Overexpression of manganese superoxide dismutase prevents alcohol-induced liver injury in the rat

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ABSTRACT

Mitochondria are thought to play a major role in hepatic oxidative stress associated with alcohol-induced liver injury. Thus, the hypothesis that delivery of the mitochondrial isoform of superoxide dismutase (Mn-SOD) via recombinant adenovirus would reduce alcohol-induced liver injury was tested. Rats were given recombinant adenovirus containing Mn-SOD (Ad.SOD2) or β-galactosidase (Ad.lacZ) and then fed alcohol enterally for four weeks. Superoxide dismutase expression and activity of Ad.SOD2 in liver mitochondria of infected animals was increased nearly 3-fold compared to Ad.lacZ infected controls. Mitochondrial glutathione levels in Ad.lacZ-infected animals were decreased after four weeks of chronic ethanol as expected, but were unchanged in Ad.SOD2 infected animals. Alanine aminotransferase was elevated significantly by ethanol, an effect that was prevented by Ad.SOD2. Moreover, pathology (e.g., the sum of steatosis, inflammation, and necrosis) was elevated dramatically by ethanol in Ad.lacZ treated rats. This effect was also blunted in animals infected with Ad.SOD2. Neutrophil infiltration was increased about 3-fold in livers from both Ad.lacZ and Ad.SOD2 infected rats by ethanol treatment. Moreover, ESR-detectable free radical adducts in bile were increased about 8-fold by ethanol. Using $^{13}$C-labeled ethanol, it was determined that nearly 60% of total adducts were due to the $\alpha$-hydroxyethyl radical adduct. This increase in radical formation was blocked completely by Ad.SOD2 infection. Furthermore, apoptosis of hepatocytes was increased about 5-fold by ethanol, an effect also blocked by Ad.SOD2. Interestingly, TNF$\alpha$ mRNA was elevated to the same extent in both Ad.lacZ- and Ad.SOD2-infected animals following ethanol exposure. These data suggest that hepatocyte mitochondrial oxidative stress is involved in alcohol-induced liver damage and likely follows Kupffer cell activation, cytokine production and neutrophil infiltration. These results also support the hypothesis that mitochondrial oxidant production is a critical factor in parenchymal cell death caused by alcohol.
INTRODUCTION

Alcoholic liver disease results from the dose- and time-dependent exposure to alcohol (1), but precise mechanisms of pathology are still largely unknown. Endotoxin and Kupffer cells have been implicated in the mechanism of early alcohol-induced liver injury using the enteral feeding model of Tsukamoto-French (2). For example, endotoxin derived from the gut activates Kupffer cells in the liver (3). In support of this idea, gut sterilization with non-absorbable antibiotics or inactivation of Kupffer cells by gadolinium chloride (GdCl$_3$) prevents alcohol-induced liver injury in this model (4,5). Furthermore, Kupffer cells, which release effectors and cytokines, are a major source of TNF$\alpha$ in the liver (6). Indeed, TNF$\alpha$ messenger RNA in liver increased after four weeks of treatment with ethanol (7). Moreover, early alcohol-induced liver injury was attenuated by anti-TNF$\alpha$ antibodies and largely prevented in TNF receptor 1 knockout mice (8,9). Thus, it is clear that TNF$\alpha$ plays a critical role in early alcohol-induced liver injury.

Reactive oxygen species generated during chronic alcohol exposure may also be a major factor in liver damage (10,11). $\alpha$-Hydroxyethyl free radicals were increased as a result of alcohol treatment (12) and were diminished by destruction of Kupffer cells with GdCl$_3$ (13). Furthermore, both production of TNF$\alpha$ and $\alpha$-hydroxyethyl free radicals were decreased in livers of NADPH oxidase knockout mice, effects which correlated with a reduction in pathology (14). Moreover, delivery of cytosolic superoxide dismutase (Cu/Zn-SOD) by adenovirus reduced early alcohol-induced liver injury as well as blunted NF$\kappa$B activation and TNF$\alpha$ production (15). However, it is not clear whether oxidants act as direct toxicants to hepatocytes or as signals to produce TNF$\alpha$ or other cytokines by Kupffer cells.

Under normal conditions, the mitochondrial isoform of superoxide dismutase balances the production of excess superoxide from electron transport. Hydrogen peroxide, a product of the superoxide dismutase reaction, is further reduced to water by glutathione (GSH) peroxidase using mitochondrial GSH (16). Mitochondrial glutathione is diminished in livers of animals exposed to chronic ethanol, supporting the hypothesis that mitochondrial oxidant production plays a role in early alcohol-induced liver injury.
Moreover, treatment with the glutathione precursor L-2-oxothiazolidine-4-carboxylic acid (OTC) blunted alcohol-induced liver injury in the enteral model (19). Thus, it is hypothesized that mitochondria may be a critical source of oxidants due to chronic ethanol consumption.

The role of Mn-SOD and mitochondrial oxidative stress in alcohol-induced liver injury is controversial. A recent report showed that homozygous mutations in the SOD2 gene which may lead to an increase in mitochondrial localization of SOD is a risk for severe alcoholic liver disease in humans (20). However, other reports have demonstrated decreases in Mn-SOD expression and activity in liver due to ethanol in rats (21-23). Thus, the hypothesis that overexpression of mitochondrial Mn-SOD would prevent alcohol-induced liver injury was tested here by delivering human mitochondrial Mn-SOD via recombinant adenovirus (Ad.SOD2). Indeed, overexpression of Mn-SOD reduces liver injury induced by alcohol in the enteral feeding model, suggesting that mitochondrial redox state is important in ethanol-induced liver injury.
MATERIALS AND METHODS

Animals and Diets.

Male Wistar rats weighing 280 to 310 g were housed in an AAALAC-approved facility on a 12-hour light/dark cycle under institutional guidelines for the humane treatment of laboratory animals. Intragastric cannulas were inserted as described by Tsukamoto et al (2). Cannulas were tunneled subcutaneously to the dorsal aspect of the neck and connected to infusion pumps by means of a spring-tether device and swivel, allowing rats to move freely in metabolic cages. Rats received a liquid diet described by Thompson and Reitz (24) which is composed of corn oil as fat (34% of total calories), protein (23%) and carbohydrate (43%), plus minerals and vitamins and supplemented with lipotropes as described by Morimoto et al (25). For the ethanol diet, maltose-dextrin was replaced isocalorically with ethanol. In each group, rats received either high-fat control diet or ethanol-containing diet by continuous infusion through an intragastric tube. The daily amount of ethanol given was gradually increased to 12.0 g/kg/day in the first week. Values were then increased progressively up to 14.5 g/kg/day.

Adenoviral synthesis and preparation.

Recombinant adenoviral vectors containing the transgene for either β-galactosidase (Ad.lacZ) or Mn-SOD (Ad.SOD2) were prepared as described elsewhere (26,27). Briefly, the plasmid shuttle vector pAd5-CMV-lacZ was constructed using standard cloning protocols. The adenoviral shuttle plasmids were transfected into the permissive HEK 293 host cell line to generate recombinant Ad.lacZ adenovirus. The Ad.SOD2 viral seed stock was a kind gift from Dr. John Engelhardt, Univ. of Iowa. Virus isolates were plaque-purified and propagated in HEK 293 cells, isolated, concentrated, and titered by plaque assay to stock titers of greater than 1×10^{11} plaque forming units (pfu). Rats were divided into two groups randomly and injected with Ad.lacZ or Ad.SOD2 at a concentration of 1×10^{9} pfu in 1 mL of lactated Ringer’s solution via the penile vein three days before liquid diet feeding was initiated.
Urine collection and ethanol assay.

Ethanol concentrations in urine (UAC) were measured daily. Rats were housed in metabolic cages and urine was collected over 24 hours in bottles with mineral oil to prevent evaporation. Samples were stored at -20°C for later analysis of ethanol. Ethanol concentration was determined by measuring absorbance at 366 nm resulting from the reduction of NAD⁺ to NADH by alcohol dehydrogenase (28).

Clinical chemistry.

Blood was collected via the abdominal aorta at sacrifice. Serum was stored at -20°C in microtubes until alanine aminotransferase (ALT) was assayed using standard enzymatic procedures (28). For measurement of plasma endotoxin, blood was taken from the portal vein and the abdominal aorta in pyrogen-free heparinized syringes during laparotomy after 4 weeks of treatment with ethanol. Blood kept in pyrogen-free glass tubes was centrifuged at 1,200 rpm for 10 minutes, and plasma was stored at -80°C also in pyrogen-free glass tubes until measurement of endotoxin with a Limulus Amebocyte Lysate test kit (Kinetic QCL, Bio Whittaker, Walkersville, MD). The levels of total glutathione (GSH + GSSG) and oxidized glutathione (GSSG) in whole liver and in mitochondria were measured as described elsewhere (29).

Measurement of α-hydroxyethyl free radical adducts in bile.

Rats were anesthetized with pentobarbital (75 mg/kg) and a 24 G catheter was inserted into the left femoral vein for injection of the spin trapping agent α-(4-pyridyl-1-oxide)-N-tet-butyl nitrotrone (POBN, 1 g/kg; Sigma St. Louis, MO). Laparotomy was performed, PE-20 tubing was inserted into the common bile duct, and the spin trap dissolved in normal saline was injected through the intravenous catheter. Bile was collected for three hours in an Eppendorf tube containing 35 µl of 5 mM deferoxamine mesylate (Sigma St. Louis, MO) to prevent ex vivo radical formation. During this procedure, 1 mL of normal saline was injected through the catheter every 30 minutes to compensate for loss of fluids. Samples were frozen immediately on dry ice and stored at -80°C until ESR analysis.

The ESR spectra of radical adducts was detected using a Varian E-109 spectrometer in an E-238 TM110 microwave cavity with instrument setting of 20 mW microwave power, 0.5 G modulation amplitude,
0.66 second of conversion time, 0.33 time constant, 80 G scan width, and 9.785 GHz microwave frequency. Spectra were recorded on an IBM-compatible computer interfaced to the spectrometer, and hyperfine coupling constants were determined with a spectral simulation program (30).

**Pathologic evaluation.**

Autopsy was performed after 4 weeks of treatment with ethanol. Livers were fixed in formalin, embedded in paraffin, and stained with hematoxylin and eosin for the assessment of pathology. Liver pathology was scored as described by Nanji et al. (31) as follows: steatosis (the percentage of hepatocytes containing fat), <25%, 1+; <50%, 2+; <75%, 3+; >75%, 4+; inflammation and necrosis: 1 focus per low-power field, 1+; 2 or more foci, 2+. Pathology was scored in a blinded manner by one of authors.

**Apoptosis and infiltrating neutrophils.**

Apoptosis was expressed as % of apoptotic hepatocytes showing condensation of chromatin and nuclear fragmentation per 2,000 hepatocytes in high power fields (×400) selected randomly (32). Caspase-3 activity was also evaluated using the chromogenic substrate DEVD-pNA (BioSource, CA) as recommended by the manufacturer. The degree of infiltrating neutrophils was expressed per 100 hepatocytes because fat accumulation causes ballooning of hepatocytes, making sinusoidal spaces narrow, which affect the numbers of hepatocytes and sinusoidal space in each field. Values were determined by counting polymorphonuclear cells in 5 high power fields (×400) per slide followed by counting the number of hepatocytes in each field. Mean values were used for statistical analysis.

**Isolation of mitochondria.**

Whole liver tissue was homogenized in 10 mL of buffer (40 mM Tris, 140 mM NaCl, and a protease inhibitor cocktail including aprotinin, leupeptin, phenylmethylsulfonyl fluoride, and dithiothreitol). Supernatant was collected after centrifugation at 900 × g for 7 min and was centrifuged at 17,000 × g for 10 min to pellet mitochondria. The mitochondrial pellet was washed and resuspended in buffer, and protein concentration was measured using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA).
**Hepatocyte isolation, infection and culture.**

Rat hepatocytes from normal, untreated rats were isolated by collagenase perfusion and Percoll centrifugation as previously described (11) and cultured at 1x10^6 cells/ well in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum and antibiotics. After 4 hours of culture, hepatocytes were infected with either Ad.lacZ (100 viral infectious units/ cell) or Ad.SOD2 and were cultured for 18 hours to allow transgene expression to occur. Cells were then incubated in ethanol (2-200 mM) or saline for 12 hours.

**Measurement of SOD activity.**

SOD activity in freshly isolated mitochondria was measured by the reduction of ferricytochrome c with modification of a method described elsewhere (33). Mitochondria were isolated by differential centrifugation as described above and added to a solution containing 50 mM K$_2$HPO$_4$, 0.1 mM Na$_2$EDTA, 0.5 mg/mL cytochrome c, and 165 mM xanthine, and superoxide was generated by the addition of 0.004 units of xanthine oxidase. The reaction proceeded at room temperature for 10 min. The absorption of cytochrome c was measured at 550 nm and SOD activity was calculated based on the millimolar extinction coefficient of 18.5.

**Western blotting.**

Mitochondria were isolated by differential centrifugation as described above. Mitochondrial protein (50 µg) was suspended in Laemlli buffer, heated at 95°C for 5 minutes, and resolved by electrophoresis using 12% SDS-PAGE. Samples were transferred to nitrocellulose and blotted with sheep anti-human Mn-SOD antibody (Oxis, Portland OR), followed by horseradish peroxidase conjugated anti-sheep IgG secondary antibody. β-galactosidase was immunoblotted using a mouse anti-β-galactosidase antibody (Chemicon, Temecula CA) followed by horseradish peroxidase conjugated anti-mouse secondary antibody. Protein was visualized by autoradiography using ECL Western Detection Reagent (Amersham Life Science, England).
Catalase Activity

Catalase activity was measured in liver homogenate as described by Aebi et al. (34) with some modifications. Briefly, homogenate (10 µg) was adjusted to a final volume of 50 µL with phosphate buffer. The reaction was initiated by adding 3.0 mL of 12.5 mM H₂O₂ in phosphate buffer, and the change in absorbance at 240 nm was measured at 25°C for 1 min. Based on a millimolar extinction coefficient for H₂O₂ of 34.9, catalase activity was defined as micromoles of H₂O₂ consumed per min per mg protein.

Glutathione peroxidase activity

Glutathione peroxidase activity was determined by adding 10 µL of liver homogenate to 850 mL of buffer containing 0.1 mM NaPO₄, 4 mM reduced glutathione, 0.1 mM nicotinamide adenine dinucleotide phosphate (NADPH) and 2 U of glutathione reductase (35). After 30 sec, 10 µl of 1.2 mM t-butyl-hydroperoxide was added to initiate the reaction. The rate of decrease in absorption of NADPH at 340 nm was measured and the amount of NADPH consumed was calculated using a millimolar extinction coefficient of 6.22.

RNase protection assay for TNFα.

Total RNA was isolated from whole liver using RNA STAT 60 (Tel-Test, Friendswood, TX). TNFα and the housekeeping gene GAPDH were detected by RNase protection assay using a mouse cytokine RNA probe template set (rCK-1, Pharmingen, San Diego, CA). Riboprobes were synthesized in the presence of [³²P]UTP to yield labeled antisense RNA probes, and RNase protection assays were performed on 20 µg of RNA using a RiboQuant™ multi-probe RNase Protection Assay Kit (Pharmingen). Protected samples were separated on 5% acrylamide-bisacrylamide (19:1) urea gels, dried, and exposed to X-Ray film.

Statistics.

Data are presented as means ± SEM. Results were compared using analysis of variance (ANOVA) followed by Student-Neuman-Keuls post-hoc test as appropriate. For comparison of pathology scores, the Mann-Whitney rank sum test was used. A p value of < 0.05 was selected prior to the study as the level of significance.
RESULTS

Body and liver weight.

A tendency for weight loss during the one week recovery time after surgery was observed in all groups (data was not shown). Body weight increased steadily after feeding diets for 4 weeks, and there were no significant differences among the groups at sacrifice (Table 1). However, liver to body weight ratios were increased 50 - 60 % by ethanol in both Ad.lacZ and Ad.SOD2 infected animals as expected (Table 1).

Urine alcohol concentration.

In animals infected with Ad.lacZ and Ad.SOD2, alcohol levels fluctuated in a cyclic pattern with a periodicity of 5 to 6 days even though ethanol was infused continuously as reported previously (2) The cyclical pattern was recently described to be due to fluctuations in body temperature and thyroid hormone levels which alter ethanol metabolism (36). There were no significant differences in mean urine alcohol concentrations between Ad.lacZ– and Ad.SOD2-infected rats (Table 1).

Western blotting and activity of SOD in mitochondria.

To test the hypothesis that adenoviral gene delivery of Mn-SOD resulted in localized expression, animals were infected with adenovirus (1 x 10^9 plaque forming units) containing the transgenes for human mitochondrial Mn-SOD (Ad.SOD2) or the bacterial reporter gene β-galactosidase (Ad.lacZ) as control. Livers were harvested at the end of ethanol treatment, subcellular compartments (i.e., mitochondria and cytosol) were isolated by differential centrifugation and transgene expression was determined by Western analysis using antibodies against human Mn-SOD or β-galactosidase (Figure 1A). β-galactosidase was detected in the cytosol of Ad.lacZ infected animals as expected. Moreover, recombinant Mn-SOD was primarily expressed in the mitochondrial compartment of the liver and was nearly 4-fold higher than the expression of endogenous Mn-SOD. A small amount of Mn-SOD was detected in the cytosol, but this is most likely due to either mitochondrial destruction during the isolation procedure or to unprocessed protein that had not been transported into the mitochondria. Enzymatic activity was also evaluated to ensure that
recombinant Mn-SOD had functional activity. The activity of SOD in whole liver extracts of Ad.SOD2 infected rats was increased about 2-fold compared to Ad.lacZ infected rats (Figure 1B).

**Serum ALT and Histology.**

Serum ALT levels in rats fed high-fat control diet were around 20 U/L after 4 weeks (Figure 2). Ethanol treatment increased values in Ad.lacZ transfected rats about 5-fold but only caused a 2-fold increase in Ad.SOD2 infected animals.

Figures 3 and 4 show representative photomicrographs and pathology scores of livers after 4 weeks of high-fat control diet with or without ethanol. There were no pathological changes in Ad.lacZ or Ad.SOD2-infected rats fed high-fat control diet, except for very mild steatosis (Figure 3A). In contrast, severe steatosis, inflammation and necrosis were observed in Ad.lacZ infected rats after ethanol treatment yielding a total pathology score of 7.8 ± 0.2 (Figure 4). High magnification photomicrographs of Ad.lacZ infected animals fed ethanol are also shown to illustrate focal infiltrate consisting largely of neutrophils and lymphocytes, necrosis and apoptosis (Figure 3B). In Ad.SOD2-infected animals, steatosis, inflammation and necrosis were blunted by nearly 50%.

**Glutathione, glutathione peroxidase, and catalase levels.**

Total glutathione levels of whole liver and mitochondria in Ad.lacZ transfected rats were decreased significantly by ethanol treatment (Table 2), confirming earlier work done with virus-free animals (17,18). In contrast, glutathione levels in Ad-SOD2 infected rats were maintained near control levels. The ratio of reduced glutathione to oxidized glutathione (GSH/GSSG) was also decreased by ethanol in Ad.lacZ infected animals but not in Ad.SOD2 infected rats (Table 2).

Since ethanol inhibits GSH transport into mitochondria (37), one possible mechanism for the protection against mitochondrial GSH depletion is that overexpression of Mn-SOD inhibits the effect of ethanol on GSH transport. To test this hypothesis, hepatocytes from untreated rats were isolated and infected with either Ad.lacZ (100 infectious units/cell) or Ad.SOD2 as described in Materials and Methods. Cells were then incubated in the presence of ethanol (2-200 mM) or saline. After 12 hours, cells were harvested
and mitochondrial glutathione levels were measured. Ethanol (200 mM) caused 78 ± 12 % depletion of mitochondrial GSH in Ad.lacZ-infected animals compared to hepatocytes incubated with vehicle alone (*, p <0.05, four individual experiments). These findings are consistent with previously published work where high concentration of ethanol (<100 mM) caused a similar loss of mitochondrial GSH (38,39). In Ad.SOD2 infected rats, mitochondrial GSH levels were depleted to a similar extent by ethanol.

Glutathione peroxidase and catalase activity was measured in whole liver extracts from Ad.lacZ and Ad.SOD2 infected rats fed either high-fat control diet or diet containing ethanol for four weeks (Table 2). Glutathione peroxidase activity was similar in all treatment groups. In contrast, catalase activity was increased significantly by ethanol; however, because of its high activity, this change probably has no physiological significance. Interestingly, overexpression of mitochondrial Mn-SOD had no effect on the increase in catalase due to ethanol. Importantly, these findings are consistent with other reports (40,41).

**Radical adducts in bile.**

Free radical adducts were detected by electron spin resonance spectroscopy in the bile of rats (Figure 5 and 6). Ethanol treatment in Ad.lacZ infected animals for 4 weeks resulted in nearly an 8-fold increase in radical adduct intensity. This increase was blunted by about 65 % in animals infected with Ad.SOD2. Computer simulation identified the α-hydroxyethyl radical ($\alpha^N = 15.69$ G and $\alpha_H^B = 2.72$ G) similar to the adduct described previously (12). Using $^{13}$C-labeled ethanol, it was determined that nearly 60% of the total adduct was hydroxyethyl. However, lipid-derived radical adducts could not be distinguished from α-hydroxyethyl radical adducts under these conditions.

**mRNA level of TNF$\alpha$.**

Since TNF$\alpha$ is critically involved in early alcohol-induced liver injury (9) and it stimulates mitochondrial oxidative stress (42-44), TNF$\alpha$ and IL-1 mRNA levels in animals treated with ethanol were evaluated by RNase protection assay (Figure 7). TNF$\alpha$ mRNA was increased about 3-fold in Ad.lacZ infected rats after 4 weeks of ethanol diet, compared to control animals which received high-fat control diet. Treatment with Ad.SOD2 did not significantly alter ethanol-induced increases in TNF$\alpha$ mRNA levels.
Neutrophil infiltration

Ethanol causes a significant increase of inflammatory cell influx in liver. Indeed, it was demonstrated above that ethanol caused a significant increase in inflammation which was blunted by ~50% in Ad.SOD infected animals. However, neutrophil infiltration was increased greater than 2-fold by ethanol and was not affected by overexpression of Mn-SOD (Figure 8A). To verify this finding, myeloperoxidase (MPO) activity, an enzyme expressed predominantly in neutrophils, was determined (Figure 8B). Indeed, ethanol caused a significant and similar increase in MPO activity in both Ad.lacZ and Ad.SOD2 infected animals, confirming the histological data.

Ethanol-induced Apoptosis.

Apoptosis was identified from condensed and fragmented nuclei in hematoxylin and eosin stained sections. Apoptosis was increased about 4-fold after four weeks of ethanol exposure in Ad.lacZ infected liver compared to tissue from high-fat control animals (Figure 9A). Delivery of Ad.SOD2 blunted ethanol-induced apoptosis by greater than 60%. In addition, caspase-3 activity was measured in liver extracts from Ad.lacZ and Ad.SOD2-infected animals after three weeks of control or ethanol-containing diet (Figure 9B). Caspase activity determined by cleavage of the chromogenic substrate DEVD-pNA was increased nearly 50% due to ethanol in Ad.lacZ-treated animals; however, this effect was largely blunted in Ad.SOD2 infected animals, suggesting that Mn-SOD overexpression indeed protects against ethanol-induced apoptosis in hepatocytes.
DISCUSSION

Delivery of mitochondrial SOD to the liver

In normal liver, mitochondria efficiently reduce oxidants under normal conditions predominantly via antioxidant mechanisms including glutathione and superoxide dismutase (45). Recently, it was shown that deletion of mitochondrial SOD by about 50% results in a functional decline of oxidative phosphorylation, an increase in oxidative stress, and increased rates of apoptosis in an age-dependent manner (46), and depletion of mitochondrial GSH (47), suggesting that Mn-SOD is important for balance of mitochondrial redox state. Moreover, these data suggest that minor changes in Mn-SOD may have a significant impact on antioxidant status of mitochondria and support the hypothesis that overexpression of Mn-SOD may be protective against mitochondrial oxidative stress. On the other hand, a recent report showed that homozygous mutations in the SOD2 gene which may lead to an increase in mitochondrial localization of SOD is a risk for severe alcoholic liver disease in humans (20), which is in contrast to the hypothesis proposed here. Whether or not these mutations are causal is very difficult to conclude since little biochemical evidence has been presented. However, these correlation studies indeed introduce important questions about the relationship of Mn-SOD levels to early ethanol-induced liver disease. Despite a number of other studies (17,48,49), the roles of Mn-SOD and mitochondrial oxidative stress in alcoholic liver disease are still unclear. Here, delivery of adenovirus containing the transgene for human Mn-SOD resulted in a significant increase in SOD almost exclusively in the mitochondrial compartment of liver (Figure 1A). Importantly, overexpression of Mn-SOD by about 3-fold (Figure 1B) nearly completely blocked ethanol-induced liver injury, suggesting that mitochondrial oxidative stress is involved in the mechanism of injury.

Mitochondrial oxidative stress plays a role in alcohol-induced liver injury

Ethanol is known to deplete mitochondrial glutathione levels (38,17,18,50) consistent with the hypothesis that ethanol causes oxidative stress in the mitochondria. Ethanol inhibits the mitochondrial glutathione transporter, which shuttles GSH into the mitochondria from the cytosol where it is generated (50,51). There are a number of possible mechanisms by which ethanol generate oxidants in mitochondria.
It has been shown that ethanol-induced TNFα increases mitochondrial oxidant production by inhibiting complex II in the electron transport chain leading to the reduction of oxygen (42,43,52). Moreover, ethanol, through activation of Kupffer cells, increases respiration via prostaglandin E2 which may exacerbate oxidant generation in mitochondria (53,54). Indeed, ethanol feeding decreased mitochondrial glutathione in this study (Table 2). Depletion of mitochondrial GSH in vivo could occur by elimination of mitochondrial oxidants through glutathione peroxidase at the expense of GSH or by inhibition of glutathione transport from the cytosol. When mitochondrial superoxide dismutase (Mn-SOD) was elevated nearly three-fold by gene delivery using adenovirus, chronic ethanol did not reduce glutathione levels (Table 2), suggesting that overexpression of Mn-SOD prevents oxidant production in vivo. It was also demonstrated here that overexpression of Mn-SOD in isolated hepatocytes did not influence depletion of mitochondrial GSH (Table 2). Why Mn-SOD protected against the depletion of GSH in vivo but not in isolated hepatocytes is not completely understood; however, this apparent paradox is most likely due to the differences in ethanol concentrations between the in vivo and in vitro experiment and/or factors that promote mitochondrial oxidant production by increasing respiration or uncoupling electron transport (e.g., TNFα and PGE2). Importantly, these data may also suggest that the defect in GSH transport during chronic ethanol exposure is a consequence of mitochondrial oxidative stress. Mitochondrial oxidative stress would lead to both GSH depletion and impaired transport and then further GSH depletion and oxidative stress. Thus, SOD2 would prevent mitochondrial GSH depletion in vivo by preventing mitochondrial oxidative stress under these conditions.

Direct evidence that Mn-SOD blunted oxidative stress comes from the fact that the increase in ESR-detectable free radical adducts due to ethanol were also blunted significantly by overexpression of Mn-SOD (Figures 5 and 6), which also implicates mitochondria as a critical source of oxidants in early ethanol-induced liver injury. It is known that oxidant generation from mitochondria is an important factor in triggering apoptosis (55). Indeed, overexpression of Mn-SOD blunted both radical adduct formation as well
as the increase in apoptosis due to ethanol (Figure 9). These data strongly support the hypothesis that mitochondrial redox status is an important factor in the pathogenesis of early alcoholic liver disease.

An interesting point is that Mn-SOD converts superoxide to H$_2$O$_2$, which is eliminated primarily by GSH and catalase. Since mitochondrial GSH is not depleted in Ad.SOD2 infected animals after ethanol exposure, it is possible that catalase is important in reducing H$_2$O$_2$ levels under these conditions. Cederbaum et al. recently showed that overexpression of catalase in either mitochondria or cytosol was equally protective against oxidants generated in mitochondria by rotenone or antimycin A (56). These data suggest that H$_2$O$_2$ readily diffuses into the cytosol most likely down a concentration gradient to catalase which is extremely abundant and highly active in hepatocytes. Moreover, others have shown (40), (41) and it is also demonstrated here that catalase activity is increased due to chronic ethanol (Table 2), supporting the conclusion that catalase levels are sufficient to metabolize H$_2$O$_2$ in liver.

**Mitochondrial oxidative stress occurs subsequent to TNFα production**

The role of TNFα in early alcohol-induced liver injury is well established (57). Recently, it was shown that mice deficient in TNFα receptors were resistant to ethanol-induced liver injury (9). Since Kupffer cells are the major source of TNFα in liver, it is hypothesized that Kupffer cells are activated by gut-derived endotoxin to produce TNFα as well as other inflammatory cytokines and mediators such as prostaglandins. TNFα induces the expression of adhesion molecules (e.g., ICAM-1) which is involved in neutrophil adhesion in the liver (58). In this study, ethanol increased mRNA levels of TNFα in both Ad.lacZ and Ad.SOD2 infected animals to the same extent (Figure 7). This most likely explains the same degree of neutrophil infiltration observed in livers from these animals (Figure 8) and is consistent with the hypothesis that Mn-SOD acts subsequent to Kupffer cell production of TNFα and recruitment of neutrophils.

As mentioned above, TNFα is also important in the production of radicals in hepatocyte mitochondria (42,43,59). Since TNFα levels and neutrophil infiltration are similar in Ad.lacZ and Ad.SOD2 infected animals but ethanol-induced free radical adduct formation and depletion of glutathione is minimized
by Ad.SOD2 *in vivo*, it is hypothesized that mitochondrial oxidative stress occurs as a result of TNFα signaling. Moreover, it is hypothesized that these downstream events of mitochondrial oxidative stress are critical to alcohol-induced parenchymal cell death. This hypothesis could explain why apoptosis induced by ethanol as a result of TNFα was blunted in Ad.SOD2 infected animals since mitochondria are involved in the mechanism of this process (55). The injury due to ethanol, however, is due largely to necrosis. This is reflected in pathology where most of the damage in the liver is primarily necrotic cell death with apoptosis contributing only slightly (~ 1% of the cells). Interestingly, Mn-SOD overexpression blunted both mechanisms of cell death suggesting indeed that mitochondrial oxidative stress is critically involved.

In conclusion, mitochondria have a pivotal role in the mechanism in alcohol-induced liver injury. Hepatocyte mitochondria most likely generate oxidants in response to Kupffer cell TNFα and prostaglandin production, leading to cell death. Overexpression of Mn-SOD by gene delivery is effective against hepatocyte mitochondrial oxidative stress and maintenance of mitochondrial GSH and is protective against alcohol-induced liver injury. Thus, preventing the accumulation of oxidants in mitochondria may be an important strategy to reduce ethanol-induced liver injury.
REFERENCES


FIGURE LEGENDS

Figure 1. Effect of ethanol on Mn-SOD expression and activity.

(A) Mn-SOD protein expression in cytosol (cyto) and mitochondria (mito) isolated by homogenation and differential centrifugation of livers of animals infected with Ad.lacZ or Ad.SOD2 was evaluated by Western blotting as described in Materials and Methods. Crude extract from human embryonic kidney (HEK) cells transduced with Ad.SOD2 (100 plaque forming units / cell) was used as a positive control. Data are representative of three individual experiments. (B) SOD activity in cytosol and mitochondria from fresh liver tissue was measured as described in Materials and Methods. Data are presented as means ± SEM (n = 6). #, p < 0.05 using ANOVA and Student-Newman-Keuls post-hoc test.

Figure 2. Effect of Ad.SOD2 on ethanol-induced increases in serum ALT.

Rats were infected with recombinant adenovirus encoding Ad.lacZ and Ad.SOD2. Blood samples were collected from the abdominal aorta at sacrifice after 4 weeks feeding of high-fat control diet (CON) or diet containing ethanol (EtOH) as described in Materials and Methods. Data are presented as means ± SEM (n = 6). #, p < 0.05 as compared with rats fed high-fat control diet; *, p < 0.05 as compared with Ad.lacZ infected rats given ethanol using ANOVA and Student-Newman-Keuls post-hoc test.

Figure 3. Representative photomicrographs of livers from Ad.lacZ and Ad.SOD2 infected animals after chronic ethanol.

(A) Photomicrographs are of hematoxylin and eosin-stained liver sections from Ad.lacZ or Ad.SOD2-infected animals fed either a high-fat control diet or diet containing ethanol for 4 weeks (original magnification 100×, H&E stain). (B) Representative photomicrographs with higher magnification demonstrating typical inflammation, necrosis and apoptosis (pointed out by white arrows) in livers from Ad.lacZ infected rats given ethanol (original magnification 400×, H&E stain).

Figure 4. Effect of Ad.lacZ or Ad.SOD2 on pathology score. Pathology was scored as described in Materials and Methods. Data are presented as means ± SEM. #, p < 0.05 compared with rats fed high-fat
control diet and *, $p < 0.05$ compared with Ad.lacZ infected rats given ethanol using the Mann-Whitney rank sum test.

**Figure 5. Representative electron spin resonance (ESR) spectra.** After four weeks of feeding high-fat control diet or ethanol-containing diet, $\alpha$-(4-pyridyl-1-oxide)-$N$-tert-butyl nitrone (1 g/kg, i.v.) was administered to rats infected with Ad-lacZ and Ad-SOD2, then bile was collected into 5 mM deferoxamine mesylate. ESR spectra of $^{13}$C ethanol was obtained by administration of $^{13}$C ethanol through the feeding tube after the breath ethanol level reached undetectable levels. Data are representative of five individual experiments.

**Figure 6. Effect of Ad.SOD2 on ethanol-induced $\alpha$-hydroxyethyl radical adduct formation.** After four weeks of feeding high-fat control diet (CON) or ethanol-containing diet (EtOH) in rats infected with Ad.lacZ and Ad.SOD2, free radical adducts were measured as described in Figure 5. The amplitude of the first peak on the ESR radical signal in each spectra was used for comparison of intensity. Data are presented as means ± SEM. #, $p < 0.05$ compared with rats fed high-fat control diet; *, $p < 0.05$ as compared with Ad-lacZ infected rats given ethanol using ANOVA and Student-Newman-Keuls post-hoc test.

**Figure 7. Effect of Ad.SOD2 and ethanol on expression of TNF$\alpha$ in liver.**

Total mRNA was prepared from livers of Ad.lacZ and Ad.SOD2 infected animals four weeks after feeding high-fat control (CON) or ethanol-containing diet (EtOH). TNF$\alpha$ mRNA was measured by RNase protection assay as described in Materials and Methods. mRNA levels of TNF$\alpha$, IL-1 and IL-6 are expressed relative to the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and L32.

**Figure 8. Effect of Ad.SOD2 on neutrophils in the liver.** (A) Infiltrating neutrophils were expressed as number of neutrophils per 100 hepatocytes. Values were determined by counting polymorphonuclear cells in 5 high power fields ($\times400$) per slide followed by counting the number of hepatocytes in each field. (B) Whole liver extract was evaluated for myeloperoxidase (MPO) activity as described in the Materials and
Methods. Data are expressed as units per mg protein and presented as means ± SEM. #, p < 0.05 compared with rats fed high-fat control diet using ANOVA and Student-Newman-Keuls post-hoc test.

**Figure 9. Effect of Ad.SOD2 on apoptosis.** After four weeks of feeding high-fat control diet (CON) or ethanol-containing diet (EtOH) in rats infected with Ad.lacZ and Ad.SOD2, apoptosis was evaluated as described in Materials and Methods. Two thousands hepatocytes were counted and the percentage of hepatocytes exhibiting apoptosis (i.e., nuclear fragmentation and condensation) was determined. (B) Whole liver extracts were evaluated for caspase-3 activity as described in the Materials and Methods. Data are expressed as units per mg protein and presented as means ± SEM. #, p < 0.05 compared with rats fed high-fat control diet; *, p < 0.05 compared with Ad.lacZ infected rats given ethanol using ANOVA and Student-Newman-Keuls post-hoc test.

**Scheme 1. Working hypothesis.**

(A) Kupffer cells are activated by gut-derived endotoxin and release inflammatory cytokines such as TNFα. TNFα causes an increase in neutrophil infiltration in liver most likely via up-regulation of important adhesion molecules such as ICAM-1 on endothelial cells. TNFα also stimulates mitochondrial oxidant production in hepatocytes through an increase in respiration as well as an inhibition of electron transport. It is proposed that mitochondrial oxidant production is a critical point leading to injury and cell death. (B) Overexpression of SOD in mitochondria prevents oxidative stress and protects hepatocytes from ethanol-induced injury.
Table 1: Effect of ethanol on body and liver weight and urine alcohol concentration.

<table>
<thead>
<tr>
<th>Virus Treatment</th>
<th>Diet</th>
<th>Initial body weight (g)</th>
<th>Body weight at 4 weeks (g)</th>
<th>Liver to Body weight ratio (%)</th>
<th>UAC (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad.lacZ</td>
<td>High-fat</td>
<td>282 ± 8</td>
<td>385 ± 15</td>
<td>3.3 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>Ad.lacZ</td>
<td>Ethanol</td>
<td>294 ± 7</td>
<td>366 ± 21</td>
<td>4.8 ± 0.3*</td>
<td>278 ± 14</td>
</tr>
<tr>
<td>Ad.SOD2</td>
<td>High-fat</td>
<td>295 ± 15</td>
<td>375 ± 5</td>
<td>3.0 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Ad.SOD2</td>
<td>Ethanol</td>
<td>293 ± 7</td>
<td>380 ± 15</td>
<td>4.7 ± 0.3*</td>
<td>263 ± 23</td>
</tr>
</tbody>
</table>

Rats were infected with recombinant adenovirus encoding the transgene β-galactosidase (Ad.lacZ) or Mn-SOD (Ad.SOD2) and fed high-fat control diet with or without ethanol for 4 weeks. Urine alcohol concentrations (UAC) were determined as described in Materials and Methods. Data are presented as means ± SEM (n = 6). *, p < 0.05 compared with appropriate group of rats fed high-fat diet without ethanol using ANOVA and Student-Newman-Keuls post-hoc test.
Table 2: Effect of Ad.SOD2 and ethanol on glutathione in whole liver and mitochondria.

<table>
<thead>
<tr>
<th></th>
<th>Ad. lacZ</th>
<th>Ad.SOD2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High-fat control</td>
<td>Ethanol</td>
</tr>
<tr>
<td><strong>Total Glutathione</strong>&lt;br&gt;Whole liver (nmol/ mg)</td>
<td>125 ± 6</td>
<td>92 ± 9&lt;sup&gt;#&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>16.0 ± 1.5</td>
<td>7.3 ± 1.2&lt;sup&gt;#&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>GSH/ GSSG</strong>&lt;br&gt;Mitochondria (nmol/ mg)</td>
<td>9.6 ± 0.7</td>
<td>4.8 ± 1.3&lt;sup&gt;#&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Catalase activity</strong>&lt;br&gt;(units/ mg)</td>
<td>2.9 ± 0.4</td>
<td>4.5 ± 0.6&lt;sup&gt;#&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>GSH. Peroxidase</strong>&lt;br&gt;(units/ mg)</td>
<td>1.6 ± 0.1</td>
<td>1.3 ± 0.3</td>
</tr>
</tbody>
</table>

Glutathione, glutathione peroxidase and catalase activity was assayed using fresh liver tissue after continuous feeding of high-fat control diet with or without ethanol for 4 weeks as described in Materials and Methods. Data are presented as means ± SEM. #, p < 0.05 compared with rats fed high-fat diet and with Ad.SOD2 infected rats given ethanol using ANOVA followed by Student-Newman-Keuls post-hoc test.
Figure 1
Figure 2

ALT (U/L)

<table>
<thead>
<tr>
<th></th>
<th>Ad.lacZ</th>
<th>Ad.SOD2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con</td>
<td><img src="chart" alt="" /></td>
<td><img src="chart" alt="" /></td>
</tr>
<tr>
<td>EtOH</td>
<td><img src="chart" alt="" /></td>
<td><img src="chart" alt="" /></td>
</tr>
</tbody>
</table>

* p < 0.05

# p < 0.01
Figure 3, Nakagami, et al
Figure 4

Steatosis

Inflammation

Necrosis

Total

Pathology Score

Legend:
- Con
- EtOH
- Ad.lacZ
- Ad.SOD2

Legend for pathologies:
- #
- # *

Legend for total score:
- #
- # *
Figure 5
Figure 6
Figure 7
Infiltration of Neutrophils

(Number/100 Hepatocytes)

A

MPO Activity

(U/mg protein)

B

<table>
<thead>
<tr>
<th></th>
<th>Con</th>
<th>EtOH</th>
<th>Con</th>
<th>EtOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad.lacZ</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ad.SOD2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 8
Figure 9

A. Apoptotic Nuclei (% of hepatocytes)

B. Caspase-3 Activity (% of control)

- Con
- EtOH
- Ad.lacZ
- Ad.SOD2

* and # symbols indicate statistical significance compared to control and other groups, respectively.
Overexpression of manganese superoxide dismutase prevents alcohol-induced liver injury in the rat
Michael D. Wheeler, Mikio Nakagami, Blair U. Bradford, Takehiko Uesugi, Ronald P. Mason, Henry D. Connor, Anna Dikalova, Maria Kadiiska and Ronald G. Thurman

J. Biol. Chem. published online July 26, 2001

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