

# Role of CYP2E1 in Ethanol-Induced Oxidant Stress, Fatty Liver and Hepatotoxicity

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## Key Words

CYP2E1 · Chronic ethanol administration · Fatty liver · Oxidative stress · Hepatotoxicity

## Abstract

**Background/Aims:** Several pathways contribute to mechanisms by which ethanol induces oxidant stress. While some studies support a role for cytochrome P450 2E1 (CYP2E1), others do not. There is a need to develop oral models of significant ethanol-induced liver injury and to evaluate the possible role of CYP2E1 in ethanol actions in such models. **Methods:** We evaluated chronic ethanol-induced liver injury, steatosis and oxidant stress in wild-type (WT) mice, CYP2E1 knockout (KO) mice and in humanized CYP2E1 knockin (KI) mice, where the human 2E1 was added back to mice deficient in the mouse 2E1. WT mice and CYP2E1 KO and KI mice (both provided by Dr. F. Gonzalez, NCI) were fed a high-fat Lieber-DeCarli liquid diet for 3 weeks; pair-fed controls received dextrose. **Results:** Ethanol produced fatty liver and oxidant stress in WT mice, but liver injury (transaminases, histopathology) was minimal. Ethanol-induced steatosis and oxidant stress were blunted in the KO mice (no liver injury) but restored in the KI mice. Significant liver injury was produced in the ethanol-fed KI mice with elevated transaminases and necrosis. This liver injury in the KI mice was associated with elevated oxidant stress and elevated levels of the human CYP2E1 compared to levels of the mouse 2E1 in WT mice. Activation of JNK was observed in the ethanol-fed KI mice compared to the other groups. Fatty liver in WT and KI mice was associated with lower levels of lipolytic PPAR- $\alpha$ . No

such changes were found in the ethanol-fed KO mice. **Conclusions:** These results show that CYP2E1 plays a major role in ethanol-induced fatty liver and oxidant stress. Restoring CYP2E1 in the CYP2E1 KO mice restores ethanol-induced fatty liver and oxidant stress. Copyright © 2011 S. Karger AG, Basel

## Introduction

The ability of acute and chronic ethanol treatment to increase production of reactive oxygen species and enhance peroxidation of lipids, proteins, and DNA has been demonstrated in a variety of systems, cells, and species, including humans. The mechanism(s) by which alcohol causes cell injury is (are) still not clear. A major mechanism that is a focus of considerable research is the role of lipid peroxidation and oxidative stress in alcohol toxicity. Many pathways have been suggested to play a key role on how ethanol induces 'oxidative stress' [1–4]. Our laboratory has focused on ethanol induction of CYP2E1 [3, 5–7]. It is likely that several, indeed many, systems contribute to the ability of ethanol to induce a state of oxidative stress.

CYP2E1 is of interest because of its ability to metabolize and activate many toxicologically important substrates including ethanol, carbon tetrachloride, acetaminophen, and N-nitrosodimethylamine to more toxic products [8–13]. Major interest in CYP2E1 reflects the ability of this enzyme to oxidize ethanol, to generate reactive products from ethanol oxidation, e.g. acetaldehyde

and the 1-hydroxyethyl radical, to activate various agents (CCl<sub>4</sub>, acetaminophen, benzene, halothane, halogenated alkanes, and alcohols) to reactive products, to generate reactive oxygen radical species and to be induced by ethanol. In the intragastric infusion model of ethanol administration, the ethanol-induced liver pathology has been shown to correlate with CYP2E1 levels and lipid peroxidation [14–18]. Inhibitors of CYP2E1 prevented the elevation of lipid peroxidation and the ethanol-induced liver pathology [19, 20]. How CYP2E1 may be mediating liver injury beyond oxidative stress is still not clear from these studies.

Understanding the biochemical and toxicological properties of CYP2E1 is important for many reasons, even besides its role in contributing to alcohol-induced liver injury since CYP2E1 is induced under a variety of pathophysiological conditions such as fasting, diabetes, obesity and high-fat diet [21–24] and by drugs [8, 10–13]. Non-alcohol-induced steatohepatitis (NASH) causes steatosis, liver cell injury, inflammation and variable necrosis. NASH is associated with obesity, type 2 diabetes and hyperlipidemia, conditions in which CYP2E1 is induced [25, 26].

Thurman and colleagues [27–29] have suggested that CYP2E1 may not play a role in alcohol liver injury based on studies with gadolinium chloride or CYP2E1 knockout (KO) mice or a broad-spectrum P450 inhibitor aminobenzotriazole. Induction by ethanol of other CYPs or of the reductase in the CYP2E1 KO mice might have served as alternative sources of oxidative stress, especially in the absence of CYP2E1 [30]. Clearly, further studies are necessary to resolve the above discrepancies. As mentioned earlier, it is likely that several mechanisms contribute to alcohol-induced liver injury, and that ethanol-induced oxidant stress is likely to arise from several sources, including CYP2E1, mitochondria and activated Kupffer cells. Bradford et al. [31] reported that CYP2E1, but not NADPH oxidase, is required for ethanol-induced oxidative DNA damage in rodent liver, and concluded that CYP2E1 is required for the induction of oxidative stress to DNA and may play a key role in ethanol-associated hepatocarcinogenesis.

Almost all the above studies assessing the role of CYP2E1 in alcoholic liver disease involve the intragastric infusion model where significant liver injury occurs [14–17, 27–29, 31]. Although much valuable information has been gained from this model, it is complex, requires special expertise, is stressful, and is not accessible to most researchers. There is a need to develop oral models of ethanol treatment which result in the production of signifi-

cant liver injury. Rodents administered Lieber-DeCarli alcohol-containing liquid diets [32] developed steatosis and oxidative stress in the liver, but significant liver injury beyond steatosis generally does not occur. A modified low-carbohydrate ethanol liquid diet caused mild liver injury in rats [33–36]. Nanji's group [37] recently reported the development of alcoholic liver disease in a voluntary feeding regimen consisting of fish oil and ethanol in female rats. In all these oral models, ALT levels were elevated about 2-fold by the chronic ethanol feeding. Although CYP2E1 was elevated by ethanol in all these models, whether CYP2E1 played a role in the elevated ALT and oxidative stress or the steatosis was not directly evaluated.

Fatty liver is a uniform and early response of the liver to ethanol consumption. Previously, fatty liver was considered benign, however, it is now known that fatty liver can increase sensitivity to hepatotoxins such as lipopolysaccharide (LPS) and high levels of fatty acids promote lipotoxicity [38–40]. Hence, there is a need to understand the mechanisms responsible for fatty liver production by ethanol. Early hypotheses for mechanisms responsible for fatty liver production by ethanol included redox state changes (elevated NADH) when ethanol was metabolized by alcohol dehydrogenase, elevated formation of acetyl-CoA from ethanol/acetaldehyde oxidation and impairment of  $\beta$ -oxidation of fatty acids [41]. Recent studies by Crabb, You and colleagues [42–45] have identified new mechanisms which regulate the synthesis and the oxidation of fatty acids as being central to how ethanol produces fatty liver. Ethanol has been shown to elevate levels of SREBP-1c, a master transcriptional regulator of lipogenic enzymes, but to lower levels of PPAR- $\alpha$ , a master transcriptional regulator of lipolytic enzymes. Decreased activation of AMPK by ethanol plays a role in these effects. Is there a role for CYP2E1 in ethanol-induced fatty liver? In the IG infusion model, Kono et al. [28] reported that after feeding ethanol for 4 weeks, there was no difference in ethanol-induced steatosis between CYP2E1 KO mice and wild-type (WT) mice. Similarly, in this model, Wan et al. [46] reported that ethanol infusion for 21 days promoted fat accumulation in CYP2E1 KO mice but not in the WT mice. No explanation or mechanism was provided for these unusual observations. In contrast, the CYP2E1 inhibitor chlormethiazole (CMZ) was reported to blunt ethanol-induced fatty liver in the IG model [20]. We are not aware of any studies providing direct evidence for or against a role for CYP2E1 in fatty liver produced in oral models of ethanol consumption. Studies described below were designed to further evaluate a pos-

sible role of CYP2E1 in ethanol-induced fatty liver and to assess downstream factors which may play a role in the molecular mechanisms by which CYP2E1 promotes development of fatty liver by ethanol.

## Methods

### *Animals and Ethanol Treatment*

SV129-background CYP2E1 KO [47] and humanized CYP2E1 transgenic (KI) mice [48, 49] were kindly provided by Dr. Frank J. Gonzalez (Laboratory of Metabolism, National Cancer Institute, Bethesda, Md., USA), and breeding colonies established at Mount Sinai. Female SV129 WT mice were purchased from Charles River Laboratory. All mice were housed in temperature-controlled animal facilities with 12-hour light/12-hour dark cycles and were permitted consumption of tap water and Purina standard chow ad libitum until being fed the liquid diets. The mice received humane care, and experiments were carried out according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals and with approval of the Mount Sinai Animal Care and Use Committee.

Female WT, KO, and KI mice were initially fed the control liquid dextrose for 3 days to acclimate them to the liquid diet. Afterward, the mice were fed either the liquid ethanol diet or the control liquid dextrose diet, as described by Lieber and DeCarli [50] for 3 weeks. The content of ethanol was gradually increased every 3–4 days from 10% (1.77% [v/v]) of total calories to 20% (3.54% [v/v]), 25% (4.42% [v/v]), 30% (5.31% [v/v]), and finally 35% of total calories (6.2% [v/v]). The control mice were pair-fed the control dextrose diet on an isoenergetic basis. The ethanol-fed mice had access to their rations ad libitum, and the conditions of WT, KO, and humanized transgenic mice were comparable. The amount of food consumed by CYP2E1 KO mice, KI mice and WT mice was approximately the same.

No mice died in any group after 3 weeks of feeding with the control or ethanol-containing diet. Before being sacrificed, the mice were fasted overnight and body weight was measured. Blood was collected and serum was separated. Serum ethanol levels were similar between the three groups of ethanol-fed mice at sacrifice. Whole liver was removed and liver weight measured; the liver was rapidly excised into fragments and washed with cold saline, and one aliquot of tissue was placed in 10% formalin solution for paraffin blocking. Another piece was placed in Tissue-Tek OCT compound, and was frozen and cut into 10- $\mu$ m frozen sections for Oil Red O staining and for immunohistochemistry assays. The remaining aliquots were stored at  $-80^{\circ}\text{C}$  for further assays.

### *Assays*

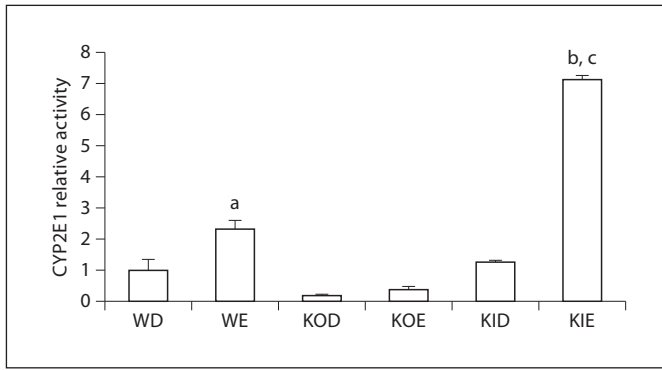
Liver sections were stained with HE for pathological evaluation. Steatosis was quantified as the percentage of cells containing fatty droplets. Necroinflammation was quantified as the number of clusters of  $\geq 5$  inflammatory cells/mm<sup>2</sup>. Five 200 $\times$  fields per liver were examined (one 200 $\times$  field area = 0.95 mm<sup>2</sup>). Serum ALT, AST, and ethanol levels, hepatic GSH, thiobarbituric acid reactive substances (TBARS), triglyceride and free fatty acid levels, immunohistochemistry, immunoblots, microsomal oxidation of *p*-nitrophenol (PNP) and other biochemical assays were carried out as previously described [51, 52].

## Results

SV129 WT mice, CYP2E1 KO mice and humanized CYP2E1 KI mice were fed the high-fat Lieber-DeCarli ethanol liquid diet over a 3-week period. Mice were pair-fed with dextrose controls. There was no change in body weight in any group over the 3-week feeding period. Because of concerns of extrapolating results with mouse enzymes to human enzymes and to overcome species differences in P450 expression, and specificity between rodents and humans, transgenic humanized mouse models expressing major P450 enzymes were developed by Cheung and Gonzalez [49]. The human P450 transgene was introduced onto the corresponding null mouse background [47] to create a humanized P450 mouse in the absence of the corresponding mouse P450 [48]. Therefore, functional activities and differences between human and mouse CYPs can be directly compared with this model. In the humanized CYP2E1 KI mouse [48], some differences were observed compared to the WT mice expressing mouse CYP2E1 such as rates of PNP (but not chlorzoxazone) oxidation and sensitivity to toxicity by acetaminophen. A major goal of the current report was to validate that any changes in fatty liver or oxidant stress produced by chronic ethanol feeding in CYP2E1 KO mice was indeed due to the deficiency in CYP2E1 by evaluating whether such actions by ethanol are restored when CYP2E1 is restored in the KO mice. Another important goal was to study whether human CYP2E1 could effectively mimic mouse CYP2E1 in contributing to ethanol-induced fatty liver, oxidant stress and liver injury and thus allow assessment of the human CYP2E1 to modulate actions of chronic ethanol administration in a whole animal model system.

### *Levels of CYP2E1*

Oxidation of PNP to *p*-nitrocatechol, a representative reflection of CYP2E1 catalytic activity, was increased about 2-fold in the ethanol-fed WT mice compared to dextrose-fed mice (fig. 1). As expected, rates of PNP oxidation were low in the CYP2E1 KO mice (the low rates are probably a reflection of PNP oxidation by other CYPs, e.g. CYP3A). Rates of PNP oxidation were highest in the ethanol-fed CYP2E1 KI mice, being about 7-fold higher than the dextrose-fed CYP2E1 KI mice and about 3-fold higher than the ethanol-fed WT mice (fig. 1). Immunoblot analysis showed the expected low levels of CYP2E1 in the KO mice, an approximate 2-fold increase in the level of CYP2E1 (CYP2E1/ $\beta$ -actin ratio) in ethanol-fed WT compared to dextrose-fed WT mice, and highest

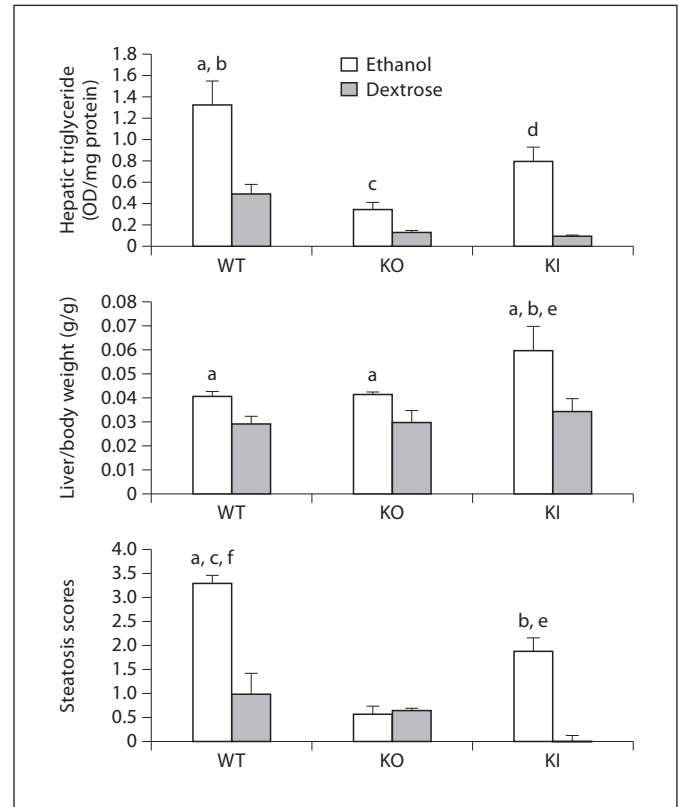


**Fig. 1.** Catalytic activity of CYP2E1. Activity was assayed from the oxidation of PNP by microsomes isolated from WT dextrose-fed (WD) and ethanol-fed (WE) mice, or from KO dextrose-fed (KOD) and KO ethanol-fed (KOE) mice or from KI dextrose-fed (KID) and KI ethanol-fed (KIE) mice. Results are from 3 to 6 pairs of mice in each group. <sup>a</sup>  $p < 0.01$  compared to WD; <sup>b</sup>  $p < 0.01$  compared to KID; <sup>c</sup>  $p < 0.01$  compared to WE.

levels of CYP2E1 in the ethanol-fed CYP2E1 KI mice (CYP2E1/ $\beta$ -actin ratio of 6). Immunohistochemistry paralleled the results on PNP oxidation and the immunoblots (not shown). The chronic ethanol feeding elevated CYP2E1 in WT mice, and more strikingly in KI mice, in the pericentral zone of the liver acinus. No CYP2E1 was detectable in the ethanol- or dextrose-fed KO mice.

#### Ethanol-Induced Fatty Liver

HE staining showed that ethanol produced fatty liver in the WT mice after the 3-week feeding period. This ethanol-induced steatosis was blunted in the CYP2E1 KO mice but at least partially restored in the CYP2E1 KI mice. Oil Red O staining showed that prominent lipid droplets were observed in frozen liver sections from WT mice fed ethanol whereas only a few lipid droplets were present in the ethanol-fed CYP2E1 KO mice. Oil Red O staining was restored in the CYP2E1 KI mice (data not shown). Triglyceride levels were elevated in livers from all ethanol-fed mice compared to dextrose-fed mice (fig. 2). Triglyceride levels were highest in the ethanol-fed WT mice, followed by the ethanol-fed CYP2E1 KI mice, and lowest in the ethanol-fed CYP2E1 KO mice (fig. 2). Levels of free fatty acids were also highest in livers from the ethanol-fed WT mice, lowest in the ethanol-fed CYP2E1 KO mice and intermediate in the ethanol-fed CYP2E1 KI mice. The ethanol feeding modestly elevated levels of free fatty acids in the WT and CYP2E1 KI mice but not in the CYP2E1 KO mice. The steatosis score confirmed that ethanol produced fatty liver in the WT and the KI mice



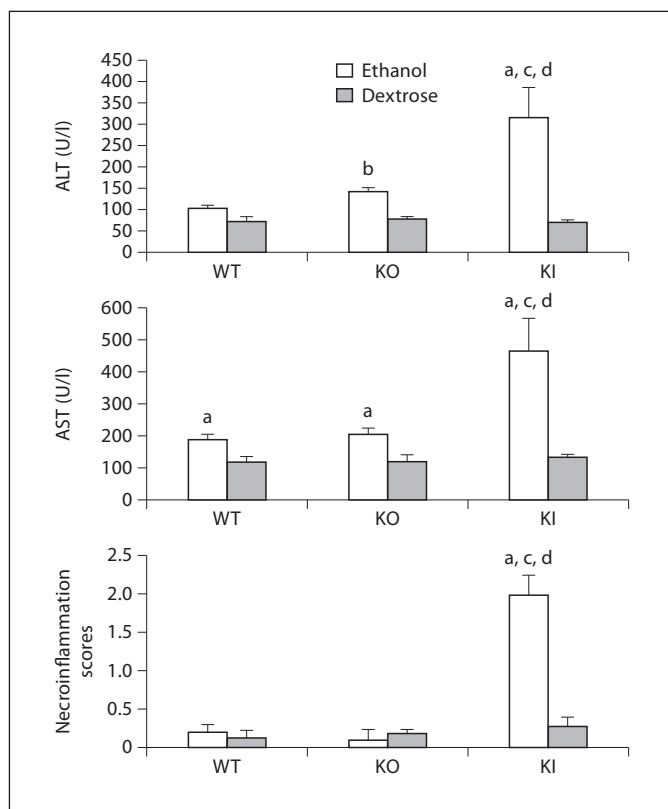
**Fig. 2.** Ethanol-induced steatosis. Hepatic triglycerides were assayed in livers from dextrose-fed and ethanol-fed WT, KO and KI mice. The steatosis score was derived in a blind fashion from the histopathology. <sup>a</sup>  $p < 0.01$  compared to dextrose-fed; <sup>b</sup>  $p < 0.01$  compared to ethanol-fed KO; <sup>c</sup>  $p < 0.05$  compared to dextrose-fed KO; <sup>d</sup>  $p < 0.05$  compared to ethanol-fed WT; <sup>e</sup>  $p < 0.05$  compared to ethanol-fed KO; <sup>f</sup>  $p < 0.05$  compared to ethanol-fed KI.

but not the KO mice (fig. 2). These results are supportive of a role of CYP2E1 in ethanol-induced steatosis.

#### Role of CYP2E1 in Ethanol-Induced Hepatotoxicity

Serum ALT and AST levels were increased 50–100% in the ethanol-fed WT and CYP2E1 KO mice but were elevated about 4-fold in the ethanol-fed CYP2E1 KI mice (fig. 3). HE staining (not shown) showed lipid droplets in the ethanol-fed WT mice compared to the dextrose-fed mice but no cellular necrosis or degeneration. No lipid droplets were found in the ethanol-fed CYP2E1 KO mice and pathology was normal. Steatosis reappeared in the ethanol-fed CYP2E1 KI mice; importantly, areas with cell swelling, focus necrosis and inflammatory infiltration were observed in the ethanol-fed KI mice. Necroinflammatory scores were elevated only for the ethanol-fed KI mice (fig. 3). We did not observe any increase in the activ-



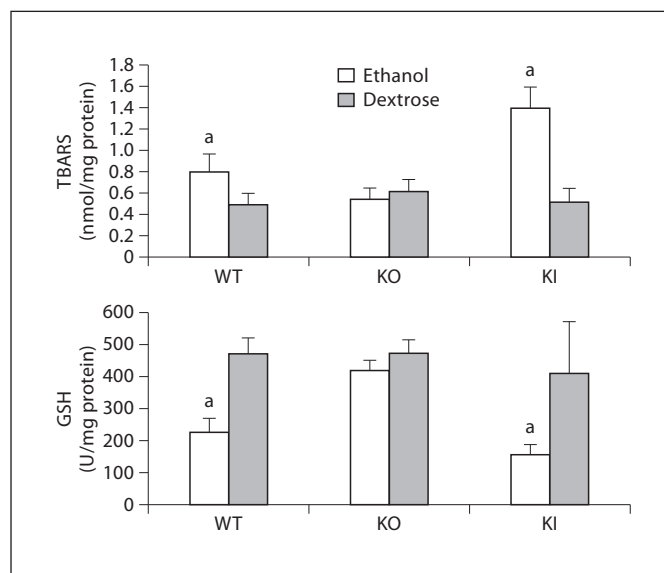


**Fig. 3.** Ethanol-induced hepatotoxicity. ALT and AST were assayed in the serum derived from dextrose- and ethanol-fed WT, KO and KI mice. <sup>a</sup>  $p < 0.05$ ; <sup>b</sup>  $p < 0.01$  compared to dextrose-fed mice; <sup>c</sup>  $p < 0.05$  compared to WT ethanol-fed mice; <sup>d</sup>  $p < 0.05$  compared to KO ethanol-fed mice.

ity of caspase 3 or caspase 9 in the ethanol-fed CYP2E1 KI mice compared to dextrose control nor was there a significant increase in TUNEL staining suggesting necrotic but not apoptotic liver injury was occurring in the ethanol-fed humanized CYP2E1 KI mice. Interestingly, while hepatotoxicity was highest in the CYP2E1 KI mice, fatty liver was highest in WT mice followed by the CYP2E1 KI mice.

#### Ethanol-Induced Oxidant Stress

CYP2E1 plays a central role in how ethanol produces oxidant stress in the liver. Chronic ethanol consumption by WT mice elevated hepatic TBARS levels (a reflection of lipid peroxidation) about 2-fold, whereas hepatic GSH levels were decreased about 50% (fig. 4). TBARS were not elevated nor was GSH depleted in the ethanol-fed CYP2E1 KO mice (fig. 4). The increase in lipid peroxidation was 'restored' in the ethanol-fed CYP2E1 KI mice

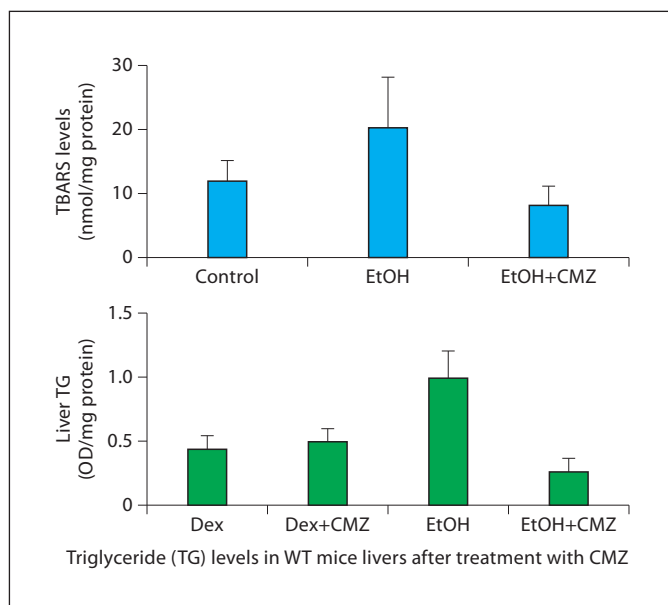


**Fig. 4.** Ethanol-induced oxidative stress. Levels of TBARS and glutathione (GSH) were assayed in liver lysates from dextrose- and ethanol-fed WT, KO and KI mice. Results are from 3 to 6 pairs of mice in each group. <sup>a</sup>  $p < 0.05$  compared to dextrose-fed mice.

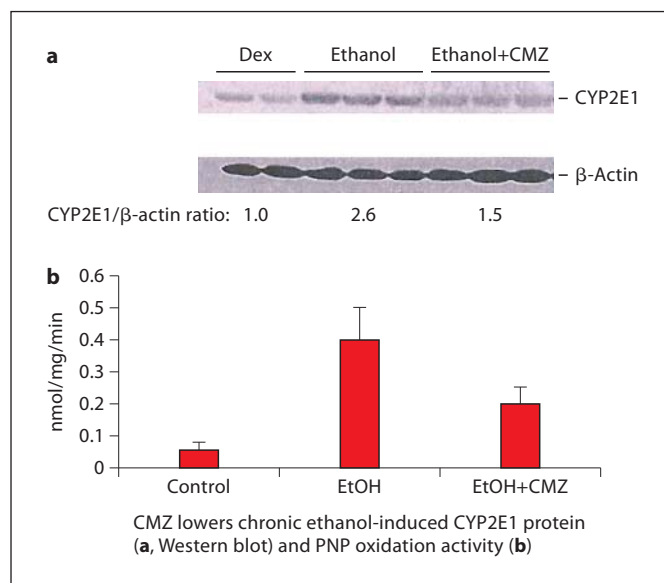
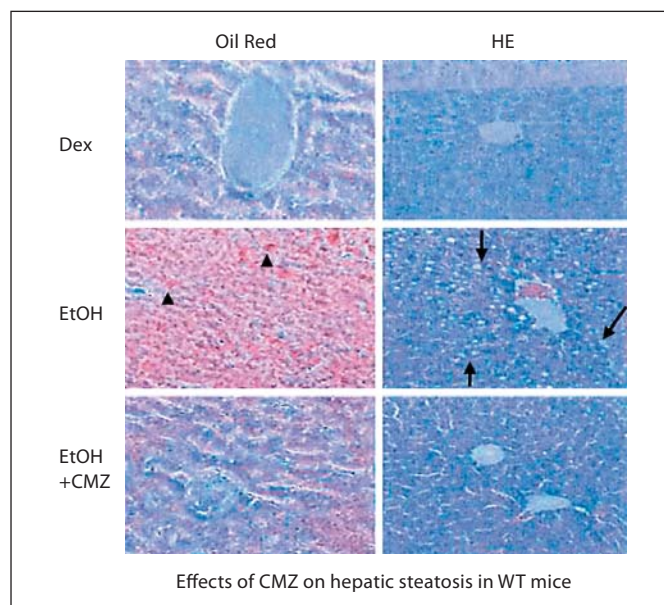
(2.8-fold increase in TBARS) as was the depletion of GSH (65% decrease) (fig. 4). The elevation of TBARS and the decline in GSH were greater in the CYP2E1 KI mice than the WT mice, which may reflect the elevated level of CYP2E1 in the ethanol-fed KI mice. Formation of 3-nitrotyrosine protein adducts is one assay for detection of peroxynitrite formation [28]. Low levels of 3-nitrotyrosine (3-NT) staining were observed in livers from the ethanol-fed WT and KO mice, but a more intense staining was observed in livers from the ethanol-fed KI mice. 4-Hydroxynonenal (4-HNE) protein adducts as detected by immunohistochemistry were elevated in WT mice and to a greater extent in KI mice as compared to dextrose controls. Thus, levels of oxidant stress induced by ethanol appeared to follow a similar pattern of induction of CYP2E1 by ethanol, being highest in the CYP2E1 KI mice, intermediate in the WT mice, and lowest in the CYP2E1 KO mice. We believe that exacerbation of ethanol-induced oxidant stress plays a key role in the hepatotoxicity and contributes, at least in part, to the steatosis found in the ethanol-fed CYP2E1 KI mice.

#### Ethanol Activates JNK in WT and CYP2E1 KI Mice but Not CYP2E1 KO Mice

Activation of JNK has been shown to be important in many models of hepatotoxicity [53–57] including



**Fig. 5.** Effect of CMZ, a CYP2E1 inhibitor, on ethanol-induced oxidant stress, steatosis and CYP2E1. Levels of TBARS, liver triglycerides and CYP2E1, CYP2E1 catalytic activity and fatty liver formation (Oil Red O staining, HE) were assayed in WT mice fed ethanol for 2 weeks in the absence or presence of CMZ.



CYP2E1 potentiation of LPS or TNF- $\alpha$  liver injury [58–61]. There are few studies evaluating the role of JNK, if any, in ethanol-induced hepatotoxicity and steatosis. JNK2 (54 kDa) was elevated 50% (0.6–0.9 pJNK2/JNK2 ratio) in WT mice chronically fed ethanol as compared to pair-fed controls. This activation of JNK2 did not occur in the CYP2E1 KO mice fed ethanol. However, activation of JNK2 was restored, and even magnified (3-fold) in the CYP2E1 KI mice fed ethanol. JNK 1 (46 kDa), which was not activated in the ethanol-fed WT or CYP2E1 KO mice, was activated 5-fold in the ethanol-fed CYP2E1 KI mice.

While preliminary, the significance of this activation of JNK2 or of JNK 1 in ethanol hepatotoxicity or hepatic fatty liver, if any, will be an important goal of future studies. We did not observe any activation of p38 MAP kinase by ethanol in any group.

#### *Effect of CMZ on Ethanol-Induced Fatty Liver, CYP2E1 and Oxidant Stress*

The CYP2E1 inhibitor CMZ was used to evaluate the role of CYP2E1 in ethanol-induced fatty liver and oxidant stress. SV129 WT mice were fed the dextrose or ethanol

Lieber-DeCarli diet for 2 weeks. CMZ (Sigma, 50 mg/kg i.p.) was injected every other day over the 2-week experiment; controls were injected with saline. Moderate steatosis was found after 2 weeks of ethanol feeding as detected by HE or Oil Red O staining (fig. 5). Liver triglycerides were elevated about 2-fold (fig. 5). CMZ treatment lowered the ethanol-induced steatosis and decreased triglyceride levels. CMZ treatment lowered the elevated levels of CYP2E1 and the increased CYP2E1 catalyzed oxidation of PNP produced by the ethanol feeding (fig. 5). Feeding ethanol to WT mice for 2 weeks increased hepatic TBARS levels 2-fold and this increase was completely prevented by CMZ (fig. 5). To further substantiate the effect of CMZ on ethanol-induced oxidative/nitrosative stress, immunohistochemical analysis of 3-NT and 4-HNE protein adduct formation was evaluated. The ethanol feeding produced an increase in formation of 3-NT and 4-HNE protein adducts and CMZ treatment inhibited these increases (data not shown). These results show the effectiveness of the CYP2E1 inhibitor CMZ in blunting ethanol-induced oxidative stress and ethanol-induced fatty liver.

## Discussion

As expected, ethanol produced fatty liver in WT mice using the high-fat Lieber-DeCarli model. This fatty liver was blunted in CYP2E1 KO mice as compared to WT mice as evident by histopathology, Oil Red O staining and biochemical assay of hepatic triglyceride levels in livers from WT mice fed ethanol compared to CYP2E1 KO mice fed ethanol. We believe two important new points arise from these results. First, it is the absence of CYP2E1 in the KO mice and not secondary effects associated with the KO that plays a major role in the blunting of the ethanol-induced fatty liver in the KO mice since restoring the presence of CYP2E1 in these mice restores the fatty liver. Second, human CYP2E1 was the CYP2E1 which restores the ethanol-induced steatosis, which indicates that human CYP2E1 can potentiate development of fatty liver in an oral model of chronic ethanol administration. This suggests that results on fatty liver extrapolated from rodent models of ethanol intake can be extrapolated to human models of ethanol intake with respect to effects of CYP2E1.

Associated with the fatty liver induced by ethanol was an increase in oxidative/nitrosative stress as reflected by the increase in TBARS, 3-NT and 4-HNE staining, and the decrease in GSH levels in the WT mice fed ethanol.

CYP2E1 is suggested to be a major contributor to ethanol-induced oxidant stress. Ethanol caused oxidative stress in WT mice, but the ethanol-induced oxidative stress was decreased in CYP2E1 KO mice. It is the absence of CYP2E1 in the CYP2E1 KO mice that plays a major role in blunting of the ethanol-induced oxidant stress since restoring CYP2E1 restores oxidative/nitrosative stress. As discussed above for fatty liver, human CYP2E1 was the CYP2E1 which restores the oxidative/nitrosative stress indicating that human CYP2E1 can potentiate development of oxidative/nitrosative stress in an oral model of chronic ethanol administration.

It appears that ethanol-induced steatosis may be related to oxidative stress as shown by Diluzio [62] who reported that antioxidants can prevent the ethanol-induced fatty liver, and studies showing that overexpression of the manganese superoxide dismutase-2 can partially reduce the ethanol steatosis [63]. CYP2E1-generated ROS contributes to the ethanol-induced oxidant stress which may explain its role in the ethanol-induced steatosis. However, mechanisms for ethanol-induced steatosis are likely to be more complex than increases in CYP2E1/cytokines/oxidant stress, e.g. ethanol did not induce fatty liver in C1q KO mice despite elevating CYP2E1, TNF- $\alpha$ , IL-6 and oxidant stress, and the authors suggested a role for the classical complement pathway in contributing to the pathogenesis of ethanol-induced liver injury [64]. Blockade of PPAR- $\alpha$  function by chronic ethanol administration plays a key role in the mechanism of ethanol-induced steatosis [65] as PPAR- $\alpha$  KO mice fed ethanol develop extensive fatty liver and liver injury [66], whereas PPAR- $\alpha$  activating ligands such as clofibrate [67] or WY14643 [65] decrease the ethanol fatty liver. We found that levels of PPAR- $\alpha$ , and a downstream target of PPAR- $\alpha$ , acyl-CoA oxidase, were decreased more than 50% by ethanol administration to WT mice and CYP2E1 KI mice, but no effect was found with the CYP2E1 KO mice. This suggests the ethanol-induced decline in PPAR- $\alpha$  may be due, at least in part, to CYP2E1, or more likely, CYP2E1-derived ROS.

Significant liver injury generally does not occur in most oral models of ethanol intake, and there is a need to develop such models for mechanistic and preventive/therapeutic designs. ALT, AST and necroinflammatory scores were higher in ethanol-fed CYP2E1 KI mice compared to WT and CYP2E1 KO mice. CYP2E1 levels and activity were about 3-fold higher in the CYP2E1 KI mice compared to the WT mice after the ethanol feeding, suggesting the elevated liver injury may be due to the elevated content of CYP2E1, although the possibility that the

human CYP2E1 is more reactive than the mouse CYP2E1 in promoting this injury remains to be evaluated. The elevated liver injury is associated with an elevated oxidative/nitrosative stress in the ethanol-fed CYP2E1 KI mice and we speculate that the latter plays a key role in the former. This will be evaluated in future studies examining the effects of antioxidants such as N-acetylcysteine, vitamins E and C, and inhibitors of the inducible nitric oxide synthase, e.g. 1400W. The elevated liver injury is also associated with an increase in activation of the JNK mitogen-activated protein kinase. Activation of JNK has been shown to be important in many models of hepatotoxicity [53–57], including CYP2E1 potentiation of LPS or TNF- $\alpha$  liver injury [58–61]. Future experiments will evaluate the ability of an inhibitor of JNK activation such as SP600125 to prevent the chronic ethanol-induced liver injury in the CYP2E1 KI mice. SP600125 was effective in preventing the CYP2E1 potentiation of LPS or TNF- $\alpha$  liver injury [59]. We speculate the elevated oxidative/nitrosative stress in ethanol-fed KI mice may play a role in the activation of JNK, e.g. the upstream MAPKKK ASK-1 is activated when its inhibitor thioredoxin-1 is oxidized by ROS, and dissociates off ASK-1, which then allows ASK-1 to activate downstream MAPKKs and subsequently JNK

[68, 69]. ROS also inactivate MAPK phosphatases which dephosphorylate activated MAPK such as JNK [70]. Although other models of hepatotoxicity produced by overexpression of CYP2E1 have been reported, e.g. a transgenic model of CYP2E1 overexpression [71, 72], or administration of an adenovirus expressing CYP2E1 to mice [73], the advantage of the CYP2E1 KI model is that the actions of the human CYP2E1 can be studied in the total absence of the mouse CYP2E1, thus allowing assessment of the role of human CYP2E1 in ethanol-induced oxidative stress and liver injury.

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### Disclosure Statement

There are no conflicts of interest to disclose.

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