Review

CYP2E1, oxidative stress and MAPK signaling pathways in alcohol-induced hepatotoxicity

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Abstract: In this review, we discuss studies on the role of CYP2E1 in alcohol-induced oxidative stress and how MAPK signaling cascades are involved in alcohol induced liver injury and fat accumulation. Many pathways have been suggested to play a role in how alcohol induces oxidative stress. Considerable attention has been given to alcohol elevated production of lipopolysaccharide (LPS) and TNF\textsubscript{\alpha} and to alcohol induction of CYP2E1. These two pathways are not exclusive of each other, however, associations and interactions between them, especially in vivo, have not been extensively evaluated. We review our results that increased oxidative stress from induction of CYP2E1 in vivo sensitizes hepatocytes to LPS and TNF\textsubscript{\alpha} toxicity and that oxidative stress, activation of p38 and JNK MAP kinases, induction of the inducible nitric oxide synthase and mitochondrial dysfunction are downstream mediators of this CYP2E1-LPS/TNF\textsubscript{\alpha} potentiated hepatotoxicity. Binge alcohol drinking induces liver steatosis. The mechanism of acute alcohol induced steatosis and the role of CYP2E1 and MAPK is not clear. Increasing oxidative stress by alcohol can activate the JNK MAP kinase signaling pathway, and JNK might be a target for prevention of alcohol induced steatosis. In wild type (WT) mice, acute alcohol activated CYP2E1, produced fatty liver and increased oxidative stress, which increased activation of the JNK signaling pathway. Acute alcohol--induced fatty liver, JNK activation and oxidative stress were blunted in CYP2E1 knockout mice. The fatty liver, elevated oxidative stress and JNK activation in WT mice were prevented by a JNK inhibitor and by the antioxidant N-acetylcysteine. Thus, mechanistically, acute alcohol elevation of CYP2E1, oxidative stress and activation of JNK interact to cause fatty liver and liver toxicity.

Keywords: CYP2E1 (cytochrome P4502E1), TNF\textsubscript{\alpha}, fatty liver, oxidative stress, alcohol liver injury, p38MAP kinase, JNK, MAPK signaling pathways

Introduction

Alcohol acts through numerous pathways to affect the liver and other organs and to lead to the development of alcoholic liver disease (ALD) [1-3]. Many mechanisms act in concert, reflecting the spectrum of the organism’s response to a myriad of direct and indirect actions of alcohol. One factor playing a central role in many pathways of alcohol--induced damage is the excessive generation of molecules called free radicals, which can result in a state called oxidative stress [4-6]. Reactive oxygen species (ROS) can damage or cause complete degradation (i.e., peroxidation) of essential complex molecules in the cells, including lipids, proteins, and DNA. Both acute and chronic alcohol exposure can increase production of ROS and enhance peroxidation of lipids, protein, and DNA, as has been demonstrated in a variety of systems, cells, and species, including humans [4-6]. Many processes and factors are involved in causing alcohol--induced oxidative stress (Fig. 1), as described below:
Changes in the NAD+/NADH ratio in the cell as a result of alcohol metabolism. Alcohol dehydrogenase converts alcohol to acetaldehyde, a toxic and reactive molecule. Aldehyde dehydrogenase converts the acetaldehyde to acetate. Each of these reactions leads to formation of one molecule of NADH, thereby providing reducing equivalents and thus enhanced activity of the respiratory chain, including heightened O2 use and ROS formation.

- Production of acetaldehyde during alcohol metabolism, which through its interactions with proteins and lipids also can lead to radical formation and cell damage.
- Damage to the mitochondria resulting in decreased ATP production.
- Effects on cell structure and function caused by alcohol’s interactions with either membrane components (phospholipids) or enzymes and other proteins.
- Alcohol–induced oxygen deficiency (i.e., hypoxia), especially in the pericentral region of the liver where extra oxygen is required to metabolize alcohol.
- Alcohol’s effects on the immune system, which lead to altered production of certain signaling molecules, cytokines, which activate biochemical processes.
- Alcohol–induced increase in the ability of the bacterial product endotoxin to enter the bloodstream and liver, where it can activate certain immune cells.
- Alcohol–induced increases in cytochrome P450 2E1 (CYP2E1), which metabolizes alcohol and other molecules and generates ROS in the process.
- Alcohol–induced increases in the levels of free iron which can promote ROS generation.
- Effects on antioxidant enzymes and chemicals, particularly glutathione (GSH).
- Biochemical reactions generating an alcohol–derived radical (i.e., the 1–hydroxyethyl radical).
- Conversion of the enzyme xanthine dehydrogenase into a form called xanthine oxidase, which can generate ROS.

Many of these processes operate concurrently, and it is likely that several, indeed many, systems contribute to the ability of alcohol to induce a state of oxidative stress. More details on some of these pathways will be discussed below.

**Alcohol, oxidative stress, and cell injury**

What is the evidence that alcohol–induced oxidative stress plays a role in cell injury, particularly damage to the liver cells; some evidence is shown in Fig. 2 and discussed below. Many studies have demonstrated that alcohol increases lipid peroxidation as well as the modification of proteins; however, it is not always clear if these changes are the causes rather than consequences of alcohol–induced tissue injury. Nevertheless, numerous investigations have found that administering antioxidants, agents that reduce the levels of free iron, or agents that replenish GSH levels can prevent or ameliorate the toxic actions of alcohol. For example, in the intragastric infusion model, the antioxidant vitamin E; the chemical ebselen, which mimics the actions of glutathione peroxidase; and the antioxidant vitamins C and E can prevent liver damage.

**Effect on GSH levels; antioxidant defense**
- Iron mobilization
- XDH → XO conversion; hypoxia
- Release of chemoattractants
- Activation of Kupffer cells, cytokines, and LPS
- Mitochondrial injury; decreased ATP
- Metabolic effects - Redox state (NADH/NAD+), acetaldehyde
- Formation of 1-hydroxyethyl radical (CH3C’HOH)
- Induction of CYP2E1

Fig. 1. Suggested mechanisms for alcohol (ethanol)-induced oxidative stress. Major pathways and enzymes which contribute to the elevated oxidative stress produced by alcohol are listed. Further discussion can be found in the text.

**Chronic alcohol increases production of ROS, lipid peroxidation in many cellular systems, in-vivo, in-vitro**
- Antioxidants usually protect against this injury – SAM, GSH precursors, SOD1/2, Ebselen, GSH precursors-NAC
- Iron chelators can be protective, adding iron potentiates the alcohol toxicity
- Alcohol toxicity is elevated in antioxidant knockout mice e.g. SOD1 null, TrxR null
- PUFA potentials, while saturated fats lower alcohol liver injury
- Addition of ethanol to liver, neuronal and other cell lines elevates ROS and cell injury
- Liver cell lines expressing ADH, CYP2E1 show ethanol-induced oxidative stress and injury
- Human alcoholics show increased markers of oxidative damage

Fig. 2. Role of oxidative stress in alcohol-induced cell injury. The figure lists data from the literature providing evidence that alcohol induces oxidative stress and that such stress plays a role in cell injury. Further discussion can be found in the text.

**Mitochondrial respiratory chain – Complex I, III**
- NADPH oxidases
- Cytochrome P450s – CYP2E1
- Xanthine dehydrogenase → Xanthine oxidase
- Oxidative enzymes – MAO, Urate, glycolate, D-aa oxidases
- Peroxosomal fatty Acyl CoA oxidase, PG synthase
- Autoxidizable biochemicals – Catecholamines, flavins, thb
- Autoxidizable hemes – hemoglobin, myoglobin
- External agents – drugs, redox cycling agents, smoke radiation, ozone
- Metal overload – iron, copper
- Inducible NOS → NO : NO + O2− → ONOO·
- Peroxynitrite

Fig. 3. Systems producing ROS in biological systems. The figure lists some of the major pathways by which ROS are generated by enzymes, autoxidizable cellular constituents and by external agents.
of glutathione peroxidase; the copper–zinc or manganese SODs; or a GSH precursor—all prevented ALD [7-11].

In the intragastric infusion model, ALD was associated with enhanced lipid peroxidation, protein modification, formation of the 1–hydroxyethyl radical and lipid radicals, and decreased in the hepatic antioxidant defense, particularly GSH levels [8-11]. Moreover, changes in the animals’ diets that helped promote or reduce oxidative stress led to corresponding changes in the extent of liver injury. For example, when polyunsaturated fats were replaced with saturated fats or medium–chain triglycerides, lipid peroxidation as well as ALD was reduced or prevented completely, indicating that both alcohol and polyunsaturated fats must be present for ALD to occur [12]. The extent of the ALD was further exacerbated when iron—which, as mentioned earlier, is required for the generation of the hydroxyl radical and therefore promotes oxidative stress—was added to these diets [13, 14]. Conversely, the addition of antioxidants such as vitamin E, SOD, or GSH precursors prevented the development of ALD, as mentioned above.

Oxidative stress in human alcoholics

There are many reports that various parameters of oxidative stress are elevated by alcohol in humans. An early study assaying for the 9,11 linoleic acid isomer in blood reported that levels were higher in chronic alcoholics immediately after withdrawal and was evidence for increased free radical activity in alcoholics [15]. Ethane is a fragmentation product of the lipid peroxidation cascade and was elevated 5-fold in alcohol abusers [16]. The ethane exhalation was correlated to the daily ethanol intake prior to hospitalization and with steatosis, and was decreased during abstinence. Similarly, plasma phosphatidylcholine hydroperoxide, a direct peroxidation product of oxidized polyunsaturated fatty acids, was 3-fold higher in alcoholic patients than in controls [17]. These levels decreased during abstinence. 4-hydroxy-2-nonenal (HNE), another lipid peroxidation fragmentation product react with proteins to generate HNE-protein adducts. Strong HNE-protein adduct immunohistochemical staining was observed in patients with ALD compared to those with viral liver disease [18]. There was a correlation in the alcoholic patients between HNE adducts and iron suggesting that the latter catalyze lipid peroxidation and HNE production. Meagher et al. [19] measured urinary excretion of isoprostanes, which are free radical-catalyzed products of arachidonic acid. Urinary isoprostanes increased in a time and dose dependent manner in volunteers receiving acute ethanol. Urinary isoprostanes markedly increased in patients with acute alcoholic hepatitis and in cirrhosis. The antioxidant vitamin C reduced urinary isoprostane generation by alcoholics with liver disease.

Besides lipid peroxidation, there are other indices of alcohol-induced oxidant stress in humans. Formation of 2,3-dihydroxybenzoic acid (DHBA) from aspirin is a hydroxyl radical catalyzed reaction. After IV administration of aspirin, there was an increase in DHBA levels in peripheral blood from patients with alcohol dependence compared to control, suggesting elevated hydroxyl radical generation [20]. Mitochondrial (Mt) DNA deletions reflect oxidative damage to mt DNA. Diverse mt DNA deletions were observed in alcoholic patients especially in alcoholic patients with microvesicular steatosis [21]. The 1-hydroxyethyl radical,HER, can react with proteins to generate HER protein adducts. The sera of alcoholic cirrhotos contained antibodies which recognized HER-protein adducts; no such antibodies were found in controls or non-alcoholic cirrhotos [22]. Protein carbonyls were elevated in the serum of patients with chronic alcoholism compared to controls [23]. Besides reports of increased oxidative damage to lipids, mtDNA and proteins by alcohol, antioxidant defense appears to be lowered. Depressed serum selenium and vitamin E levels in alcoholic populations have been reported [24], as well as reduced hepatic α-tocopherol levels [25]. While many of the above and other studies show increased oxidative stress in human alcoholics, the functional significance of these changes is not known. HNE adducts and 80HdG levels were higher in livers from patients with ALD; the location of the HNE adducts and the nuclear expression of 8-hydroxy-deoxyguanine was mainly in hepatocytes with active inflammation [26]. The grade of necro-inflammation activity and presence of Mallory bodies and severity of steatosis were associated with the expression of HNE adducts and 80HdG. It appears that lipid peroxidation and oxidative DNA damage correlate with the severity of steatosis and liver injury, leading to the conclusion that oxidative stress plays a major role in the pathogenesis of human alcoholic liver disease. Markers of lipid peroxidation, antioxidant status, hepatic fibrogenesis and liver function were measured in blood and urine from 24 patients with established alcoholic liver cirrhosis and 49 matched controls. In the alcohol group, markers of lipid peroxidation, 8-isoprostane and MDA were significantly increased as was the GSSG/GSH ratio, while antioxidants selenium, GSH and vitamins A, C and E were decreased. Markers of hepatic fibrogenesis correlated with the elevated lipid peroxidation and lowered antioxidants [27]. Several studies have shown the presence of antibodies against MDA, 4-HNE and oxidized phospholipids in serum of patients with alcohol liver disease but not in patients with fatty liver only [28]. Elevations in circulating IgG against MDA-albumin or oxidized cardiolipin were higher in heavy drinkers with ALD than in heavy drinkers without ALD or healthy controls [29]. The elevation of these antibodies was associated with higher circulating levels of IL-2 and TNF-α, but not IL-6 or IL-8. The authors concluded that immune responses towards oxidative stress-derived antigens promoted TNF-α production and contributed to liver damage in alcohol abusers. Albano has recently reviewed free radical mechanisms in immune reactions associated with alcoholic liver disease [30]. Mitochondria are also an important contributor to ROS generation and alcoholic liver injury [31,
Fig. 4. Model for ethanol-induced liver toxicity via induction of CYP2E1. This increase in CYP2E1 by ethanol results in enhanced formation of ROS such as the superoxide anion radical and hydrogen peroxide. In the presence of transition metals such as non-heme iron, more powerful oxidants such as the hydroxyl radical (OH), ferryl and perferryl oxidants (FeO) and the 1-hydroxyl ethyl radical are generated. These oxidants readily peroxodize polyunsaturated fatty acids (PUFAs), destroying biological membranes and producing reactive lipid aldehydes such as malondialdehyde (MDA) and 4-hydroxy-nonenal (HNE) and reactive lipid hydroperoxides (LOOH). Mitochondria are a downstream target of these oxidants eventually resulting in bioenergetic failure and cell toxicity.
occurs and in which significant liver injury is produced. In these models, large increases in microsomal lipid peroxidation have been observed and the ethanol-induced liver pathology has been shown to correlate with CYP2E1 levels and elevated lipid peroxidation [13, 38]. Importantly, a decrease in CYP2E1 induction was found to be associated with a reduction in alcohol-induced liver injury. CYP2E1 inhibitors such as dialyl sulfide, phenethyl isothiocyanate and chloromethiazole blocked the lipid peroxidation and ameliorated the pathologic changes in ethanol-fed rats [39-42]. Strong association between CYP2E1 and ethanol induced liver injuries was observed. In recent years, in vitro and in vivo studies on cell lines over-expressing CYP2E1, or on CYP2E1 knockout or CYP2E1 gene overexpression mice, have been major tools for studying the mechanism of alcoholic liver diseases.

HepG2 cell lines that express CYP2E1 were established in our lab either by retroviral infection methods (MV2E1-9 cells, or E9 cells) or by plasmid transfection methods (E47 cells) [43, 44]. The addition of acetaminophen, ethanol, CCl4 or arachidonic acid resulted in cytotoxicity to the HepG2 cells that expressed CYP2E1 but not to the control HepG2 cells (MV-5 or C34 cells), which do not express CYP2E1 [45-47]. These data validate the utility of the HepG2 cell model to study CYP2E1-dependent toxicity, since acetaminophen, ethanol and CCl4 are known substrates of CYP2E1. Concentrations of iron, arachidonic acid, or ethanol that were toxic to CYP2E1 expressing cells were not toxic to control cells. Toxicity was found when GSH was depleted by 1-buthionine sulfoximine in CYP2E1 expressing cells [48]. When CYP2E1 expressing cells were treated with iron, arachidonic acid, or ethanol, there was increased production of ROS, and these increases were prevented by treating cells with antioxidants such as vitamin E, vitamin C or trolox [48, 49]. When CYP2E1 expressing cells were treated with alcohol, increased accumulation of lipids was found compared to C34 cells, and this accumulation of lipids was related to a decrease of autophagy in HepG2 cells expressing CYP2E1 [50]. In addition, low concentrations of arachidonic acid or iron that by themselves are not toxic to CYP2E1 expressing cells can potentiate cell toxicity when added together at these low concentrations [50]. A HepG2 cell culture model in which CYP2E1 and alcohol dehydrogenase were both expressed has been utilized to study ethanol-CYP2E1-proteasome function, interferon gamma induction of the proteasome and interferon gamma signaling [51-53]. Figure 4 is a model by which we propose that ethanol induction of CYP2E1 and subsequent formation of ROS results in mitochondrial dysfunction and cellular toxicity.

CYP2E1 knockout mice were developed by Gonzalez and colleagues to determine the role of CYP2E1 in xenobiotic metabolism and toxicity [54, 55]. The development of the CYP2E1 knockout mouse has been of great value in establishing the role of CYP2E1 in the metabolism and toxicity of various hepatotoxins. Studies in our lab [56] also showed that CYP2E1 plays a role in experimental alcoholic fatty liver in an oral ethanol-feeding model. After 4 weeks of ethanol feeding to wild type (WT) mice, ethanol produced fatty liver and elevated liver triglyceride levels as compared to the dextrose pair-fed controls. However, the chronic ethanol feeding did not elevate the steatosis score in CYP2E1 knockout (KO) mice. Accumulation of triglycerides in liver, induction of CYP2E1 by ethanol, and increased oxidative stress were observed in wild-type mice but not in CYP2E1-knockout mice [56]. An inhibitor of CYP2E1, chloromethiazole, lowered macrovesicular fat accumulation, inhibited oxidative stress, and up-regulated PPAR-α protein level in wild-type mice fed ethanol [56]. Despite the increased steatosis and oxidative stress, the chronic ethanol feeding did not elevate necrosis or inflammation in WT or KO mice [56]. These results indicate that CYP2E1 contributes to experimental alcoholic fatty liver in this model and suggest that CYP2E1-derived oxidative stress may inhibit oxidation of fatty acids by preventing up-regulation of PPAR-α by ethanol, resulting in fatty liver [56].

A CYP2E1 transgenic mouse model containing human CYP2E1 cDNA was developed that overexpressed CYP2E1. When treated with ethanol, the CYP2E1 overexpressing mice displayed higher transaminase levels and histological damage compared with the control mice [57]. Humanized CYP2E1 knockin mice were recently developed in which the human CYP2E1 transgene was introduced into the corresponding CYP2E1 null mouse background [58]. This CYP2E1 knockin mouse allows functional activities of the human CYP2E1 to be studied and validates that decreased actions by ethanol in CYP2E1 knockout mice can be restored by reintroduction of the human CYP2E1 [58, 59]. When we fed ethanol to CYP2E1 KO mice which were “knocked in” to express human CYP2E1 (KI mice) [60], liver steatosis was restored compared to no increase in fat accumulation in the KO mice. Ethanol also elevated oxidative stress and caused necroinflammation in the K1 mice [60]. These results support a role for CYP2E1 in alcohol-induced liver injury. However, studies by others have suggested that CYP2E1 does not contribute significantly to alcohol liver injury [61, 62]. As discussed elsewhere [60,63], induction by ethanol of other CYPs or of the reductase in the CYP2E1 knockout mice might have served as alternative sources of oxidative stress in these mice, especially in the absence of CYP2E1. Bradford et al using CYP2E1 and NADPH oxidase knockout mice concluded that CYP2E1 was required for ethanol induction of oxidative stress to DNA, whereas NADPH oxidase was required for ethanol-induced liver injury [64].

LPS/TNFα-CYP2E1 interactions

Yin et al and Iimuro et al have presented powerful support for a role for TNFα in alcohol-induced liver injury via a TNFR1-dependent pathway in the intragastric infusion
model [65, 66]. One complication in this central role for TNFα is that hepatocytes are normally resistant to TNFα induced toxicity. This led to the hypothesis that besides elevating TNFα, alcohol somehow sensitizes or primes the liver to become susceptible to TNFα. Combined treatment with ethanol plus TNFα is more toxic to hepatocytes and HepG2 E47 cells which express high levels of CYP2E1 than control hepatocytes with lower levels of CYP2E1 or HepG2 C34 cells which do not express CYP2E1 [67]. RALA hepatocytes with increased expression of CYP2E1 were sensitized to TNFα mediated cell death [68]. These results suggest that increased oxidant stress from CYP2E1 may sensitize isolated hepatocytes to TNFα-induced toxicity. To link induction of CYP2E1 and TNFα hepatotoxicity, we showed that induction of CYP2E1 with pyrazole potentiated LPS and TNFα induced oxidant stress and hepatotoxicity in wild type by not in CYP2E1 knockout mice [63, 69]. Administration of 50 ug/Kg of TNFα to saline-treated SV129 mice produced only minor increases in serum ALT and AST levels. However, administration of TNFα to mice which were pretreated with pyrazole to elevate CYP2E1 strikingly elevated serum transaminase levels [63, 69]. Pyrazole treatment in the absence of TNFα did not elevate ALT or AST levels. The combined pyrazole plus TNFα treatment produced extensive necrosis of hepatocytes in the wild type mice but not in the CYP2E1 KO mice. The combination of pyrazole and TNFα potentiated ROS formation e.g increased lipid peroxidation, decreased GSH levels, compared to treatment with pyrazole alone or TNFα alone in the WT mice. No increases in oxidant stress were found with the CYP2E1 knockout mice. Thus, two independent risk factors believed to be important in alcoholic liver injury, CYP2E1 and TNFα, interacted with each other to potentiate liver injury [63, 69].

MAPK signaling pathway mediates CYP2E1 plus TNFα induced liver injury

Mitogen-activated protein kinases (MAPKs) are serine-threonine kinases that mediate intracellular signaling associated with a variety of cellular activities including cell proliferation, differentiation, survival, death, and transformation. The mammalian MAPK family consists of extracellular signal-regulated kinase (ERK), p38 MAPK, and c-Jun NH2-terminal kinase (JNK) also known as stress-activated protein kinase or SAPK) [70]. The MAPK signaling cascade consists of three distinct members of the protein kinase family, including MAP kinase (MAPK), MAP kinase kinase (MAPKK), and MAP kinase kinase kinase (MAPKKK). MAPKKK phosphorylates and thereby activates MAPKK, and the activated form of MAPKK in turn phosphorylates and activates MAPK. Activated MAPK may translocate to the cell nucleus and regulate the activities of transcription factors and thereby control gene expression [71, 72].

Apoptosis signal regulating kinase-1 (ASK-1) is a member of the MAPKKK family that activates JNK and p38 MAPK in response to various stresses including oxidative stress and TNFα [72, 73]. Overexpression of ASK-1 induced apoptotic cell death, and ASK-1 was activated in cells treated with TNFα. Moreover, TNFα–induced apoptosis was inhibited by a catalytically inactive form of ASK-1 [72]. Studies in ASK-1 knockout mice have shown that this MAPKKK is selectively required for the sustained activation of JNK and p38 MAPK signaling cascades in response to oxidative stress or TNFα [74]. The mechanism of ASK-1 activation by oxidative stress is thought to involve several molecular events, which have been partially elucidated. In the resting state, ASK-1 resides in a multimolecular complex associated with the reduced form of its endogenous inhibitor, thioredoxin. When cells are subjected to oxidative stress, the thioredoxin is oxidized and the oxidized form of thioredoxin dissociates from ASK-1, resulting in the autophosphorylation-dependent activation of the kinase. Activated ASK-1 leads to the recruitment/activation of MKK-3/6 and MKK-4/7, which in turn activate p38 MAPK and JNK, respectively [75]. The JNK and p38 MAPK signaling pathways are activated by proinflammatory cytokines such as TNFα and IL-1β or in response to cellular stresses such as genotoxic, osmotic, hypoxic, or oxidative stress. Activation of MAPK is a common mediator of hepatocyte injury from a variety of stimuli, including alcohol, LPS/TNFα, bile acids, acetaminophen [76-79].

In either in vivo or in vitro models of alcoholic liver disease, an increase of gene expression of the MAPK pathway was found [80,81]. Compatible data in protein expression levels were seen in many studies. Intraperitoneal injection of alcohol to rats induced rapid phosphorylation of p38 MAPK and JNK after only 1 hr of ethanol injection and this was accompanied with apoptosis of the liver [82]. In human stellate cells, increased phosphorylation of p38 MAPK and JNK was found to be associated with ethanol induced stellate cell activation, toxicity and apoptosis [83]. JNK and p38 MAPK may become activated simultaneously, while some studies have shown that JNK and p38 MAPK may even react in the opposite way according to the specific treatments. In one study, after chronic alcohol feeding, LPS stimulation of Kupffer cells increased p38 MAPK activity, whereas it decreased JNK activity [84]. In human monocytes, acute alcohol exposure increased JNK phosphorylation, while chronic alcohol exposure decreased JNK activity [85]. Further studies are needed to clarify why MAPK can react differently depending on the stimuli or in different cell types.

In recent years, the role of CYP2E1 in the induction of oxidative stress and in the activation of MAPK signaling pathway has gained more appreciation. This has been shown by studies in which CYP2E1 was activated in mice or in cell culture followed by challenge with different stimuli. Ethanol exposure was found to enhance TNFα induced cell toxicity in HepG2 cells, especially CYP2E1 expressing E47 cells.
This increase was due to the increased activation of p38 MAPK signaling, which increased the activation of caspase 3, mitochondrial depolarization and cytochrome C release into the cytosol [79]. The idea that CYP2E1 plays an important role in the activation of MAPK pathways was further confirmed by studies in which increasing levels of CYP2E1 in mice by pyrazole treatment, followed by challenge with either TNFα or LPS or Fas agonistic JO2 antibody resulted in elevations of pJNK/JNK or pp38/p38 MAPK ratios accompanied by severe liver injuries [69,78,86,87]. LPS treatment alone did not cause significant JNK activation or p38 MAPK activation as reflected by the low pJNK and pp38 MAPK levels relative to total JNK and p38 MAPK levels. Similar low ratios were found for the saline or the pyrazole alone treated mice [63, 69, 86]. However, both JNK and p38 MAPK were activated in livers of the pyrazole plus LPS treated mice. Similar results were found with TNFα replacing LPS [78]. ERK was not altered by either TNFα alone or pyrazole plus TNFα treatment. To evaluate the significance of these changes in MAPK activation, the effect of SP600125, an inhibitor of JNK, and SB203580, an inhibitor of p38 MAPK on the hepatotoxicity was determined [69,78]. The TNFα plus pyrazole elevation of transaminases was blunted by administration of SP600125 (15mg/kg) or SB203580 (15mg/kg). The p38 and JNK MAPK inhibitors also lowered the necrosis and partially

Fig. 5. Scheme for TNFα plus CYP2E1 activation of the MAP kinase kinase kinase ASK-1, MAP kinase kinases MKK3/6 or MKK4/7, and MAP kinases JNK or p38 and the role of CYP2E1–generated ROS in removal of inhibitory thioredoxin from its complex with ASK-1. The scheme is discussed in the text.
blocked the increased oxidative stress produced by the pyrazole plus TNFα treatment, but had no effect on CYP2E1 activity or protein levels. These results suggest the CYP2E1 elevation of TNFα liver injury and oxidative stress is MAPK dependent. Activation of JNK in the pyrazole plus TNFα group was blocked by SP600125 but not SB203580 whereas activation of p38 MAPK was blocked by SB203580 but not SP 600125.

We hypothesized [69, 78] that mitochondria are an eventual target for developing liver injury induced by TNFα when CYP2E1 is elevated by pyrazole. Initiation of a mitochondrial permeability transition was determined by assessing mitochondrial swelling in the absence and presence of 100uM calcium. Succinate (10mM) was the respiratory substrate. The addition of 100uM calcium caused a low rate of swelling by mitochondria isolated from the saline treated or TNFα alone treated mice. Mitochondrial swelling was somewhat elevated in mitochondria from the pyrazole alone treated mice. Swelling was very rapid without any lag phase with the mitochondria isolated from the pyrazole plus TNFα-treated mice. Importantly, this rapid swelling was blocked by cyclosporine A (2uM), a classic inhibitor of the mitochondrial permeability transition. The mitochondrial permeability transition induced by the combined TNFα plus pyrazole treatment was blunted by the JNK and p38 MAPK inhibitors SP600125 and SB203580. Thus, the mitochondrial injury induced by elevated CYP2E1 plus TNFα was MAPK-dependent. To validate that the activation of JNK and p38 MAPK by CYP2E1 plus TNFα was due to elevated oxidant stress, the effect of N-acetylcyesteine (NAC), an antioxidant and a precursor of GSH was determined. NAC blocked the liver injury and the elevated oxidant stress produced by pyrazole plus TNFα, and also blunted the activation of JNK and p38 MAPK. These results suggest that elevated oxidative stress is central to the activation of JNK and p38 MAPK and to the liver injury produced by TNFα plus pyrazole.

Activated MAPKs become deactivated when dephosphorylated by phosphatases. Major MAPK phosphatases (MKP) include MKP-1, 2, 5 and 7 [88-90]. ROS promote TNFα induced death and sustained JNK activation by inhibiting MKPs as critical cysteine residues are oxidized. GSH, thioredoxin and peroxiredoxins reduce oxidized MKPs back to the active reduced state [88-90]. The activity of MKP-1 was found to be decreased in association with the phosphorylation of JNK and p38 MAPK after treatment of mice with pyrazole and TNFα [69].

**ASK-1 activation**

To determine how JNK and p38 MAPK are being activated after the treatment with pyrazole plus TNFα, activation of the upstream MAPKKK, ASK-1 was studied [69]. In mice treated with pyrazole for 3 days to increase CYP2E1 and then challenged with TNFα, ASK-1 was activated at 4h after the administration of TNFα. Activation of ASK-1 was associated with dissociation of its inhibitor thioredoxin at 4 h after TNFα administration. This was followed by the activation of MKK-3/6 and MKK-4/7 at 4-8 and 12 hrs. JNK and p38 MAPK were phosphorylated at 8-12 hrs. These time points confirmed that the signaling cascade of MAPK was first the phosphorylation of ASK-1, then MAPKK, and subsequently the downstream JNK and p38 MAPK were activated. Blockage of the JNK and p38 MAPK cascade activation by MAPK inhibitors prevented CYP2E1 plus TNFα induced liver injury. This MAPK signaling cascade was not found in CYP2E1 knockout mice. This indicates that CYP2E1 induced oxidative stress is critical in the activation of the MAPK signaling pathway, which is critical in the CYP2E1 plus TNFα induced liver injury. Indeed, the liver injury occurred between 8 and 12 hrs after TNFα administration, a time after the activation of ASK-1 (4 hrs) and MAPKK (4-8 hrs).

A scheme summarizing these results is shown in Fig 5. Upon binding to its receptor, TNFα activates MAPK signaling via complex I and caspases via complex II. ROS produced from CYP2E1 causes dissociation of the inhibitory thioredoxin from its complex with ASK-1, resulting in activation (phosphorylation) of ASK-1. Activated ASK-1 then activates downstream MAP kinase kinases MKK3/6 and MKK 4/7 which in turn activate JNK and p38 MAPK. Activation of the MAP kinases may also be promoted by inhibition of MAPK phosphatases such as MKP-1. Increases in ROS from CYP2E1 and TNFα and activated MAPK ultimately cause damage to the mitochondria followed by liver injury.

**Thioredoxin**

Thioredoxin is an endogenous multifunctional protein with a redox-active disulfide/dithiol within the conserved active site sequence:-Cys-Gly-Pro-Cys- [91]. Thioredoxin-1 is located in the cytosolic fraction and thioredoxin-2 is found in the mitochondria. Thioredoxin is a stress-inducible protein, the expression of which is enhanced by various types of stresses, e.g. viral infection, exposure to ultraviolet light, X-ray irradiation, hydrogen peroxide, and ethanol [92-94]. The thioredoxin/peroxiredoxin system is considered to act as a critical endogenous antioxidant system in addition to the glutathione (GSH)/ GSH peroxidase system [95, 96]. In addition to its anti-oxidant effect, intracellular thioredoxin shows redox regulatory functions in signal transduction. As mentioned above, ASK-1 is a mitogen activated protein kinase kinase kinase and binds to thioredoxin under normal conditions. When thioredoxin is oxidized, ASK-1 dissociates from thioredoxin, the free ASK-1 becomes activated, and interacts with TRAF2/6, inducing the eventual phosphorylation of JNK and p38 MAPK to transduce the signal of apoptosis [97, 98]. Dissociation of ASK-1 from thioredoxin was observed in TNFα induced hepatotoxicity in CYP2E1 expressing mice [69].
In in vitro experiments, overexpression of thioredoxin decreases hepatic fibrosis, decreases TNFα induced apoptosis in HepG2 cells, and decreases hepatic stellate cell proliferation and DNA synthesis [99]. In addition, thioredoxin is also involved in protection against the toxicity of chemicals such as cisplatin. In E47 cells expressing CYP2E1, knocking down thioredoxin by thioredoxin siRNA increased cell toxicity by cisplatin [100]. In fact, thioredoxin and glutathione cooperate in the protection against oxidative stress induced by cisplatin. In general, thioredoxin via its antioxidant activity, had been shown to suppress oxidative stress. Although it is well known that CYP2E1 is an inducer of oxidative stress, it is not known whether thioredoxin can protect cells from CYP2E1 induced oxidative stress. By SiRNA approaches, knock down of thioredoxin-1 or thioredoxin-2 or both in E47 or C34 cells caused a comparable decreased viability of E47 cells but no loss of viability occurred in C34 cells [101]. The decreased viability in E47 cells was accompanied by an increase of ROS production in the cells [101]. Further mechanisms for the loss of protection from oxidative stress caused by CYP2E1 with thioredoxin knockdown are under investigation. IP injection of human recombinant thioredoxin to mice chronically fed ethanol decreased oxidative stress, inflammatory cytokine expression and apoptosis [102].

**Activation of NF-kB**

TNFα induces the activation of NF-kB in the liver and this induction results in the upregulation of several protective enzymes which prevent against the toxicity produced by TNFα [103,104]. We evaluated the effect of NF-kB on the toxicity produced by the combined pyrazole plus TNFα toxicity [105]. Treatment with TNFα alone increased the levels of IkBα and Ikββ 2.1 and 2.6 fold, respectively, as compared to saline controls. However, combined treatment with pyrazole plus TNFα lowered these levels to 0.5 and 0.3 of the saline controls. The consequences of this were that TNFα alone lowered levels of Ikβα and Ikββ whereas pyrazole plus TNFα elevated these about 2.6 fold. This resulted in NF-kB DNA binding activity being increased by TNFα alone, with a subsequent increase in formation of protective enzymes such as the manganese SOD2, whereas NF-kB DNA binding activity was decreased by the TNFα plus pyrazole combined treatment with a subsequent decline in levels of protective enzymes such as SOD2. It is likely that CYP2E1 plus TNFα elevated ROS coupled to a decline in NF-kB signaling to produce protective factors potentiate cellular toxicity as summarized in the scheme shown in Fig. 6.

**Fig. 6.** Proposed scheme for inhibition of NF-kB signaling by combined treatment with pyrazole plus TNFα. (A) shows sites where CYP2E1 plus TNFα elevation of ROS is hypothesized to inhibit the activation of NF-kB and thereby prevent the subsequent production of cellular protective factors. (B) shows the overall scheme for the role of oxidative and nitrosative stress in the activation of JNK and p38 MAPK and inactivation of NF-kB resulting in mitochondrial dysfunction and liver injury.
Protective actions of MAP kinases

Although most studies showed that activation of MAPK signaling pathways initiate apoptosis and induce cell death, there are studies showing that the phosphorylation of MAPK may be involved in cellular protective mechanisms. Acute alcohol consumption, increased IL-10 and heme oxygenase-1 in monocytes and this was accompanied with increased LPS induced phosphorylation of p38 MAPK and decreased production of TNFa [106]. A p38 MAPK inhibitor, SB203580, prevented both the ethanol-induced increase in IL-10 production and the inhibitory effect of ethanol on TNFa production [106]. This suggests an anti-inflammatory mechanism by acute alcohol treatment on monocytes was mediated by MAPK. Another study showed that when human hepatocytes were treated with 100mM ethanol, there was increased oxidative stress, depletion of glutathione, and increase of release of aspartate aminotransferase and lactate dehydrogenase. Addition of Quercetin protected human hepatocytes from these effects. However, inhibitors of p38 MAPK, JNK and ERK blocked the quercetin induced protective effects [107]. This suggests that the MAPK signaling cascade mediated the protective effect by quercetin.

In the ERK signaling pathway, ERK1 or ERK2 (ERK1/2) is activated by MEK1/2. ERK signaling proteins play a key role in transmission of extracellular signals into cells [108]. Similar to the dual results of JNK and p38 MAPK studies, the activation of ERK could show either harmful or beneficial effects. However, it seems that most studies suggest that inhibition of ERK activation may be involved in potentiating alcohol induced liver injuries. In E47 HepG2 cells which express CYP2E1, the expression of heme oxygenase-1 was higher than that in C34 HepG2 cells which do not express CYP2E1. The increase of heme oxygenase-1 was accompanied by an increased phosphorylation of ERK, but not p38 MAPK or JNK. PD98059, a specific inhibitor of ERK MAPK, blocked the activity of a heme oxygenase-1 reporter construct in E47 cells [109]. This indicated that ERK phosphorylation mediated the increased production of heme oxygenase-1, a protective protein in E47 cells. Chronic ethanol feeding of mice decreased activation of ERK [105-108]; this was accompanied by the formation of fatty liver and increase of liver triglycerides [109]. In addition, chronic ethanol feeding increased the 4-HNE-ERK monomer formation, and decreased the phosphorylation of ERK. Treatment of hepatocytes with 4-HNE induced a dose-dependence reduction of phosphorylation of ERK. This suggests that lipid peroxidation induced by alcohol can inhibit the phosphorylation of ERK [110]. EGF, a strong ERK1/2 activator, conferred beneficial effects on alcohol-induced fatty liver and liver injury [111].

On the other hand, chronic alcohol increased LPS-induced ERK1/2 activation which contributed to TNFα expression in macrophages [112]. Intraperitoneal injection of alcohol to rats induced rapid phosphorylation of ERK after 1 hr of ethanol injection, and the phosphorylation continued 4 hrs after ethanol injection. ERK1/2 activation by LPS was correlated with necrosis of liver cells and steatosis in rat liver [82].

Role of nitric oxide (NO)

Ethanol has been shown to increase levels of NO in the liver [113] and peroxynitrite, produced from the interaction of NO with superoxide radical plays a role in ethanol-induced toxicity in hypoxic liver [114,115]. Ethanol-induction of iNOS was shown to be required for alcohol – induced liver disease since ethanol hepatotoxicity was blunted in iNOS knockout mice [114]. We hypothesized [105] that ethanol induction of CYP2E1 would elevate superoxide production while ethanol induction of iNOS would elevate NO production; such increases would result in peroxynitrite formation and subsequently cause mitochondrial dysfunction and liver injury. iNOS was elevated 2.7 fold by the combined TNFα plus pyrazole treatment compared to each treatment alone and this increase was blunted by the iNOS inhibitor W1400 [105]. The activation of JNK and p38 MAPK by pyrazole plus TNFα was blocked by inhibition of iNOS with W1400 as well as by the general antioxidant N-acetyl-cysteine. This was associated with a decline in the pyrazole plus TNFα hepatotoxicity (elevated transaminases). N-acetyl-cysteine also blunted hepatotoxicity [105]. These results show that elevated oxidative as well as nitrosative stress play a role in the activation of JNK and p38 MAPK and the enhanced hepatotoxicity produced by pyrazole plus TNFα.

Role of JNK in acute ethanol-induced steatosis

Acute alcohol consumption induces steatosis in liver [116]. Steatosis is an early and reversible stage of alcohol induced liver injuries. However, steatosis can evolve towards steatohepatitis, which is characterized not only by lipid accumulation but also by necroinflammation and fibrosis. Previous work showed that chronic alcohol consumption induced fatty liver was, at least in part, CYP2E1 dependent [56, 60]. Chronic ethanol-induced fatty liver was significantly blunted in CYP2E1 knockout (KO) mice as compared to wild type (WT) mice [56]. Restoring CYP2E1 to the KO mice restored the chronic alcohol-induced fatty liver [60]. CYP2E1 is also critical in alcohol induced steatosis in HepG2 cells. However, whether CYP2E1 plays a role in acute alcohol induced fatty liver was unknown. Acute alcohol induced a moderate increase in the phosphorylation of JNK [82]. This may suggest that JNK plays a role in the progression of steatosis, and might be a target for the prevention of steatosis and further development of liver damage by alcohol. Below, we review our studies [117] as to whether CYP2E1 and JNK play a critical role in acute alcohol induced liver steatosis.
Acute alcohol treatment induces steatosis in liver, which involves activation of CYP2E1 and JNK

Mice were treated with a total of 4.68g/kg b.w. alcohol and sacrificed 18 hrs after the last dose of ethanol. Significant steatosis was produced in livers from wild type (WT) by acute alcohol treatment, as shown by H&E staining, Oil Red O staining, and TG levels in liver. Acute alcohol treatment also increased CYP2E1 protein expression, and CYP2E1 activity. This suggests that CYP2E1 might play a role in the acute alcohol induced steatosis. Further evidence for a role of CYP2E1 was tested by treating CYP2E1 knockout mice with acute alcohol. No steatosis was induced by acute alcohol in CYP2E1 knockout mice confirming CYP2E1 plays a role in formation of steatosis in this acute alcohol model [117].

JNK inhibitor completely blocked the acute alcohol induced steatosis [117]. We evaluated whether this blockage of steatosis could be due to inhibition of CYP2E1. The JNK inhibitor did not block either CYP2E1 protein expression or activity. This suggests that inhibition by the JNK inhibitor is not through direct inhibition of CYP2E1 but rather through inhibition of effects that are downstream of ethanol-CYP2E1 signaling as described below. Acute alcohol treatment per se or together with JNK inhibitor did not cause liver injury as reflected by ALT and AST assay.

Acute alcohol treatment induced activation of JNK

After chronic alcohol treatment, MAP kinase signaling pathways were involved in development of liver injuries [63, 69]. Based upon the blunting of acute ethanol-induced steatosis by the JNK inhibitor, we evaluated whether JNK was indeed activated, and if so, which JNK isoform, JNK1 or JNK2 or both was activated. Both JNK1 and JNK2 were activated, by acute alcohol treatment with increases in the pJNK1/JNK1 and pJNK2/JNK2 ratio of 2.4 fold and 4.7 fold as compared to saline control, respectively [117]. This activation was blocked by the JNK inhibitor. Immunohistochemistry staining also confirmed the activation of JNK by acute alcohol and the prevention of this activation by JNK inhibitor. Acute alcohol treatment also increased the activation of cJUN as shown by the elevated ratio of pcJUN/cJUN. JNK inhibitor blocked this cJUN activation.

JNK inhibitor blocks oxidative stress induced by acute alcohol treatment

Chronic alcohol treatment of mice increases oxidative stress in the liver [59, 60]. There are also reports on increased oxidative stress in acute alcohol treatment [118]. In the current binge alcohol model, oxidative stress was increased, as shown by elevated 4-HNE staining, increased TBARS levels, and decreased GSH levels compared to saline controls. The JNK inhibitor blocked this acute alcohol induced oxidative stress decreasing levels of 4-HNE and TBARS, and increasing levels of GSH in liver. To further evaluate the role of oxidative stress in steatosis formation in this acute alcohol model, mice were pretreated with the antioxidant N-acetyl-L-cysteine (NAC) prior to acute alcohol treatment. NAC pretreatment blocked oxidative stress induced by acute alcohol treatment, leading to decreasing 4-HNE protein adduct formation and TBARS levels and increasing GSH levels. The NAC treatment did not block the acute alcohol induction of CYP2E1 indicating that the blockage of oxidative stress by NAC is not due to the inhibition of CYP2E1. NAC blocked acute alcohol induced steatosis as shown by reduced H&E staining, Oil Red O staining and liver triglyceride levels. This suggests that oxidative stress indeed plays an important role in the acute alcohol induced steatosis. Inhibition of oxidative stress with NAC also blocked JNK activation. This suggests there is a reciprocal relationship between the increase of oxidative stress and activation of JNK which may play important roles in the acute alcohol-induced steatosis.

Conclusions and future perspectives

Many studies have shown that alcohol ingestion induces CYP2E1 and oxidative stress. Increased ROS production results in increased oxidative stress in liver, and consequently results in alcoholic steatosis. Although it is still not clear what the exact contributions of CYP2E1 or ADH are to the oxidative stress and endoplasmic reticulum (ER) stress produced by ethanol, studies show that CYP2E1 plays an important role in alcohol induced steatosis [56,119]. In vivo experiments with CYP2E1 knockout mice, and inhibitors of CYP2E1 confirmed its role in alcohol induced liver injury [40, 42, 56, 60].

This review summarizes research showing that activation of the JNK and p38 MAPK pathways play an important role in alcohol induced liver injury. Further studies are needed to more clearly define molecular mechanisms for the actions of MAPK in alcohol-induced liver injury, steatosis and oxidant stress. In addition, biochemical, signaling, and immunological pathways are all involved in alcohol induced liver injuries. Known mechanisms for alcohol effects include inhibition of fatty acid oxidation by preventing the up-regulation of PPAR-α by ethanol [56], increases in SREBP [120,121], which result in decreased fatty acid oxidation and increased lipogenesis, and endotoxemia and formation of cytokines such as TNFα. Alcohol potentiation of Fas-induced toxicity is also related to activation of MAPK pathways [86]. TLR4, a receptor for LPS binding, is also involved in alcohol induced liver injury. Knockout of TLR4 protected livers from injury, TNFα toxicity and oxidative stress indicating that TLR4 plays a critical role in alcohol induced liver injury [122]. Complement contributes to alcoholic liver injuries. Pritchard et al showed that the third and the fifth components of the
complement cascade are involved in triglyceride accumulation and inflammation of hepatocytes, respectively [123]. Further studies are needed to unravel specific details of these mechanisms of alcoholic liver injuries. The role of MAPK in alcohol steatosis, effects on lipolytic and lipogenic genes, TLR4 signaling, complement signaling in the liver, ER stress, mitochondrial dysfunction and CYP2E1 actions and toxicity requires further study.

Although CYP2E1 is mainly located in the membrane of the ER, it is also present in the mitochondria [124-126]. It is not clear how mitochondrial CYP2E1 vs. microsomal CYP2E1 contribute to alcoholic liver disease, oxidative stress, activation of JNK and p38 MAPK. Interestingly, a recent study by Bansal et al [127] using cells overexpressing either mitochondrial CYP2E1 or microsomal CYP2E1 showed that mitochondrial CYP2E1 is more important in depletion of GSH, and increase of cellular F2-isoprostanes, indicators of increased oxidative stress by alcohol. Moreover, overexpression of mitochondrial CYP2E1 in yeast cells caused more severe mitochondrial DNA damage than that found in microsomal CYP2E1 expressing cells [127]. Further work is needed to identify the biochemical function and significance of microsomal and mitochondrial CYP2E1. In this respect, JNK can translocate to the mitochondria and whether cytosolic or mitochondrial JNK contribute to the hepatic effects of alcohol is not known.

Acute alcohol administration produced an activation of CYP2E1, an increase in oxidative stress and activation of JNK in WT mice. CYP2E1 knockout mice treated with acute alcohol did not develop steatosis, indicating that CYP2E1 plays an important role in steatosis formation in this acute binge alcohol model. In response to acute and chronic ethanol exposure, mitogen-activated protein kinase family members including JNKs are activated [128]. These responses to ethanol translate into activation of nuclear transcription factors and altered gene expression within the liver, leading to the development of steatosis and the early stages of alcohol-induced liver injury [129]. JNK has been implicated in hepatic injury produced by alcohol, acetaminophen, TNFα, ischemia-reperfusion and hepatitis virus [130-136]. Although it has been suggested that JNK may promote cell death and JNK2 may promote proliferation and survival, it appears that depending on the toxin, tissue and cell type, either JNK1 or JNK2 or both play the major role in cell injury. To clarify a possible role of JNK on acute alcohol induced steatosis, we used a systemic JNK inhibitor (JNK inhibitor XIII) to suppress JNK prior to acute alcohol treatment. It was reported that a JNK inhibitor blunted liver toxicity by acetaminophen partly by reducing oxidative stress in mitochondria [136]. The JNK inhibitor blunted steatosis produced by acute alcohol as well as the acute alcohol elevation of oxidant stress. The role of oxidative stress in acute alcohol induced steatosis was confirmed by the fact that NAC which blocked the elevation in oxidative stress by acute alcohol also blocked the acute alcohol induced steatosis.

In conclusion, acute alcohol treatment initially induces CYP2E1 activation followed by increases in oxidative stress in the liver. Increased oxidative stress activates JNK, while activated JNK further promotes oxidative stress. We propose that the increased oxidative stress and activation of JNK result in mitochondrial dysfunction and liver injury. Interactions between CYP2E1, oxidative stress and JNK activation play a role in the mechanism by which acute alcohol treatment promotes lipid accumulation in the liver. Inhibition of CYP2E1 or of JNK or antioxidant treatment may prove useful in preventing acute alcohol-induced fatty liver.

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Competing interests

None declared.

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