Chronic Enteral Ethanol Treatment Causes Hypoxia in Rat Liver Tissue In Vivo

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It is known that ethanol increases oxygen consumption in in vitro liver models, which could lead to hypoxia. Although it was shown recently that one large dose of ethanol caused hypoxia in rat liver in vivo, whether ethanol produces hypoxia in a clinically relevant chronic model remains unclear. In the present study, therefore, the effect of chronic ethanol on hypoxia was investigated in vivo using the 2-nitroimidazole hypoxia marker, pimonidazole. Male Wistar rats (300-325 g) were exposed to enteral ethanol continuously for 4 weeks. In this model, rats develop steatosis, inflammation, and necrosis characteristic of early stages of clinical alcoholic liver disease in humans. One hour before they were killed, rats were injected with pimonidazole (120 mg/kg intravenously), and livers were surgically isolated, removed, and fixed. Protein-bound pimonidazole adducts were identified on formalin-fixed, paraffin-embedded tissue with immunohistochemistry. Ethanol administration for 4 weeks significantly increased serum aspartate transaminase levels and hepatic pathology scores for steatosis, inflammation, and necrosis, as expected. Ethanol treatment significantly increased both the extent and number of cells that stained positive for pimonidazole compared with control animals given an enteral diet without ethanol. Quantitative image-analysis of pimonidazole binding showed that 4 weeks of ethanol administration nearly doubled the pimonidazole-positive area in tissue. Ethanol also increased pimonidazole binding significantly at 7 days, long before inflammation and necrosis could be detected. These results indicate that chronic ethanol causes hypoxia at the cellular level in rat liver in vivo and lend support to the hypothesis that hypoxia is involved in mechanisms of early alcoholic liver injury. (HEPATOLOGY 1997;25:920-926.)

Hypoxic injury to tissue, especially in heart disease, is well known, and many studies indicate that hypoxia plays an important role in the pathophysiology of injury to other tissues, such as the liver. Chronic ethanol consumption leads to liver damage in oxygen-poor pericentral regions of the liver lobule in both humans and in animal models. Isreal et al. first postulated that ethanol caused hypoxia based on increased oxygen consumption in liver slices isolated from rats exposed chronically to ethanol. Subsequently, Yuki et al. showed that a single, large dose of ethanol in vivo also produced a hypermetabolic state in an intact, perfused rat liver.

In that study, ethanol caused an almost twofold increase in oxygen uptake coupled with an increase in ethanol metabolism in subsequently perfused rat liver in only 2 to 3 hours. This swift increase in alcohol metabolism makes the hepatic oxygen gradient steeper and could lead to hypoxia in pericentral regions of the lobule in the perfused liver. However, these techniques require high concentrations of oxygen controlled by the investigator and cannot address possible effects of changes in blood flow. For example, ethanol increases blood flow, which could theoretically counteract hypoxia by increasing oxygen delivery.

Nitroimidazole compounds such as pimonidazole are reductively activated and covalently bind to macromolecules in cells at low oxygen concentration. Oxygen inhibits binding by competing for electrons in the first step of the reductive activation process. Half-maximal inhibition of binding occurs at oxygen concentrations around 4 μmol/L, but binding can be detected at oxygen concentrations of up to 14 μmol/L. It is known that binding intermediates incorporate both ring and side-chain moieties of the 2-nitroimidazole, and that binding is most efficient for thiol-containing molecules. The structures of adducts to thiol-containing proteins is not known, but they are assumed to be analogous to the adducts formed when small molecules such as glutathione bind to reductively activated 2-nitroimidazoles. The binding of 2-nitroimidazoles to hypoxic cells has been used to predict the hypoxic fraction in animal and human tumors as an index of resistance to radiation. It was shown recently that the binding pattern of pimonidazole in liver is determined by the hepatic oxygen gradient. Specifically, perfusing livers in the anterograde direction at flow rates that make the liver normoxic in periportal and hypoxic in pericentral regions led to pimonidazole adduct accumulation in pericentral regions. In contrast, reversing the direction of perfusion (retrograde perfusion) led to adduct accumulation in periportal regions. Based on this work, it was concluded that pimonidazole can be used in liver to study changes in hepatic tissue oxygenation.

Using hypoxia markers as tools to study hepatic oxygenation has attractive advantages over previously established in vitro and in vivo techniques for studying the effects of ethanol on hepatic oxygenation. In particular, pimonidazole adducts accumulate in intact, awake animals and measure hepatic tissue hypoxia at the cellular level without physically disturbing the liver. Previous in vivo methods to determine the effect of ethanol on hepatic oxygenation relied on oxygen measurements in the blood. Measurement of pimonidazole adducts also takes into account the effect of steep intracellular oxygen gradients on cellular oxygenation, an effect not addressed by measuring blood oxygen tension or tissue oxygen consumption in vitro.

Indeed, it was recently shown that one large dose of ethanol acutely causes tissue hypoxia directly in pericentral regions of the liver lobule in vivo using pimonidazole. However,
whether ethanol causes hypoxia in a clinically relevant model of liver injury remained unclear. Dietary manipulation and controlled enteral administration of ethanol using techniques developed by Tsukamoto and French now make it possible to produce pathological changes in the liver that resemble alterations that occur in human alcoholics (e.g., steatosis, inflammation, and necrosis). Previous work has shown that livers from rats given enteral ethanol using the Tsukamoto-French protocol have increased oxygen extraction\(^2\)\(^3\) and decreased surface oxygen tension\(^2\)\(^4\) compared with controls, supporting the hypothesis that ethanol causes hypoxia in this clinically relevant model. Therefore, in this study, pimonidazole was used to determine whether chronic ethanol causes hypoxia at the cellular level in rat liver tissue in vivo using a clinically relevant model of early alcoholic liver injury. Preliminary results of this study have been presented elsewhere.\(^2\)\(^5\)

**MATERIALS AND METHODS**

**Chemicals and Reagents.** Absolute ethanol (United States Pharmacopeia) was obtained from Aaper Chemical Co. (Shelbyville, KY), and sodium pentobarbital (Nembutal) was purchased from Aldrich (Milwaukee, WI). Pimonidazole hydrochloride was synthesized according to published procedures\(^2\)\(^6\) and characterized using standard chromatographic, elemental analysis and spectrographic techniques. Monoclonal antibodies were prepared as described previously.\(^2\)\(^4\) Chemicals used for the preparation of formalin-fixed, paraffin-embedded tissue sections were of reagent-grade purity from local suppliers. Enzymatic supplies including protease (protease E), Avidin-Biotin Complex Peroxidase Vectastain kit, avidin-biotin blocking kit, rat-adsorbed horse anti-mouse antibodies and diaminobenzidine peroxidase substrate were purchased from Vector Laboratories, Inc. (Burlingame, CA).

**Experimental Animals and Procedure.** Male Wistar rats, weighing approximately 300 g, were housed in an AAALAC-approved facility with lights on between 6 am and 6 pm. Intragastric cannulas were implanted into the stomach as described by Tsukamoto and French.\(^2\)\(^7\) A liquid diet was prepared according to Thompson and Reitz,\(^2\)\(^8\) which contained corn oil as fat (37% of total calories), protein (23%), carbohydrate (5%), minerals and vitamins, and ethanol or isocaloric maltose-dextrin (35%). During the first week of diet exposure, ethanol was increased in a stepwise fashion from 0 to 9 to 10 g/kg/d. Animals were infused continuously with the diet for 4 weeks and given humane care in compliance with institutional guidelines. Concentrations of ethanol in urine were measured daily,\(^2\)\(^9\) and serum samples were collected weekly for the measurement of aspartate transaminase. Biopsies were taken after 2 and 4 weeks; pathology was scored (steatosis, inflammation, and necrosis) using techniques described elsewhere.\(^3\)\(^0\) After 4 weeks on the enteral diet, animals were injected with pimonidazole (120 mg/kg intravenously) 1 hour before killing. Liver biopsies were formalin-fixed, embedded in paraffin, and sectioned. To investigate whether hypoxia is an early event in the progression of alcoholic liver damage, an additional group of animals received an enteral diet with or without ethanol for 7 days and given pimonidazole as described above.

**Analysis of Tissue-Bound Pimonidazole Using Immunohistochemistry.** Bound pimonidazole was detected in formalin-fixed, paraffin-embedded tissues at 25× magnification with a biotin-streptavidin-peroxidase indirect immunostaining method modified for rat liver.\(^2\)\(^9\) Sections were hydrated and treated briefly with 0.1% pronase (protease E) and exposed to monoclonal anti-pimonidazole immunoglobulin G antibody in phosphate-buffered saline—TWEEN for 2 hours at 37℃. Rat-adsorbed horse anti-mouse antibody was then applied to sections for 30 minutes. Once the antibody-biotin-peroxidase complex was formed, diaminobenzidine chromogen was added as the peroxidase substrate. After completing the immunostaining procedure, a counterstain of hematoxylin was applied, followed by mounting with crystal mount solution.

**Quantitation of Immunohistochemistry.** A Universal Imaging Corp. Image-1/AT image acquisition and analysis system (Chester, PA) incorporating an Axioskop 50 microscope (Carl Zeiss, Inc., Thornwood, NY) was used to capture and analyze the immunostained tissue sections at 25× magnification. Color-detection ranges were set for the red-brown color of the diaminobenzidine chromogen based on an intensely labeled point. The extent of pimonidazole labeling in the liver lobule was defined as the percent of the field area within the default color range determined by the software. Because fatty accumulation tends to cause hepatocyte hypertrophy, it is possible that the increase in area of pimonidazole binding would not represent an actual increase in hypoxic cells, but just increased cell size. Therefore, the number of pimonidazole-positive cells was also counted in pericentral regions of the liver lobule. The number of brown nuclei were counted in a radius of about 15 cell widths around the periluminal lumen (≈350 cells per field) at 400×. This radius was chosen to incorporate most of the pericentral region where pimonidazole labeling predominated. Data from each tissue section (5 fields per section) were pooled to determine means.

**RESULTS**

**Effect of Chronic Ethanol Administration on Weight Gain and Urine Ethanol Levels.** Body weights tended to decline during the first week of enteral feeding, but then stabilized and remained constant for the following 3 weeks. Mean body weights at the time of killing were not significantly different between groups (control, 319 ± 5 g; ethanol, 307 ± 10 g). Ethanol administration was gradually increased to 9 to 10 g/kg/d during the first week after surgery, and was held in a range between 10 and 12 g/kg/d for 3 weeks. Figure 1 shows a representative plot of daily urine ethanol concentration in an ethanol-fed rat. As observed previously,\(^2\)\(^9\) ethanol levels fluctuate in a cyclic pattern from near zero to greater than 400 mg/dL, even though ethanol was infused at constant rates; the mechanism of this cycling pattern is currently unknown. Mean urine ethanol concentrations over the 4 weeks of study were 245 ± 22 mg/dL. Ethanol concentrations in serum collected from rats at the time of killing ranged from 87 to 255 mg/dL.

**Effect of Chronic Ethanol Treatment on Liver Injury: Histology and Serum Aspartate Transaminase.** Histology was normal in control rats with little or no fatty infiltration, inflammation, or focal necrosis (Fig. 2A, 2C, and 2E). In comparison, the enteral ethanol diet dramatically increased fatty infiltration (Fig. 2B, 2D) and caused inflammation and focal necrosis (Fig. 2F). The hepatic pathology score is summarized in Fig.
Effects of Chronic Ethanol Administration on Sublobular Patterns of Pimonidazole Binding in Rat Liver. Figure 4 shows patterns of binding of pimonidazole (brown) against a hematoxylin counterstain (blue) in livers from representative control-treated (Fig. 4A and 4B) and ethanol-treated (Fig. 4C and 4D) rats after 4 weeks. Pimonidazole binding in livers from controls (Fig. 4A) was localized predominantly in pericentral regions a few cell widths from the terminal hepatic venule (Fig. 4B). In contrast, 4 weeks of enteral ethanol increased the extent of pimonidazole staining compared with controls (Fig. 4C), with binding extending into midzonal regions of the hepatic lobule (Fig. 4D).

Figure 5 summarizes the results of quantitation of pimonidazole immunohistochemistry with image-analysis (Fig. 5A) and cell counting (Fig. 5B). The extent of pimonidazole staining in livers from ethanol-treated rats was significantly greater than high-fat enteral-diet controls by a factor of about 1.5 at both 7 days and 4 weeks (Fig. 5A). Ethanol treatment also increased the percent of intensely labeled cells in pericentral areas significantly over enteral-diet controls by a factor of 1.5 (Fig. 5B). The high-fat enteral diet without ethanol had no effect on either of the above parameters compared with chow-fed controls (Fig. 5A, 5B)

**DISCUSSION**

**Ethanol Causes Hypoxia in a Clinically Relevant Model of Alcoholic Liver Injury.** Recently, Arteel et al.\(^24\) showed that acute ethanol causes hypoxia in rat liver in vivo using pimonidazole, confirming conclusions drawn from previous in vitro experiments investigating the effects of acute ethanol on hepatic oxygen consumption.\(^5,6,23\) Importantly, selectively destroying Kupffer cells, which has been shown to prevent the hypermetabolic state caused by ethanol in perfused liver,\(^21\) also blocked hypoxia caused by acute ethanol in vivo.\(^21\) However, acute ethanol did not cause pathophysiological changes associated with alcoholic liver injury; therefore, conclusions regarding a possible role of hypoxia in the progression of alcoholic liver disease could not be made from that study.

In the present study, chronic ethanol administration significantly increased the binding of pimonidazole in liver tissue (Figs. 4 and 5) in a clinically relevant model of early alcoholic liver injury. Moreover, the increase in binding was localized in oxygen-poor pericentral regions of the liver lobule (Figs. 4 and 5B). Ethanol treatment increased both the extent of pimonidazole binding (Fig. 5A) and the number of hypoxic cells (Fig. 5B). Therefore, it is concluded that the observed increase in the extent of pimonidazole binding caused by chronic ethanol treatment is not simply a result of an increase in cell size caused by fatty accumulation, but reflects a true increase in tissue hypoxia.

Although previous studies have investigated the effects of chronic ethanol on hepatic blood oxygenation in vivo,\(^21,32,34\) the results of the present study showed clearly for the first time that such treatment causes hypoxia at the cellular level in pericentral regions of rat liver tissue in vivo. Previously, Tsukamoto and Xi\(^21\) detected a significant increase in hepatic oxygen extraction in rats on the Tsukamoto-French protocol,
a model that develops clinically significant liver injury. However, other authors failed to observe increases in oxygen consumption in rats fed a liquid diet containing ethanol chronically in a model that only develops fatty liver. Reasons for these differences remain unclear. In this study, we showed that ethanol increases hypoxia marker binding after only 7 days of an enteral diet, before any overt liver pathology in this model (e.g., inflammation and necrosis) (Fig. 5). It is possible that hypoxia plays a key role in the progression of alcoholic liver injury; this could explain the lack of damage in models that do not exhibit increases in hepatic oxygen extraction. There are studies, however, in which oxygen extraction was not increased after ethanol in models in which liver injury develops. For example, Lieber et al. reported impairment of hepatic oxygen extraction in baboons exposed chronically to an ethanol diet. In that study, however, animals were fasted overnight. Fasting blocks the hypermetabolic state caused by ethanol in the rat by depleting glycogen reserves, and thereby could prevent increases in oxygen consumption after ethanol.
Possible Role of Hypoxia in Alcoholic Liver Injury. While the present study showed that chronic ethanol causes hepatic hypoxia in rat liver in vivo, mechanisms responsible still remain unclear. It is possible that chronic ethanol causes a hypermetabolic state similar to that observed after acute ethanol\(^6,24\); this idea is supported by observed increases in hepatic oxygen extraction.\(^{21,28}\) In addition, ethanol may have vasoactive actions leading to decreased hepatic blood flow, which could cause hypoxia in downstream regions of the hepatic lobule. For example, Oshita et al.\(^{37}\) found that ethanol infusion increased portal pressure, a measure of microvascular tone, in isolated, perfused rat liver. The levels of ethanol required to increase portal pressure in perfused liver are comparable with the higher blood ethanol levels observed with rats on the Tsukamoto-French protocol in this study (≈400 mg/dL).

Ethanol-induced hypoxia in rats on the Tsukamoto-French protocol may fluctuate in a dose-dependent manner with the cycling pattern of blood ethanol concentrations characteristic of this model (Fig. 1). In fact, some studies support this hypothesis. For example, both hypermetabolism\(^6\) and increased portal pressure\(^37\) caused by ethanol have been shown to correlate with alcohol levels in perfused liver. Furthermore, Knecht et al.\(^{39}\) showed a dose-dependent decrease in hepatic surface oxygen tension in vivo in rats given ethanol via the Tsukamoto-French protocol. In the present study, blood alcohol levels in the serum were quite variable in rats at the time of killing but correlated significantly with the number of hypoxic cells in pericentral regions ($r^2 = .86$), suggesting that there is a dose response for ethanol on hypoxia. On the other hand, alcohol affects breathing, which could influence systemic oxygen tension. However, blood alcohol at the time of pimonidazole administration ranged from 87 to 258 mg/dL, values at which Tsukamoto and Xi\(^{21}\) did not observe significant differences in arterial $P_O_2$ between control rats and rats given enteral ethanol. Indeed, even at the lowest blood alcohol concentration studied here, the number of hypoxic cells was approximately 50% higher than control values. Therefore, we conclude that the hypoxia observed in this model is a true physiological response and is not caused by the effect of ethanol on breathing.

For over 25 years, the potential that hypoxia plays a causal role in liver injury has been recognized.\(^2\) While hypoxia can damage hepatocytes, reoxygenation following hypoxia can paradoxically induce more damage. Upon reintroduction of oxygen, reactive free radicals (e.g., superoxide and hydroxyl radicals) could be produced, leading to the formation of $\alpha$-hydroxyethyl and lipid radicals and subsequent cell damage. The evidence that hypoxia/reoxygenation injury occurs in ethanol-induced liver injury is supported by very recent work in which $\alpha$-hydroxyethyl free radicals were detected in bile collected from rats on the Tsukamoto-French protocol.\(^39\) Therefore, the demonstration for the first time here that chronic ethanol causes hypoxia at the cellular level in rat liver tissue in vivo in a clinically relevant model of early alcoholic liver injury, and that hypoxia precedes overt liver damage, adds strength to the hypothesis that hypoxia plays a critical early step in the mechanism of alcoholic liver injury.

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Fig. 5. Effect of ethanol on hepatic pimonidazole binding: quantitation of immunohistochemistry. Controls received a high-fat enteral diet for 7 days or 4 weeks as indicated. Image-analysis (A) and cell counting in pericentral regions (≈15 cell width from terminal hepatic venules) of the hepatic lobule (B) were performed as described in Materials and Methods. The total number of cells counted per field was not significantly different between groups. Results were means ± SEM; n = 4. * P < .05 compared with controls using one-way ANOVA.
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