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ALCOHOL-INDUCED HEPATOTOXICITY: A ROLE FOR OXYGEN FREE RADICALS

M. YOUNES and O. STRUBELT

Institute of Toxicology, Medical University of Lübeck, Ratzeburger Allee 160, 2400 Lübeck, W.-Germany

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Perfusion of isolated rat livers with ethanol at a concentration of 2 g/l (‰) resulted in a release of glutamate-pyruvate-transaminase (GPT) and sorbitol dehydrogenase (SDH) into the perfusate as markers of toxicity. Inhibition of alcohol dehydrogenase by 4-methylpyrazole or of aldehyde dehydrogenase by cyanamide totally abolished ethanol hepatotoxicity despite of a severalfold increase in acetaldehyde concentration in the perfusate. Addition of superoxide dismutase or catalase clearly suppressed the ethanol-induced release of GPT and SDH, suggesting that $^{\cdot}O_2^-$ and H_2O_2 are involved in this process. Also, chelation of iron ions by means of desferrioxamine displayed a clear inhibitory action, suggesting the involvement of an iron-catalyzed Haber–Weiß-reaction leading to the formation of $^{\cdot}OH$ radicals in the hepatotoxic response to ethanol. Our data suggest that during the metabolism of acetaldehyde primary reactive oxygen species ($^{\cdot}O_2^-$, H_2O_2) are produced which may interact to yield hydroxyl or $^{\cdot}OH$-like radicals, which possibly represent the hepatotoxic principle of ethanol.

KEY WORDS: Alcohol, hepatotoxicity, oxygen radicals, superoxide dismutase, catalase, desferrioxamine.

INTRODUCTION

The mechanism of ethanol-induced liver injury is still a matter of debate. The early findings that pretreatment of alcohol-intoxicated animals with antioxidants protected against ethanol-induced fatty liver[1] suggested that oxidative mechanisms, mainly lipidperoxidative damage, might be involved in the pathogenesis of ethanol hepatotoxicity.[2] Subsequent investigations revealed controversial results with respect to the induction of lipid peroxidation by ethanol: while an increase in the exhalation of ethane,[3] in the hepatic content of conjugated dienes[4] as well as in malondialdehyde concentration and spontaneous chemiluminescence[5] upon ethanol treatment were reported, no evidence of hepatic lipid peroxidation in ethanol-induced liver damage was found by others[6,7] such lack of evidence might be related to the presence of glutathione-dependent defense mechanisms.[8] In fact, ethanol produces a significant decrease in hepatic glutathione (GSH) which can be due to GSH oxidation,[9] GSH release from the liver into the bile[9] and conjugation with acetaldehyde.[10]

On the other hand, the ethanol metabolite acetaldehyde itself was suspected to mediate alcohol hepatotoxicity via direct covalent binding to hepatic macromolecules.[11] Using an *in vitro*-preparation, in which the hepatic structure is preserved, the isolated, hemoglobin-free perfused rat liver, we studied the role of acetaldehyde as a mediator of ethanol hepatotoxicity. In a similar model, Müller and Sies[12] observed an increase in alkane formation upon application of ethanol and acetaldehyde. Furthermore, we investigated the possible involvement of reactive oxygen species in alcohol toxicity towards the liver.

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MATERIALS AND METHODS

Animals and surgical procedures

Male Wistar rats (350–450 g; breeder: Winkelmann, Borchen) were employed throughout. They had free access to feed (Altromin® standard diet) and drinking water until use.

Removal of the liver was carried out under pentobarbital anesthesia (60 mg/ml, nembutal®-solution 1 ml/kg i.p.). Following anesthesia, the rat received an injection of 500 sodium heparin in 1 ml 0.9% NaCl-solution into the saphenous vein. With the animal lying on its back, the limbs were fixed in extension on a surgical board and a vertical longitudinal midline incision was made extending from the pubis to the upper chest. The common bile duct was cannulated with a PE 10 catheter, then the portal vein, followd by the lower vena cava, were both cannulated using PE 240 catheters. 20 ml of sodium heparin solution (500 u/ml in 0.9% NaCl) were then infused through the portal vein. After ligating the upper vena cava, the liver was carefully dissected out and reinfused with 20 ml of sodium heparin solution. After rapid weighing, it was connected to the perfusion apparatus.

Liver perfusion

A recirculating liver perfusion system[13] was employed. The portal catheter was connected to the perfusate, then the liver was placed on a platform making sure that the outflow catheter was led down through a central hole of the platform extending into the neck of the bottom reservoir. The perfusion medium consisted of 250 ml Krebs–Henseleit-buffer, pH 7.4 (per 1: 118 mmol NaCl, 5 mmol KCl, 1.1 mmol $MgSO_4$, 1.2 mmol KH_2PO_4, 25 mmol $NaHCO_3$). $CaCl_2$ (2.5 mmol/l) was added to the prewarmed perfusion medium (37°C) immediately before the start of the perfusion. 50 ml of the medium were infused through the liver and discarded prior to starting the experiment. Sodium taurocholate (26.7 g/l) was continuously infused into the perfusion medium at a rate of 12 ml/h to stimulate bile secretion. The perfusion medium was continuously gassed with carbogen (95% O_2, 5% CO_2), the partial pressure of O_2 amounting to 600 mm Hg. The whole system was kept in a closed chamber at 37°C.

Viability criteria

At zero time as well as every 30 min after the start of the perfusion experiments, 1 ml samples of the perfusate were removed from directly above the portal catheter (influent perfusate) as well as from the effluent perfusate. These were injected into a Micro pH/Blood Gas Analyzer 413 (Instrumentation Laboratory) to determine pH, O_2- and CO_2-contents. Oxygen consumption was calculated from the difference in oxygen concentrations. Bile was collected in portions of 30 min.

Biochemical determinations

1 ml samples of the effluent perfusate were collected every 30 min. In these samples, the activities of glutamate-pyruvate-transaminase (GPT) and sorbitol dehydrogenase (SDH), as well as the concentrations of lactate and pyruvate were assayed using commercial assay kits of Boehringer, Mannheim.

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Application of ethanol and other reagents

Ethanol was added directly to the perfusion medium to yield a final concentration of 2 g/l after a 30 min-equilibration period. Its availability was assayed by measurement of its concentration in the perfusate at various time points, using a Hewlett-Packard gas chromatograph (HP 5710 A) equipped with a packed column (3% SP-1500 on Carbopack B, 80/120 mesh) and a flame ionization detector. Assay conditions were: column temperature 120°C, injector temperature 200°C, detector temperature 300°C and carrier gas flow (N_2) 30 ml/min. Under these conditions the retention time of ethanol was 0.85 min.

Acetaldehyde formed was determined in the perfusate by means of gas chromatography following conjugation to 2,4-dinitrophenylhydrazine. Retention time under the conditions given above was 3.0 min.

Inhibition of alcohol dehydrogenase or aldehyde oxidase was accomplished by addition of 4-methylpyrazole (0.5 mmol/l) or cyanamide (0.12 mmol/l), respectively, to the perfusate 30 min prior to the application of ethanol. Superoxide dismutase (20 μg/ml), catalase (20 μg/ml) and desferrioxamine (0.5 mg/ml), also, were dissolved directly in the perfusion medium at the start of the experiment.

RESULTS

Addition of ethanol at a concentration of 2 g/l (2‰) to the perfusion medium resulted in a measureable hepatotoxicity as evidenced by the release of the liver-specific enzymes glutamate-pyruvate-transaminase (GPT) and sorbitol dehydrogenase (SDH) into the perfusate (Figure 1).

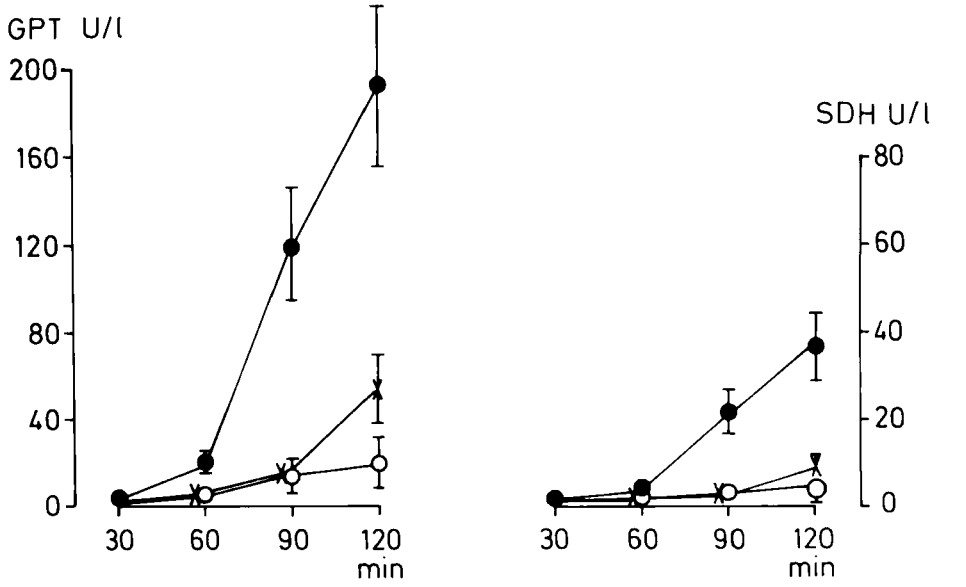

FIGURE 1 Activities of glutamate-pyruvate-transaminase (GPT) and sorbitol dehydrogenase (SDH) released from the isolated perfused rat liver into the perfusate upon application of ethanol (2 g/l). ●—●: ethanol only; ○—○: ethanol + cyanamide, 0.12 mmol/l, ×—×: ethanol + 4-methylpyrazol, 0.5 mmol/l.

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Inhibition of alcohol dehydrogenase by adding 4-methylpyrazole to the medium prior to ethanol application clearly suppressed ethanol-induced enzyme release (Figure 1) and inhibited ethanol metabolism (Figure 2). Also, the increase in the lactate/pyruvate ratio, known to occur upon ethanol treatment also *in vivo*, was totally suppressed (Figure 3), a further evidence for the inhibition of alcohol metabolism.

Cyanamide, given at a concentration which showed no hepatotoxicity of its own 30 min before ethanol application, resulted in a clear inhibition of aldehyde dehydrogenase as evidenced by the accumulation of acetaldehyde in the perfusate reflecting elevated levels in the liver also (Figure 2), a phenomenon which was also seen *in vivo*.[14] Again, release of GPT and SDH was nearly totally inhibited (Figure 1).

Addition of acetate to the perfusate instead of ethanol showed no toxicity at all (data not shown). This is in accordance with the observation that acetate did not evoke ethane formation in perfused liver.[12]

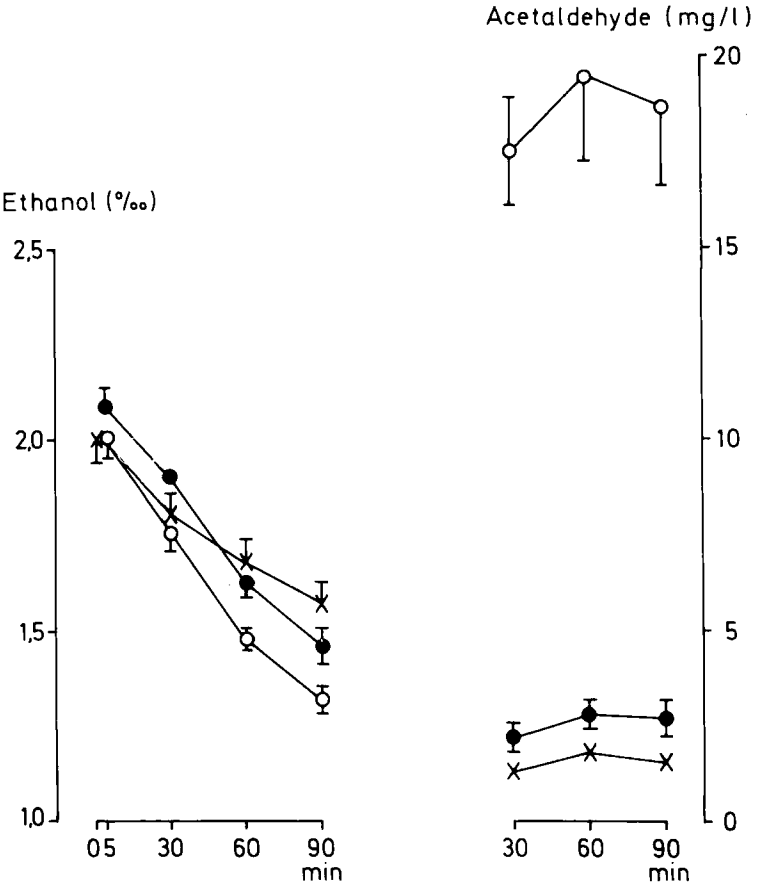

FIGURE 2 Concentrations of ethanol and acetaldehyde in the perfusate upon application of 2 g/l ethanol. ●—●: ethanol only; ○—○: ethanol + cyanamide, 0.12 mmol/l; ×—×: ethanol + 4-methylpyrazole, 0.5 mmol/l.

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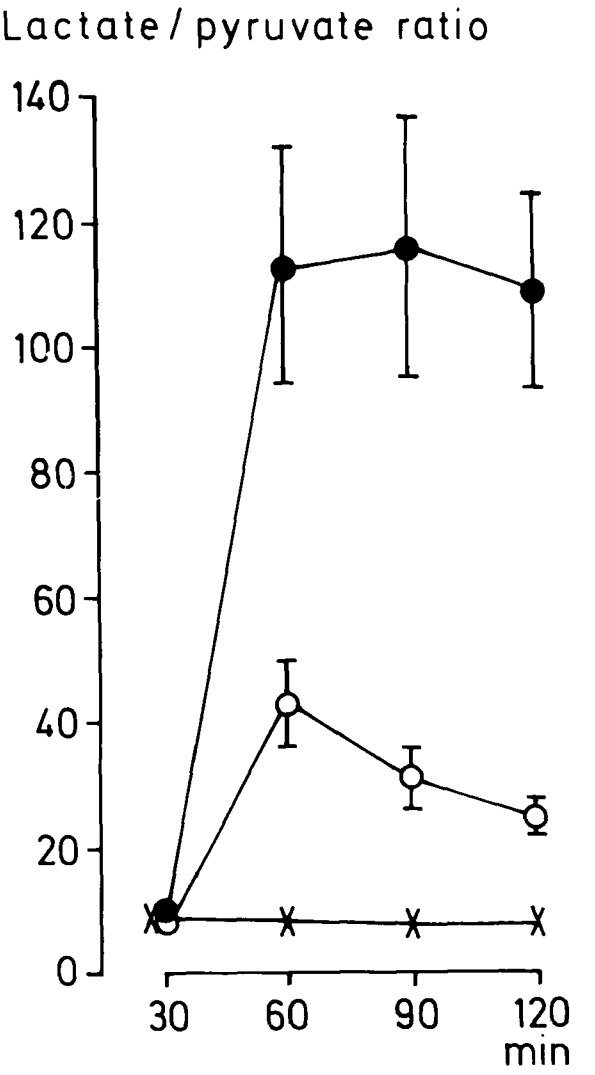

FIGURE 3 Lactate/pyruvate ratio in the perfusate upon application of ethanol at a concentration of 2 g/l. ●—●: ethanol only; ○—○: ethanol + cyanamide, 0.12 mmol/l; ×—×: ethanol + 4-methylpyrazole, 0.5 mmol/l.

The hepatotoxicity of ethanol in this model was also markedly inhibited by prior addition of superoxide dismutase, catalase or desferrioxamine to the perfusion medium (Figure 4).

Neither of the agents inhibited alcohol metabolism or led to an accumulation of acetaldehyde, suggesting that both alcohol dehydrogenase and aldehyde oxidase activities were not affected by any of the pretreatments (Figure 5), which is also substantiated by a lack of effect on the increased lactate/pyruvate ratio after ethanol addition (data now shown).

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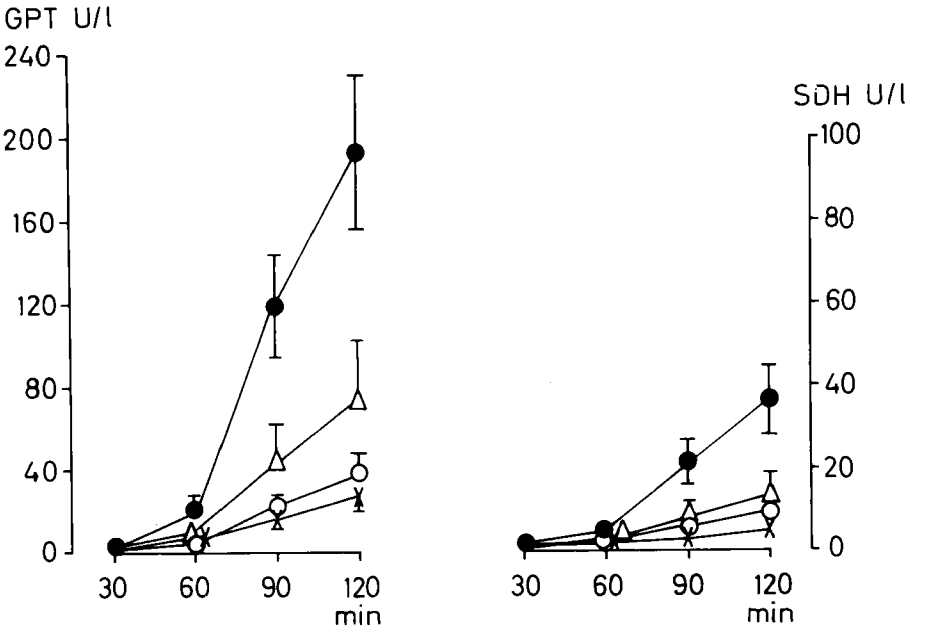

FIGURE 4 Activities of glutamate-pyruvate-transaminase (GPT) and sorbitol dehydrogenase (SDH) released from the isolated perfused rat liver into the perfusate upon application of ethanol (2 g/l): ●—●: ethanol only; ○—○: ethanol + superoxide dismutase, 20 μg/ml; ▲—△: ethanol + catalase, 20 μg/ml; ×—×: ethanol + desferrioxamine, 0.5 mg/ml.

DISCUSSION

In the model system employed in this study, the isolated perfused liver, ethanol proved capable of inducing liver damage. Inhibition of the two steps involved in ethanol metabolism, i.e. the oxidation to acetaldehyde and the further conversion to acetic acid, led to an inhibition of alcohol-induced enzyme leakage. This clearly shows that ethanol has to be metabolized in order to induce hepatic lesions. The nature of the metabolite responsible for the toxicity towards the liver has not been clearly identified. Several authors suggested that acetaldehyde itself interacted with cellular macromolecules[11,15] by forming covalent adducts. These were suspected to mediate hepatocellular damage.

In our study, however, inhibition of aldehyde oxidation by cyanamide resulting in an accumulation of acetaldehyde did not augment ethanol hepatotoxicity, neither did the addition of acetate to the perfusion medium. Thus, we conclude that neither of the stable metabolites of ethanol, acetaldehyde or acetate, are direct mediators of alcohol toxicity, as suggested previously by Müller and Sies.[16]

Both superoxide dismutase and catalase proved capable of preventing enzyme release from the liver upon ethanol treatment, without affecting ethanol or acetaldehyde metabolism. Thus, superoxide anion radicals ($^{\cdot}O_2^-$) and hydrogen peroxide (H_2O_2) seem to be involved in ethanol-induced liver damage. As chelation of iron ions by desferrioxamine also suppressed ethanol hepatotoxicity, we conclude that $^{\cdot}O_2^-$ and H_2O_2 interact in an iron-catalyzed reaction according to Haber and Weiß[17] to produce

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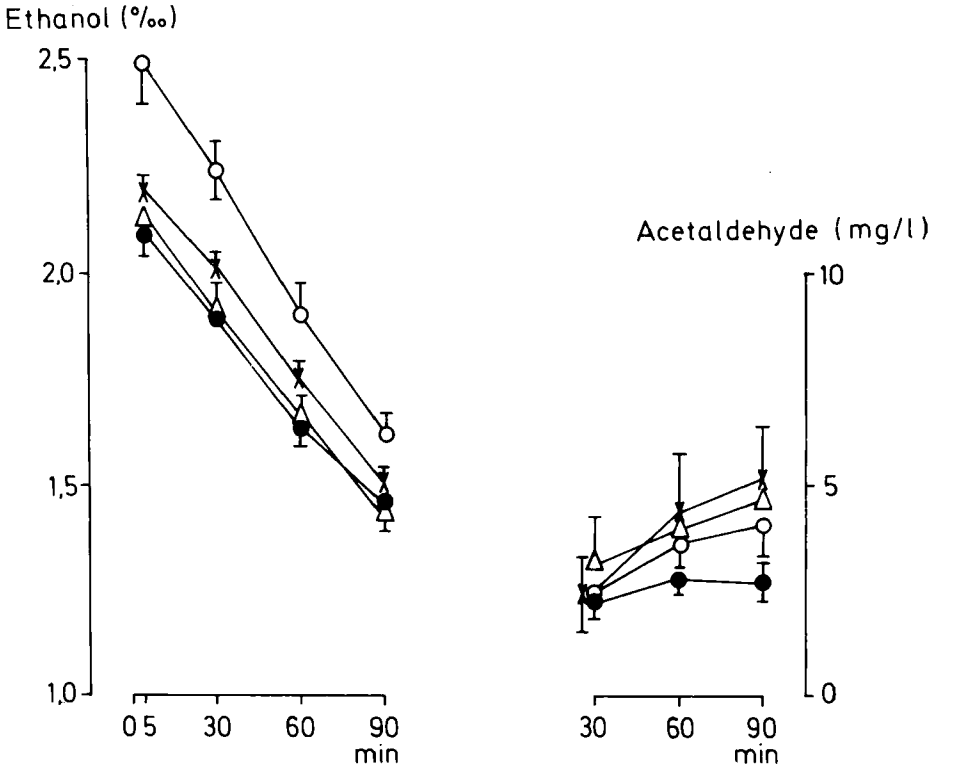

FIGURE 5 Concentrations of ethanol and acetaldehyde in the perfusate upon application of 2 g/l ethanol. ●—●: ethanol only; ○—○: ethanol + superoxide dismutase, 20 μg/ml; ▲—△: ethanol + catalase, 20 μg/ml; × — ×: ethanol + desferrioxamine, 0.5 mg/ml.

·OH- or ·OH-like radicals, which mediate alcohol toxicity towards the liver. The step of oxygen activation takes place during the metabolism of acetaldehyde, as its inhibition suppressed cytotoxicity despite of an intact ethanol oxidation.

The fact that ethanol metabolism is accompanied by oxidative stress is substantiated by the findings that ethanol treatment results in a decrease in liver soluble thiol content[4,5] due to the oxidation of glutathione and its release into the bile.[9] Furthermore, several authors have found ethanol to lead to lipid peroxidation.[1–5] Using the isolated perfused liver, as in our study, it was recently shown that ethanol resulted in a release of ethane and n-pentane as markers of lipid peroxidation.[12,18] This effect was suppressed when either alcohol dehydrogenase or aldehyde oxidation were inhibited.[12,16] The ability of ethanol to induce oxygen radical formation was substanfrom ethanol-treated rats $\cdot O_2^-$ than those isolated from control animals.[17]

In summary, our data suggest, in accordance with Müller and Sies,[16,20] that during the metabolism of acetaldehyde oxygen activation takes places. As a consequence, primary reactive oxygen species ($\cdot O_2^-$, H_2O_2) are produced which may interact in the presence of iron ions in a Haber–Weiß reaction yielding hydroxyl or ·OH-like radicals. These may represent the hepatotoxic principle of ethanol.

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Accepted by Prof. H. Sies

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