using this model, were anesthetized with Nembutal (75 mg/kg). Blood was collected from the tail vein, centrifuged, and stored frozen as serum for later AST analysis. Bile ducts were cannulated with PE10 tubing, rats were administered POBN (100 mg/kg, intraperitoneally), and bile samples were collected at 15-min intervals, for 1 hr, into 30 μl of a solution of desferroxamine mesylate (5 mM) to prevent *ex vivo* radical formation. Samples were frozen immediately on dry ice and stored at −70° until EPR analysis was performed. Liver biopsies were taken at the end of bile collection and were fixed with formalin, embedded in paraffin, and stained either with hematoxylin and eosin or with osmium. Liver pathology was scored as described by Nanji *et al.* (19) (steatosis: <25%, 1+; <50%, 2+; <75%, 3+; >75%, 4+; inflammation: 1 focus/low-power field, 1+; 2 or more, 2+; necrosis: 1 focus/low-power field, 1+; 2 or more, 2+).

Free radical detection. Bile samples were thawed and placed in a quartz flat cell for EPR analysis. A Varian E-109 spectrometer equipped with a TM_{110} cavity was used. Instrument conditions were as follows: 20-mW microwave power, 0.53-G modulation amplitude, 80-G scan width, 16-min scan, and 1-sec time constant. Data were collected with an IBM-type computer interfaced to the spectrometer. Simulations and double integrations of spectra to determine amplitude were carried out with a computer program (20). Mean values of group data were compared using analysis of variance with the Bonferroni *post hoc* test; pathology data were compared using the Kruskal-Wallis test, with Dunn's method for individual comparisons.

Liver surface oxygen tension measurement. After anesthesia with methoxyflurane, the abdomen was opened and a Clark-type platinum oxygen electrode was placed gently on the liver surface, to polarographically determine oxygen tension (21). Because the portal venule terminates 200 μm from the liver surface, this value largely reflects oxygen tension in pericentral regions of the hepatic lobule.

Results

Effects of ethanol and gadolinium chloride on radical adduct formation. Minimal radical adduct was detected in bile from rats maintained on the control diet for 2 weeks and acutely given POBN and ethanol (Fig. 1A). However, a robust six-line radical adduct spectrum was detected in the bile of rats fed the ethanol diet for similar times (Figs. 1B and 2A). Coupling constants (a^N = 15.78 G, $a^{H\beta}$ = 2.56 G) were typical of those for a carbon-centered radical adduct, but POBN/·lipid radical adducts could not be distinguished from POBN/α-hydroxyethyl radical adducts under these conditions. $GdCl_3$, a specific Kupffer cell toxicant, dramatically reduced the magnitude of the radical adduct signal (Fig. 1C).

Minimal radical adduct was detected in bile from chow-fed animals treated acutely with ethanol and spin trap (Fig. 2B), when bile samples were collected into a solution of desferroxamine mesylate. However, it was possible to form radical adduct *ex vivo* in bile samples collected without desferroxamine mesylate (Fig. 2C). Thus, desferroxamine mesylate was routinely added to all collection vials in this study.

[^{13}C]Ethanol was given to rats fed the ethanol diet, to determine whether the radical adduct was ^{13}C derived. A 12-line spectrum was detected, indicating that the α-hydroxyethyl radical adduct was indeed formed (Fig. 2D). Simulation of this spectrum indicated that about two thirds of

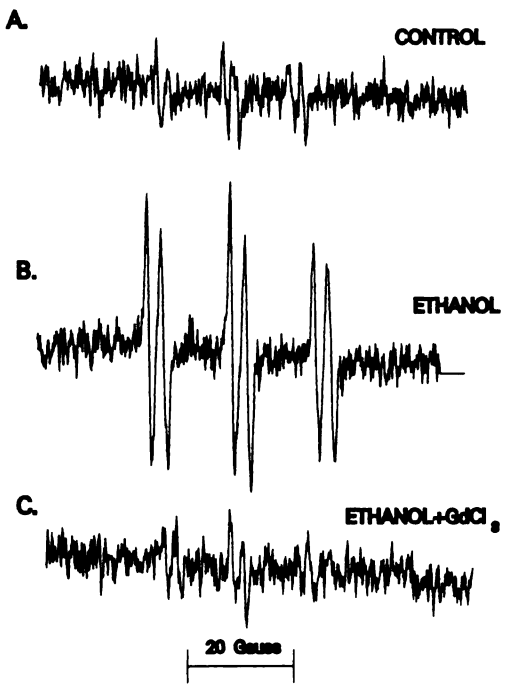

Fig. 1. EPR spectra of bile from rats. Rats were maintained for 2 weeks with continuous gastric infusion of diet containing 37% of calories as corn oil (A) or ethanol (B). Some rats were given twice-weekly injections of $GdCl_3$ (10 mg/kg, intravenously) and fed the ethanol diet (C). Bile samples were collected into desferal (desferroxamine mesylate, 0.5 mM) after administration of POBN (100 mg/kg, intraperitoneally), and analysis of EPR spectra was performed as described in Materials and Methods. Representative spectra are shown.

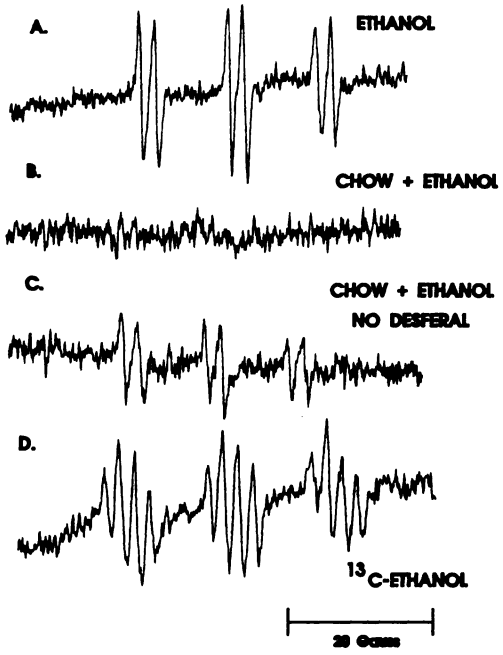

Fig. 2. EPR spectra of bile from rats treated with ethanol. Rats were maintained on an ethanol-containing diet (A and D), as described in the legend to Fig. 1, or were fed a normal chow diet and treated with ethanol (2.0 g/kg, intragastrically) 1 hr before collection of bile, as described in Materials and Methods (B and C). All bile samples were collected into desferal solution except for C, which is otherwise identical to B. The spectrum in D is from bile from a rat treated as in A; however, [^{13}C]ethanol (2.0 g/kg, intragastrically) was administered 1 hr before the collection of bile. Representative spectra are shown.

the signal was derived from [^{13}C]ethanol and the balance was most likely derived from lipid.

Average EPR signal intensity was measured as the double integral of the two low-field peaks of each spectrum (Fig. 3). For chow-fed animals, three of the seven samples showed no trace of radical adduct in bile, with only small amounts being present in the other samples. This small amount of radical adduct could have resulted from inadequate mixing of collected samples, with subsequent *ex vivo* radical adduct formation. Alternatively, it is possible that only small amounts of radical adduct were formed in the rats under these dietary conditions. Similar results were obtained with high fat-fed control rats. However, the mean radical adduct signal from rats fed the ethanol diet was significantly greater (Fig. 3). Importantly, $GdCl_3$ significantly reduced the average signal intensity.

Pathology and hypoxia. The pathology score was elevated significantly over control values by ethanol treatment and was reduced by gadolinium chloride treatment (Fig. 4). Indeed, an excellent correlation between radical adduct signal and pathology score was observed in this study (Fig. 5). Serum AST values were elevated significantly in rats fed the ethanol diet, compared with the control group (160 ± 14 versus 110 ± 12 IU/liter, $p < 0.03$). $GdCl_3$ tended to decrease AST at 2 weeks in this study (data not shown) but caused significant declines at 4 weeks in a previous study (16).

Hypoxia has been suggested to be an important component of alcohol-induced liver injury, because chronic ethanol exposure causes a hypermetabolic state (22). However, this viewpoint is controversial, because ethanol also increases blood

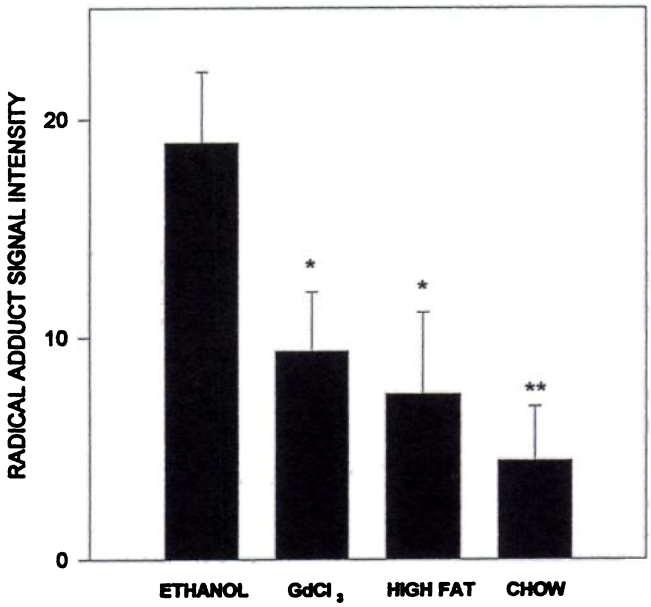

Fig. 3. Average radical adduct signal intensity from bile. EPR signal intensity was quantitated as the double integral of peaks from bile samples and was averaged for rats treated as described in the legends to Figs. 1 and 2. Statistical comparisons were made with the ethanol group by using analysis of variance with Bonferroni comparisons (n = 4–7 for all groups). *, $p < 0.05$; **, $p < 0.01$.

flow to the liver (23). Therefore, oxygen tension was measured on the liver surface *in vivo*. As can be seen in Fig. 6, a dose-dependent decrease in surface oxygen tension, indicative of hypoxia, was observed as blood ethanol levels in-

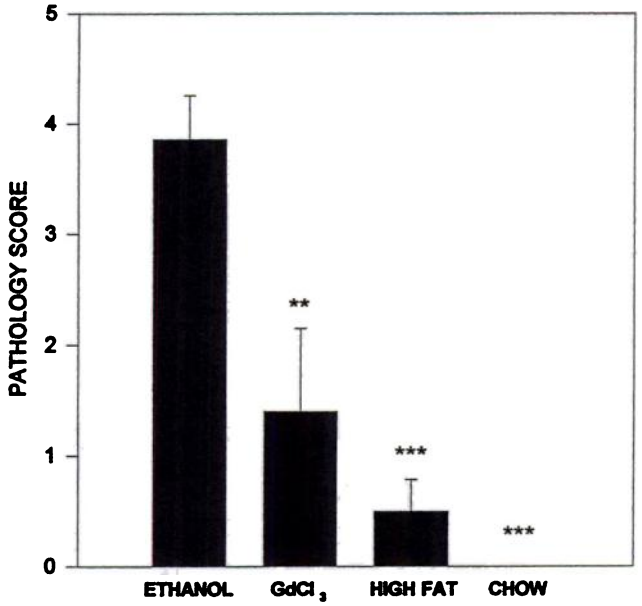

Fig. 4. Average hepatic pathology scores. Pathology scores were determined from histological specimens of liver taken after collection of bile. Sections were scored as described in Materials and Methods. Statistical comparisons were made with the Kruskal-Wallis test, with Dunn's method for individual comparisons (n = 4–7 rats for all groups). **, $p < 0.01$; ***, $p < 0.001$.

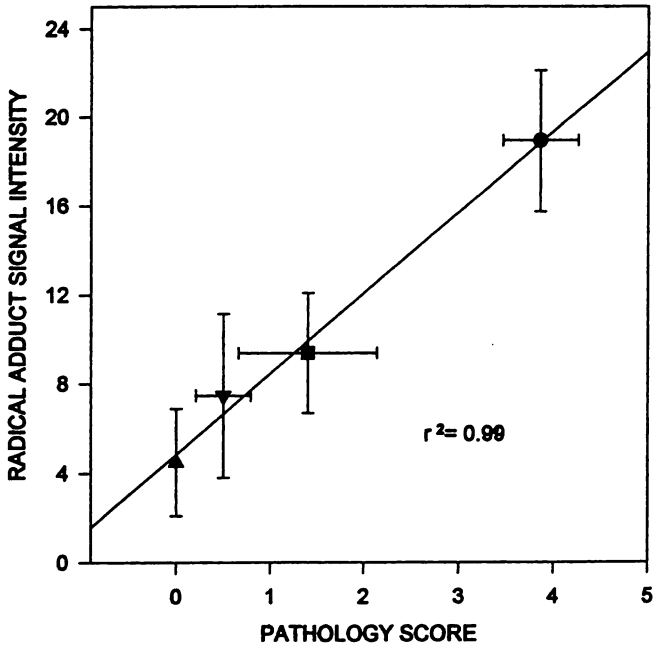

Fig. 5. Correlation between radical adduct intensity and pathological findings. Data are from Figs. 3 and 4.

creased. This curve was shifted upwards by $GdCl_3$ treatment, indicating that oxygen delivery to the liver was improved dramatically when the Kupffer cells were destroyed (Fig. 6).

Discussion

Detection of free radicals in the Tsukamoto-French model of intragastric delivery of ethanol. In this study, free radical adducts were detected in the bile of rats after continuous intragastric treatment with diets containing high

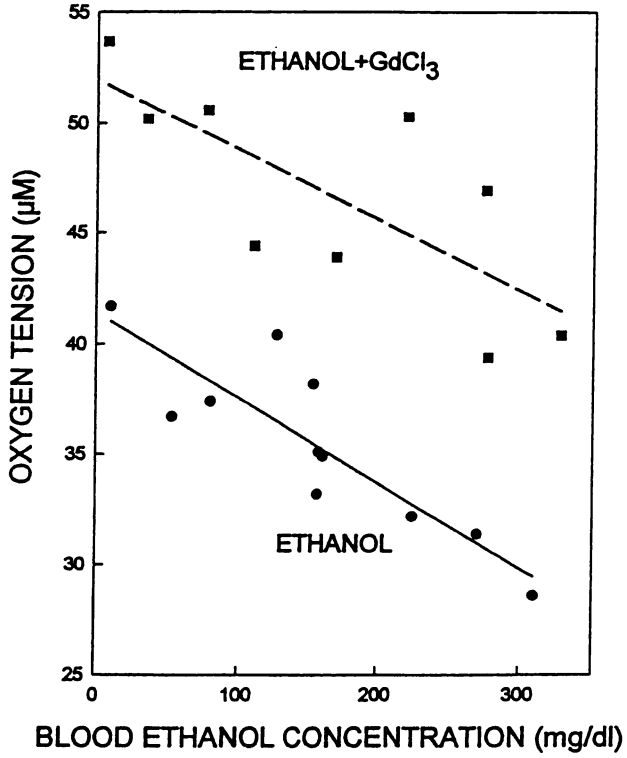

Fig. 6. Relationship between liver surface oxygen tension and blood alcohol concentration. Blood alcohol concentrations were determined by analysis of breath samples, as described in Materials and Methods. Rats were anesthetized, the abdomen was surgically opened, and oxygen tension was polarographically determined on the surface of the liver by using a Clark-type electrode. Data largely reflect pericentral oxygen tension (21). Correlations are for ethanol-fed rats (r = 0.87, $p <$ 0.001) and $GdCl_3$-treated, ethanol-fed rats (r = 0.75, $p < 0.001$). Points represent data from individual rats.

fat and ethanol, indicating that measurable oxidative stress was produced. Acute ethanol administration was not sufficient to produce radical adducts. It is true that levels of lipid-derived radicals from ethanol-fed deer mice were greater with increased blood ethanol concentrations at the time of the experiment (6), and radical adduct signal intensity was linearly proportional to breath ethanol levels in ethanol/high fat-treated rats in this study (r^2 = 0.56, n = 7). Thus, ethanol content at the time of the experiment has some effect on radical signal intensity; however, when individual blood ethanol levels were plotted versus radical adduct signal for high fat- and gadolinium-treated groups, no correlation was observed. Furthermore, the decrease in radical adduct signal intensity in $GdCl_3$-treated rats cannot be explained by ethanol content, because the breath ethanol levels of ethanol-fed animals were not decreased by $GdCl_3$ treatment. Thus, ethanol levels at the time of the experiment are not sufficient to produce radical adduct, but ethanol concentrations may modulate the intensity of the radical adduct signal that is formed.

Because radical adduct is measured in bile, there are several considerations other than radical adduct formation that could, in theory, affect the detection of radical adduct. For instance, changes in bile flow and biliary secretion might affect radical adduct signal. However, the rate and volume of bile flow in these experiments were unchanged by ethanol and/or gadolinium chloride treatment. In addition, radical

adduct can be reduced to an EPR-invisible hydroxylamine, which could affect detection. It is possible that differing hepatic oxygen concentrations in the various treatment groups could cause differences in hydroxylamine formation, leading to apparent differences in radical adduct concentration. However, hydroxylamine formation is reversible by the autooxidation of bile samples during EPR analysis (24), a procedure used in this study, making it unlikely that hydroxylamine could affect the conclusions drawn here. Finally, nitroxides can be irreversibly reduced by microsomal suspensions, in theory diminishing signal intensity (25). However, in our studies radical adduct signal was actually higher in alcohol-fed animals than in controls. Because alcohol is an inducer of microsomal cytochrome P-450 II.E.1, we might expect the opposite result if reductive metabolism were significant. Furthermore, gadolinium chloride has no effect on microsomal enzymes (26).

Both an α-hydroxyethyl radical adduct and a lipid-derived radical adduct species have been spin trapped after ethanol and high fat feeding in other rodent models (5–7). Measurement of coupling constants was useful in radical adduct identification in this study, because the observed radical adduct spectrum could be due to the POBN/α-hydroxyethyl radical adduct, a POBN/lipid-derived radical adduct species, or a mixture of the two. When [^{13}C]ethanol was used, a POBN/α-hydroxyethyl radical adduct signal was identified *in vivo* (Fig. 2). This is the first time that an ethanol-derived radical has been detected in a rodent model that resembles human alcoholism with respect to clinical findings. Furthermore, the data in this study demonstrate that Kupffer cells participate in generation of the radical.

Role of Kupffer cells in free radical formation. The decrease in radical adduct formation in rats fed the ethanol diet and treated with $GdCl_3$ is significant, because it indicates that Kupffer cells could be the primary source of free radicals (Fig. 7). Superoxide is known to be produced by Kupffer cells (10, 11), and both α-hydroxyethyl (8) and lipid-derived radicals could then arise as secondary or "marker" radicals, because oxygen-derived radical adducts are generally too unstable to be detected directly *in vivo*. This could explain how radical adducts could be produced in deer mice (6) despite the lack of radical adduct formation in liver homogenates or microsomes plus cytosol (8). Other possibilities need to be considered, because Kupffer cells are located in the sinusoidal space and radical adducts were detected in bile (Fig. 7). It is possible that radical adducts formed in the sinusoidal space could be transported to the bile. For example, radiolabel from *N-t*-butyl-α-[^{14}C]phenylnitrone was detected in bile after intraperitoneal dosing (27), and the spin probe tempol was readily detected in the bile of rats[1] and deer mice (data not shown) after intraperitoneal administration. Alternatively, Kupffer cells may produce mediators that affect parenchymal cell oxygen metabolism, leading to hypoxia followed by radical formation upon reoxygenation (28). A role for xanthine oxidase is possible in this scenario, because hypoxia was demonstrated directly in this study and was minimized when Kupffer cells were destroyed with $GdCl_3$. Furthermore, it is known that the swift increase in alcohol metabolism (22), which is a rapid activation of alcohol metabolism involving a hypermetabolic state, was blocked by

[1] P. M. Hanna, personal communication.

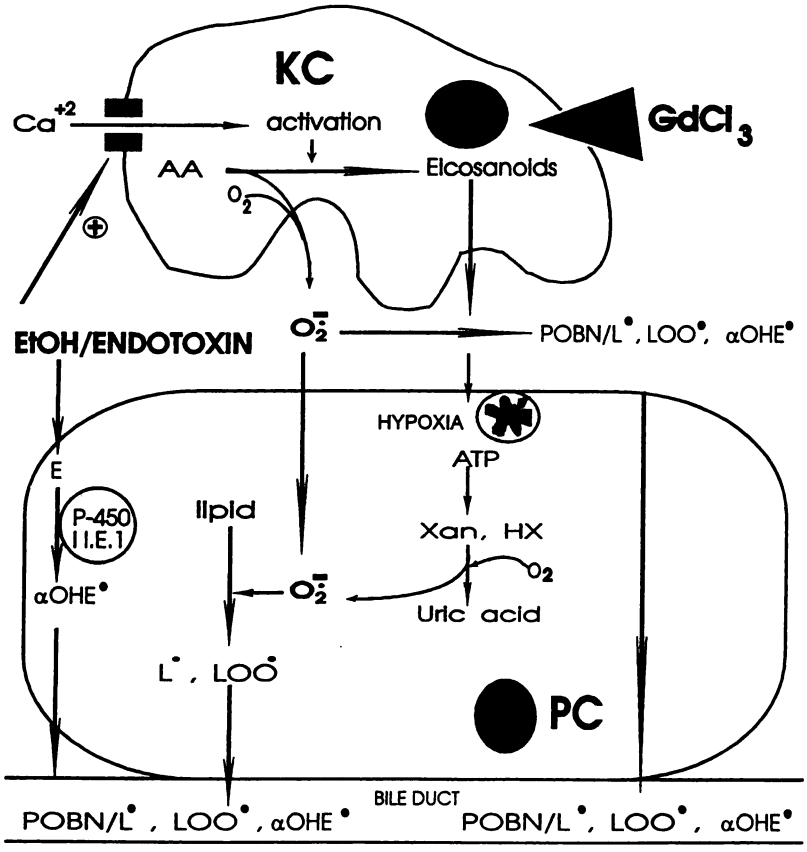

Fig. 7. Scheme depicting possible sources of free radicals. *KC*, Kupffer cell; *AA*, arachidonic acid; *E* or *EtOH*, ethanol; *Xan*, xanthine; *HX*, hypoxanthine; $O_2\cdot^-$, superoxide radicals; *L*·, lipid radicals; *LOO*·, lipid peroxide radicals; *αOHE*·, α-hydroxyethyl radicals; *PC*, pericentral region.

$GdCl_3$ treatment (29) and that Kupffer cells produce eicosanoids that stimulate parenchymal cell oxygen metabolism (30). A role for xanthine oxidase in the production of oxygen radicals is plausible, because injury in a low-flow/reflow liver perfusion model was blocked by allopurinol (31). In addition or alternatively, Kupffer cells, like other white blood cells, are known to produce oxygen radicals by respiratory burst mechanisms (32). The fact that radical adducts detected in this study were found in bile is consistent with the hypothesis that Kupffer cells are involved in the production of free radicals.

Possible involvement of free radicals in alcohol-induced liver injury. The production of free radical products in rats fed the ethanol diet accompanies the development of steatosis, inflammation, and necrosis. Furthermore, when radical production was diminished, toxicity was reduced. Indeed, a good correlation between free radical production and liver injury was observed in this study (Fig. 5), supporting a role for free radicals in ethanol-induced toxicity. Particularly exciting are results with the Kupffer cell toxicant $GdCl_3$. These results confirmed previous work from this laboratory that demonstrated that hepatic toxicity due to alcohol was decreased when Kupffer cells were destroyed (16). This strongly suggests a role for Kupffer cells in the early phase of alcoholic liver damage. Furthermore, the simultaneous decrease in free radicals is consistent with the hypothesis that reactive oxygen species produced directly from Kupffer cells or indirectly via mechanisms involving hypoxia and xanthine oxidase participate in the development of alcoholic liver injury.

It is possible, of course, that Kupffer cells exert their hepatotoxic effect by means other than the production of reactive oxygen species and that increased Kupffer cell function is merely an associated effect. However, it seems likely that any oxidative stress would at least contribute to hepatocellular injury by placing increased strain on the protective mechanisms of the cell. For example, Sipes and co-workers (33, 34) have shown that reactive oxygen species from Kupffer cells are responsible for the increase in CCl_4 toxicity that follows treatment with vitamin A, and Edwards *et al.* (26) demonstrated that CCl_4 toxicity could be prevented by treatment with $GdCl_3$. It is reasonable to conclude that free radical production involving Kupffer cells contributes to ethanol-induced hepatotoxicity.

In conclusion, α-hydroxyethyl radical adducts were observed in the bile of rats fed an ethanol-containing diet using the Tsukamoto-French model. Free radical production correlated with liver injury, and decreases in both free radical production and hepatotoxicity with $GdCl_3$ treatment suggest a role for Kupffer cells in the development of alcoholic liver damage, by production of damaging reactive oxygen species.

Acknowledgments

We gratefully acknowledge Sandra J. Jordan and Jean Corbett (National Institute of Environmental Health Sciences) for assistance with this study.

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