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CYP2E1 and Oxidative Liver Injury by Alcohol

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Abstract

Ethanol-induced oxidative stress appears to play a major role in mechanisms by which ethanol causes liver injury. Many pathways have been suggested to contribute to the ability of ethanol to induce a state of oxidative stress. One central pathway appears to be the induction of cytochrome P450 2E1 (CYP2E1) by ethanol. CYP2E1 metabolizes and activates many toxicological substrates, including ethanol, to more reactive, toxic products. Levels of CYP2E1 are elevated under a variety of physiological and pathophysiological conditions, and after acute and chronic alcohol treatment. CYP2E1 is also an effective generator of reactive oxygen species such as the superoxide anion radical and hydrogen peroxide, and in the presence of iron catalysts, produces powerful oxidants such as the hydroxyl radical. This Review Article summarizes some of the biochemical and toxicological properties of CYP2E1, and briefly describes the use of cell lines developed to constitutively express CYP2E1 in assessing the actions of CYP2E1. Possible therapeutic implications for treatment of alcoholic liver injury by inhibition of CYP2E1 or CYP2E1-dependent oxidative stress will be discussed, followed by some future directions which may help to understand the actions of CYP2E1 and its role in alcoholic liver injury.

Introduction-cytochrome P450, oxidative stress, and alcoholic liver injury

The cytochrome P450 enzymes are a superfamily of hemeproteins that serve as terminal oxidases in the mixed function oxidase system for metabolizing various endogenous substrates such as steroids and fatty acids, and xenobiotics including drugs, toxins and carcinogens (1). Many different enzymes belong to this P450 family; P450s are present in virtually all living organisms. A systematic nomenclature system was developed for the P450 family which is based on the sequence identity of the different P450 enzymes (2,3). The enzymes are named CYP for cytochrome P450, followed by an Arabic number denoting the family (more than 40% identity on the amino acid sequence level), a letter designating the subfamily (more than 55% identity) and finally an Arabic numeral representing the individual gene in the subfamily. The P450s catalyze many different chemical reactions including monooxygenation (insertion of an atom of oxygen into the substrate), peroxidation, reduction, dealkylation, epoxidation, and dehalogenation (4–6). Many different compounds of diverse structure can be metabolized by P450 enzymes. A major function of P450-catalyzed reactions is to convert a compound into a more polar metabolite that can be easily excreted directly by the organism or conjugated by phase II enzymes into more polar excretable metabolites. With some compounds e.g. carbon tetrachloride or acetaminophen, metabolism by P450 can give rise to toxic metabolites which damage cells. For P450s to function catalytically, flavoprotein reductases such as NAPDH-cytochrome P450 reductase, adrenodoxin, adrenodoxin reductase, are necessary to transfer electrons from NAPDH or NADH to reduce the heme from the ferric redox state to the ferrous

state. The latter is necessary to bind molecular oxygen to form the oxygenated P450 complex that catalyzes the diverse chemical reactions mentioned above (7). Cytochrome b5 may also play an important role in electron transfer to certain P450s.

It is important to recognize that oxygen activation by P450, necessary for the enzymes catalytic function, can also result in the production of reactive oxygen species (ROS). Small amounts of the superoxide anion radical ($O_2^{\cdot-}$) can be produced from decay of the oxygenated P450 complex, while hydrogen peroxide (H_2O_2) can form from either dismutation of $O_2^{\cdot-}$ or from decay of the peroxy P450 complex (8–10). ROS have been implicated in many of the major diseases that plague mankind, including the toxicity of O_2 itself; hyperbaric O_2; ischemia-reperfusion injury; cardiovascular diseases; atherosclerosis; carcinogenesis; diabetes; neurodegenerative diseases, including Parkinson's disease and Alzheimer's disease; toxicity of heavy metals, e.g., iron; asbestos injury; radiation injury; vitamin deficiency; drug (e.g., redox cycling agents) toxicity; aging; inflammation; smoking toxicity; emphysema; and toxicity of acute and chronic ethanol treatment (11–15). ROS can be produced from many systems in cells including the mitochondrial respiratory chain (16); the cytochrome P450s (10,17); oxidative enzymes such as xanthine oxidase, aldehyde oxidase, cyclooxygenase, monoamine oxidase, the NADPH oxidase complex (18,19); autooxidation of heme proteins such as ferrohemoglobin or myoglobin or biochemicals such as catecholamines, quinones or tetrahydrobiopterins. In addition to these cellular sources of ROS, environmental sources of ROS include radiation, UV light, smoke and certain drugs which can redox cycle. ROS are toxic to cells because they can react with most cellular macromolecules inactivating enzymes or denaturing proteins, causing DNA damage such as strand breaks, base removal or base modifications which can result in mutation, peroxidation of lipids which can result in destruction of biological membranes and produce reactive aldehydic products such as malondialdehyde or 4-hydroxynonenal (20,21). A variety of enzymatic and non-enzymatic mechanisms have evolved to protect cells against ROS, including the superoxide dismutases, which remove $O_2^{\cdot-}$; catalase and the glutathione (GSH) peroxidase system which remove H_2O_2; glutathione transferases which can remove reactive intermediates and lipid aldehydes; metallothioneins, heme oxygenase, thioredoxin which remove various ROS; ceruloplasmin and ferritin which help remove metals such as iron which promote oxidative stress reactions; non-enzymatic, low molecular weight antioxidants such as GSH itself, vitamin E, ascorbate (vitamin C), vitamin A, ubiquinone, uric acid, bilirubin (22,23). Oxidative stress or toxicity by ROS reflects a balance between the rates of production of ROS compared to the rates of removal of ROS plus repair of damaged cellular macromolecules. While excess ROS can cause toxicity, macrophages and neutrophils contain an NADPH oxidase which produces ROS to destroy foreign organisms (24), and the enzyme myeloperoxidase catalyzes a reaction between H_2O_2 and chloride to produce the powerful oxidant hypochlorite (bleach) to help destroy foreign invaders. In addition, ROS at low concentrations, especially H_2O_2, may be important in signal transduction mechanisms in cells, and thus be involved in cellular physiology and metabolism (25).

The ability of acute and chronic ethanol treatment to increase production of reactive oxygen species and enhance peroxidation of lipids, protein, and DNA has been demonstrated in a variety of systems, cells, and species, including humans. Much has been learned about alcohol metabolism, the various enzymes and pathways involved, and how alcohol, directly via its metabolism, or indirectly via its solvent-like action affecting cellular membranes impacts on cell function. Yet, despite this tremendous growth in understanding alcohol metabolism and actions, the mechanism(s) by which alcohol causes cell injury are still not clear. A variety of leading mechanisms have been briefly summarized (13–15), and it is likely that many of them ultimately converge as they reflect a spectrum of the organism's response to the myriad of direct and indirect actions of alcohol. 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 in how ethanol induces “oxidative stress” (reviewed in 13–15). Again, many of these pathways are not exclusive of one another and it is likely that several, indeed many, systems contribute to the ability of alcohol to induce a state of oxidative stress.

What is the evidence that ethanol-induced oxidative stress plays a role in cell injury? There are many studies which show that administration of antioxidants or iron chelators or GSH-replenishing agents can prevent or ameliorate the toxic action of ethanol. The most convincing data that oxidative stress contributes to alcohol-induced liver injury comes from the studies using the intragastric infusion model of alcohol administration. In these studies, alcohol-induced liver injury was associated with enhanced lipid peroxidation, protein carbonyl formation, formation of the 1-hydroxyethyl radical, formation of lipid radicals, decreases in hepatic antioxidant defense, especially GSH (26–30). Replacement of polyunsaturated fat (required for lipid peroxidation to occur) with saturated fat or medium chain triglycerides in the diets fed to rats intragastrically, lowered or prevented the lipid peroxidation, and the alcohol-induced liver injury (29,30). Thus, alcohol plus polyunsaturated fat was required for the injury to occur. Addition of iron, known to generate OH and promote oxidative stress, to these diets exacerbated the liver injury (31). Importantly, addition of antioxidants such as vitamin E, ebselen, superoxide dismutase (SOD), GSH precursors, prevented the alcohol-induced liver injury (28). Because alcohol-induced liver injury has been linked to oxidative stress, we investigated the effect of a compromised antioxidant defense system, copper-zinc superoxide dismutase (SOD1) deficiency on alcohol-induced liver injury (32,33). A rather moderate ethanol consumption promoted oxidative stress and liver injury in SOD1 knockout mice indicating that compromised antioxidant defense promotes alcohol liver injury.

In addition to these in vivo studies, in vitro studies with hepatocytes also showed that ethanol can produce oxidative stress and hepatocyte toxicity. Studies with isolated hepatocytes from control rats or chronic ethanol-fed rats indicated that ethanol metabolism via alcohol dehydrogenase results in an increase in ROS production, hepatocyte injury, and apoptosis, reactions blocked by antioxidants (34,35). Studies in our laboratory with HepG2 cell lines expressing CYP2E1 showed that addition of ethanol or polyunsaturated fatty acids or iron, or depletion of GSH, resulted in cell toxicity, increased oxidative stress and mitochondrial damage, reactions prevented by antioxidants (36) Recent reviews on the roles of oxidative stress in alcoholic liver disease can be found in (37,38). Since CYP2E1 plays a role in ethanol-induced oxidant stress and is a minor pathway of ethanol oxidation, the biochemical and toxicological properties of CYP2E1 will form the basis for much of the remainder of this review.

CYP2E1 and the microsomal ethanol oxidizing system

Alcohol dehydrogenase is the major enzyme pathway for oxidizing ethanol to acetaldehyde. The morphological observations that chronic-ethanol treatment causes proliferation of the liver smooth endoplasmic reticulum suggested that ethanol, similar to certain xenobiotics which are metabolized by cytochrome P450, may also be metabolized by P450 (39). A microsomal ethanol oxidizing system (MEOS) was characterized by Lieber and associates and shown to be dependent on P450 (40). The Km for ethanol oxidation by MEOS (about 10 mM) was about an order of magnitude greater than the Km for ethanol by alcohol dehydrogenase. Acetaldehyde is the product resulting from ethanol oxidation by MEOS, and it is clear that MEOS represents a minor pathway of ethanol oxidation, probably accounting for less than 10 percent of the liver capacity to oxidize ethanol (41). Importantly, activity of MEOS is enhanced after chronic ethanol treatment, partly due to an increased total content of P450, and partly due to induction of CYP2E1, a member of the P450 family with high catalytic activity with ethanol (40). Induction of MEOS may play an important role in the metabolic tolerance found after chronic

ethanol treatment, i.e., the increased capacity to oxidize ethanol. While there was early controversy over the nature of MEOS, the purification of an ethanol-inducible form of P450 from rabbit liver microsomes firmly established the role of P450 in MEOS (42). Ethanol-inducible P450s have been isolated from many species and while several P450s may be induced by ethanol, the major inducible P450 is now referred to as CYP2E1.

CYP2E1 substrates

CYP2E1 metabolizes a variety of small, hydrophobic substrates and drugs (reviewed in 40, 43–46). Possible physiological substrates are acetone and fatty acids such as linoleic and arachidonic acid (47). From a toxicological point of view, interest in CYP2E1 revolves around the ability of this enzyme to metabolize and activate many toxicologically important compounds such as ethanol, carbon tetrachloride, acetaminophen, benzene, halothane and many other halogenated substrates. Procarcinogens including nitrosamines and azo compounds are effective substrates for CYP2E1 e.g., CYP2E1 is a low Km dimethylnitrosamine demethylase (48). Toxicity by the above compounds is enhanced after induction of CYP2E1 e.g. by ethanol treatment, and toxicity is reduced by inhibitors of CYP2E1 or in CYP2E1 knockout mice (49). Of the substrates, chlorzoxazone is of special value as its hydroxylated product can readily be assayed in the blood and the ratio of 6-hydroxychlorzoxazone/chlorzoxazone is widely used to assess the approximate levels of CYP2E1 in humans, including alcoholics (50).

Molecular oxygen itself is likely to be a most important substrate for CYP2E1. CYP2E1, relative to several other P450 enzymes, displays high NADPH oxidase activity as it appears to be poorly coupled with NADPH-cytochrome P450 reductase (51,52). CYP2E1 was the most efficient P450 enzyme in the initiation of NADPH-dependent lipid peroxidation in reconstituted membranes among five different P450 forms investigated. Furthermore, anti-CYP2E1 IgG inhibited microsomal NADPH oxidase activity and microsomal lipid peroxidation dependent on P450, but not lipid peroxidation initiated by the action of NADPH-cytochrome P450 reductase (52). In our laboratory, we found that microsomes isolated from rats fed ethanol chronically were about twofold to threefold more reactive in generating superoxide radical and H_2O_2 and in the presence of ferric complexes, in generating hydroxyl radical and undergoing lipid peroxidation compared to microsomes from pair-fed controls (53–56). CYP2E1 levels were elevated about threefold to fivefold in the liver microsomes after feeding rats the Lieber-DeCarli diet for four weeks. The enhanced effectiveness of microsomes isolated from the ethanol-fed rats was prevented by addition of chemical inhibitors of CYP2E1 and by polyclonal antibody raised against CYP2E1, confirming that the increased activity in these microsomes was due to CYP2E1.

CYP2E1 is a minor pathway of ethanol oxidation as it catalyzes the two electron oxidation of ethanol to acetaldehyde. Interestingly, acetaldehyde is also a substrate for CYP2E1 and is oxidized to acetate, thus CYP2E1 can, at least theoretically, catalyze the oxidation of ethanol to acetate (57). However, this oxidation is likely to be negligible in the presence of ethanol, the substrate which generates acetaldehyde (58). CYP2E1 can also promote the one electron oxidation of ethanol to the 1-hydroxyethyl radical. Detection of the 1-hydroxyethyl radical in the bile after administration of ethanol to rodents has been a most valuable assay for determining ethanol-induced radical formation and oxidant stress in vivo (26,59).

Mitochondrial CYP2E1

CYP2E1 is mainly found in the liver but significant amounts are also found in most organs, including the brain (60). CYP2E1 is expressed mainly in the hepatocytes of the liver, however, significant amounts are also found in the Kupffer cells (61), and hepatocyte and Kupffer cell CYP2E1 are inducible e.g. by ethanol. CYP2E1, like other xenobiotic metabolizing P450s, is

mainly located in the membrane of the endoplasmic reticulum (ER). CYP2E1 has also been detected in other cellular compartments such as the plasma membrane (62–64). CYP2E1 located at the plasma membrane has been suggested to play a role in the immune mediated hepatotoxicity observed in patients suffering from drug toxicity and ALD (65–67). CYP2E1 was shown to be transported out of the ER to the Golgi apparatus, with subsequent transfer to the plasma membrane (68,69).

Ingelman-Sundberg and co-workers, and Avadhani and co-workers have shown that CYP2E1 is also present in the mitochondria (70–75). Essentially 2 forms of CYP2E1 are present in the mitochondria, a highly phosphorylated form mediated via cAMP-dependent protein kinase A, and a shortened 40 kDa amino terminal-truncated form which lacks the N-terminal amino acids, and which can be further NH2-terminally truncated to produce a mature mitochondrial form of CYP2E1 lacking about 100 amino acids. The phosphorylation and amino terminal truncation are hypothesized to cause conformational changes and altered interactions with molecular chaperones and signal recognition particles and direct the CYP2E1 to the mitochondria. The mitochondrial CYP2E1 is catalytically active with typical substrates but requires, as do the other mitochondrial P450s, adrenodoxin and adrenodoxin reductase (and NADPH) as electron donors (70,73). It is not clear what regulates either the phosphorylation or the amino-terminal truncation which directs CYP2E1 to the mitochondria. Importantly, the mitochondria isolated from rat liver and highly purified and essentially devoid of endoplasmic reticulum contamination, contained CYP2E1 indicating the in vivo presence of mitochondrial CYP2E1 (70,71,73). Robin et al (73) showed pyrazole treatment not only elevated microsomal CYP2E1, but also mitochondrial CYP2E1. The mitochondrial CYP2E1 was present at about 30% of the level of the microsomal CYP2E1 under basal conditions, and at 40% of the level of the microsomal CYP2E1 after pyrazole treatment (73). In a similar manner, streptozotocin-induced diabetes elevated microsomal CYP2E1 2- to 3-fold, and mitochondrial CYP2E1 5- to 6-fold (75); mitochondrial CYP2E1 protein and catalytic activity was 25 to 35% that of microsomal CYP2E1 after treatment with streptozotocin. Raza & John (76) recently reported that 4-hydroxynonenal increased CYP2E1 activity in the mitochondria and postmitochondrial supernatant of PC12 cells, in association with elevated mitochondrial oxidative stress.

To evaluate functional consequences associated with expression of mitochondrial CYP2E1, we established a HepG2 cell line which expresses CYP2E1 in the mitochondria (77). A CYP2E1 expression vector lacking the coding sequences for amino acids 2–34 was generated, cloned into a pCI-neo expression vector, transfected into HepG2 cells, and stable cell lines established by selection for G418 resistance. Western blot analysis of whole cell extracts and of isolated mitochondria, and immunofluoresence of permeabilized cells showed the presence of CYP2E1 in the mitochondrial fraction of mE10 and mE27 cells (HepG2 cells transfected with the amino terminal depleted CYP2E1) but not in pCI vector transfected HepG2 cells or E47 HepG2 cells which express CYP2E1 in the endoplasmic reticulum. Treatment with 0.1 mM BSO for 48 h to lower GSH levels caused a striking loss of cell viability in mE10 and mE27 cells which contain mtCYP2E1 but not in the pCI-neo cells. Toxicity could be prevented by antioxidants such as glutathione ethyl ester and trolox, suggesting enhanced oxidant stress plays a role in the toxicity. Indeed, ROS production (DCF fluorescence) was elevated after BSO addition to the mE10 and mE27 cells. There was an increase in 3-nitrotyrosine protein adducts and 4-hydroxynonenal protein adducts in the mE10 and mE27 cells treated with BSO compared to plasmid vector controls. Mitochondrial membrane potential slightly declined in BSO-treated pCI-neo cells but dramatically declined in the mE10 and mE27 cells. This decline in MMP was prevented by cyclosporine A, and the BSO-induced loss of cell viability in the mE10 and mE27 cells was prevented by cyclosporine A. Importantly, ethanol was shown to elevate the levels of mitochondrial CYP2E1 in addition to the well known increase in microsomal CYP2E1 (78). It is interesting to speculate that damage to mitochondrial function and membrane potential produced by mitochondrial CYP2E1 may be an early event in liver

cell injury and that mitochondrial CYP2E1 may contribute to the biochemical and toxicological effects which were previously ascribed to CYP2E1 in the endoplasmic reticulum. However, at present it is not obvious how effects contributed by mitochondrial CYP2E1 versus microsomal CYP2E1 in vivo or in primary hepatocytes can be distinguished from each other e.g. lack of specific inhibitors.

Induction and regulation of CYP2E1

Many of the substrates for CYP2E1 can induce their own metabolism. This was initially observed with ethanol, which is a substrate for CYP2E1 and elevates CYP2E1 levels (39,40). In fact, these two properties explain the ability of ethanol to inhibit the metabolism of certain substrates when the alcohol is present, i.e., ethanol and the substrate compete for oxidation by CYP2E1, and for ethanol to increase the metabolism of substrates when it is no longer present to compete, but the ethanol treatment elevated the levels of the CYP2E1 catalyst. Ethanol can be oxidized by other P450s besides CYP2E1, notably CYPs 3A and 1A, and ethanol treatment can elevate the levels of these CYPs (79,80). A variety of heterocyclic compounds such as imidazole, pyrazole, 4-methylpyrazole, thiazole, isoniazid have been shown to elevate CYP2E1 levels as do solvents such as dimethylsulfoxide, various alcohols, benzene and acetone (81–83). These low molecular weight compounds have been used in vivo or in vitro to elevated or help prevent loss of CYP2E1 under tissue culture conditions and their mode of mechanism will be discussed below.

CYP2E1 can also be induced under a variety of metabolic or nutritional conditions. For example, CYP2E1 levels were elevated in chronically obese, overfed rats and in rats fed a high-fat diet (84). Somewhat paradoxical, in rats levels of CYP2E1 were increased by fasting and by prolonged starvation (85,86). Diabetes has been reported to increase the expression of CYP2E1 mRNA and protein levels several fold (87). This may be related to actions of insulin which downregulated CYP2E1 expression at the posttranscriptional level in a rat hepatoma cell line (88,89) and in rat hepatocyte culture (90). CYP2E1 levels were elevated in liver and kidney microsomes of rats treated with streptozotocin.

CYP2E1 induction in diabetes may be associated with the elevated production of ketone bodies (91). The carbohydrate content of the diet influences CYP2E1 levels as a low carbohydrate diet increased the extent of induction of MEOS by ethanol (92) and high fat/low carbohydrate diets resulted in the highest levels of CYP2E1 induced by ethanol (93). In this respect, it is interesting that alcohol-induced liver is magnified in diets with very low levels of carbohydrate and high levels of fat (94).

Besides insulin, other hormones can affect CYP2E1 levels. Hypophysectomy and triiodothyronine increase CYP2E1 protein and mRNA levels in contrast to insulin which lowers them (89,95). In primary rat hepatocyte cultures, glucagon lowered CYP2E1 levels by accelerating turnover of the CYP2E1 protein by a cyclic AMP-dependent process (96). Testosterone increased renal but not hepatic CYP2E1 levels (97).

Considerable data have been reported elucidating the molecular mechanism of CYP2E1 regulation by exogenous compounds as well as during pathophysiologiccal conditions. CYP2E1 is regulated by multiple, distinct regulatory mechanisms (83,98,99). The CYP2E1 gene is under transcriptional control during development. In rats, immediately after birth, it is activated and is maximally transcribed within the first week. Upon fasting or induced diabetes, the mRNA for CYP2E1 is increased several fold due to posttranscriptional mRNA stabilization (100). After administration of ethanol, acetone or pyrazole to rats, Song et al found that CYP2E1 mRNA levels did not increase (81). The mechanism of induction was, therefore, suggested to be at the level of protein degradation. CYP2E1 is not transcriptionally activated

by an acute bolus dose or chronic administration of ethanol, acetone, or other exogenous inducing agents. Although elevation of CYP2E1 mRNA levels has been reported (101), most investigators have found little induction or slight reduction of CYP2E1 mRNA level after ethanol administration (81,83,102). From in vivo data of CYP2E1 turnover in rats chronically treated with acetone (103) and in vitro hepatocyte culture systems (104,105), exogenous CYP2E1 inducers such as acetone, ethanol, imidazole and 4-methylpyrazole (4-MP) were shown to increase CYP2E1 by protein stabilization. Roberts et al (106,107) reported that ethanol increases CYP2E1 by protein stabilization. This phenomenon was observed not only in the liver but also other extra-hepatic tissues such as kidney, brain and intestine. In addition, CYP2E1 protein stabilization appeared dependent on blood ethanol or acetone concentration. Furthermore, a turnover study, using in vivo radiolabeling of CYP2E1 with (^{14}C)$NaHCO_3$ and immuno-purification, demonstrated that ethanol treatment abolished the rapid phase of CYP2E1 degradation while biphasic degradation of CYP2E1 was observed in the control animals (108). Pyrazole and 4-MP elevated liver and kidney CYP2E1 immunoreactive protein and catalytic activity in the absence of an increase in CYP2E1 mRNA levels (109–111). In isolated rat hepatocyte cultures, CYP2E1 mRNA and protein levels and CYP2E1 catalytic activity rapidly declined with time in culture. Addition of pyrazole or 4-MP slowed the decline in CYP2E1 protein and activity, without any effect on CYP2E1 mRNA levels (105). Similarly, McGhee et al (112) reported the half-life of CYP2E1 in a hepatoma cell line to be 1.8 h in the absence of ethanol and 45 h in the presence of ethanol. It is clear that a major level of regulation of CYP2E1 formation appear to be posttranscriptional as various substrates and ligands increase the content of CYP2E1 by protection against rapid degradation by intracellular proteolytic pathways.

What are the proteolytic systems responsible for CYP2E1 turnover and prevented from their action on CYP2E1 by ethanol? Roberts (113) provided evidence for a role of the proteasome in the degradation of several cytochrome P450s including CYP2E1. Huan et al (114) showed that in a Hela cell line, inhibitors of the proteasome decreased the degradation of CYP2E1 and CYP2B1. They found that ubiquitination of CYP2E1 was not required for its degradation by the proteasome. However, Banerjee et al (115) using molecular models predicted a cytosolic domain of CYP2E1 which would function as a putative ubiquitination-target/substrate interaction structure. An antibody recognizing this domain (amino acids 317–340) quenched CYP2E1 ubiquinitation and inhibited CYP2E1 catalytic activity. They suggested that substrate binding shields the CYP2E1 protein from turnover by blocking the ubiquitination domain. Morishima et al (116) reported that a HSP90 inhibitor promoted CYP2E1 degradation by the proteasome. They found that purified bacterially expressed truncated CYP2E1 (Δ3–29) is ubiquitylated by the E3 ubiquitin ligase CHIP and concluded that CYP2E1 is a HSP90 “client” protein. In contrast, Huan et al (114) found that three HSP90 inhibitors had no effect on CYP2E1 turnover. We found that in an in vitro reconstituted system containing microsomes or cytosol or purified 20S proteasome, geldanamycin, an inhibitor of HSP90, decreased CYP2E1 degradation and suggested that HSP90 helps present oxidized CYP2E1 to the proteasome for degradation (117). The proteasome complex was important in the degradation of CYP2E1 in HepG2 cells, as proteasome inhibitors proved to be effective in preventing CYP2E1 degradation. Importantly, Bardag-Gorce et al (118) showed that the rapid loss of CYP2E1, which occurs in vivo after the ethanol inducer is withdrawn, could be blocked by the proteasome inhibitor PS-341, thus establishing the critical role of the proteasome in regulating CYP2E1 turnover in vivo.

CYP2E1 and alcohol-induced liver injury

Since CYP2E1 can generate ROS during its catalytic circle, and its levels are elevated by chronic treatment with ethanol, CYP2E1 has been suggested as a major contributor to ethanol-induced oxidant stress, and to ethanol-induced liver injury. Initial suggestions for a role for

CYP2E1 in alcoholic liver injury arose from studies with the intragastric model of ethanol feeding in which prominent induction of CYP2E1 occurs and in which significant liver injury occurs (29–31). 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 (29,30,119,120). Experimentally, a decrease in CYP2E1 induction was found to be associated with a reduction in alcohol-induced liver injury (121, 122). CYP2E1 inhibitors such as diallyl sulfide (DAS) (123), phenethyl isothiocyanate (PIC) (124,125) and chlormethiazole (126), blocked the lipid peroxidation and ameliorated the pathologic changes in ethanol-fed rats. Polyenylphosphatidylcholine (PPC), another compound exerting anti-CYP2E1 properties (127) was effective in opposing alcohol-induced oxidative stress (40). A strong association between dietary carbohydrate, enhanced CYP2E1 induction and hepatic necrosis was observed. No liver injury was found if carbohydrate levels were elevated (128). It was concluded that diet is an important factor in toxicity mediated by ethanol because of modulation of the levels of CYP2E1 (128). Ethanol consumption in oral liquid diets does not cause significant liver injury. However, micro and macrovesicular steatosis, occasional inflammatory foci and a three-fold increase in transaminase levels was observed in a nutritional adequate ethanol containing liquid diet with a carbohydrate content of 5.5%; no changes were found if the level of carbohydrate was elevated to 11% (94,129). Thus dietary and nutritional factors play a key role in the toxic actions of ethanol to the liver, in part, due to modulation of the levels of CYP2E1. Recently, A CYP2E1 transgenic mouse model was developed that overexpressed CYP2E1. When treated with ethanol, the CYP2E1 overexpressing mice displayed higher transaminase levels and histological features of liver injury compared with the control mice (130). We developed an adenoviral vector which expresses human CYP2E1 and showed that infection of HepG2 cells with this adenovirus potentiated acetaminophen toxicity as compared to HepG2 cells infected with a LacZ expressing adenovirus (131). Administration of CYP2E1 adenovirus in vivo to mice produced significant liver injury compared to the LacZ-infected mice as reflected by histopathology, markers of oxidative stress and elevated transaminase levels (132).

On the other hand, studies by Thurman and colleagues have presented powerful support for a role for endotoxin, activation of Kupffer cells and cytokines such as TNFα in the alcohol-induced liver injury found with the intragastric infusion model (133,134). They suggested that CYP2E1 may not play a role in alcohol liver injury based upon studies with gadolinium chloride or CYP2E1 knockout mice (135,136). Female CYP2E1 wild type mice or knockout mice were given a high fat liquid diet intragastrically with either ethanol or isocaloric maltose-dextrin for 4 weeks. Mice given ethanol had elevated transaminases, mild steatosis and slight inflammation and necrosis with no differences in pathology between the wild type and the knockouts (135). However, Bardag-Gorce et al (137) using the same model reported that ethanol-induced oxidative stress and inactivation of the proteasome complex was completely prevented in these mice. They concluded that CYP2E1 induction by chronic ethanol treatment was responsible for the decrease in proteasome activity and accumulation of oxidized proteins in the liver. They speculated the pathology found in the CYP2E1 knockouts by Kono et al (135) may be due to upregulation of NADPH-cytochrome P450 reductase and other CYPs such as CYP3A and 4A (137) (see below). As to their observations with gadolinium chloride, others have reported that gadolinium chloride does indeed decrease levels of several P450 enzymes including CYP2E1, and lowered the induction of CYP2E1 by ethanol. Moreover, Leclercq et al. (138) using the same knockout mice observed that other CYPs, notably CYP4A10 and CYP4A14, were upregulated in the CYP2E1 knockout but not the wild type mice; these CYPs were, like CYP2E1, active generators of ROS and catalysts of lipid peroxidation, and in the absence of CYP2E1 served as alternative initiators of oxidative stress. Bradford et al (139) 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. 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.

Cell lines expressing CYP2E1

To characterize the biochemical and toxicological properties of CYP2E1, several investigators have developed cell lines to express CYP2E1. The first cell line to be developed was to introduce human CYP2E1 into NIH 3T3 mouse fibroblasts via retroviral infection followed by selection via G418 resistance (140). Southern blot analysis showed the viral DNA was integrated into the cellular DNA. The transduced CYP2E1 was catalytically active, oxidizing ethoxycoumarin to 7-hydroxycoumarin, and forming labeled covalent DNA adducts after the incubation with (^{14}C)-nitroso-dimethylamine (140). The cytotoxic effect of N-nitrosodimethylamine was studied in the P450-expressing human fibroblast cell line GM2E1, which express the rat CYP2E1 (141). N-nitrosomethylamine was toxic to the cells expressing CYP2E1; toxicity was decreased by CYP2E1 inhibitors and was partially prevented by antioxidants (141). Toxicity was apoptotic in nature and could be prevented by caspase inhibitors (142). A V79 Chinese Hamster cell line expressing human CYP2E1 was used in toxicological studies involving CYP2E1-mediated activation of N-nitrosodimethylamine and p-nitrophenol and for mutagenicity studies with the former substrate (143). Human CYP2E1 was also expressed in the rat adrenal pheochromocytoma cell line, PC12 (144). CYP2E1 metabolism of several different substrates was characterized in these cells; levels of enzyme activities were about 10% that of human liver microsomes, similar to what our lab found with HepG2 cells expressing CYP2E1 (145). The PC12 cells were shown to metabolize acetaminophen, and activation of this protoxin caused a loss of viability to the cells (144). Acetaminophen toxicity was also characterized in a human hepatoman cell line, HLE, expressing human CYP2E1 (146). Treating these cells with buthionine-sulfoximine to lower GSH levels also produced a decrease in cell viability which could be inhibited by ethanol or vitamin E. This cell line has been used to examine changes of heme metabolism via assays of δ-aminoleulinic acid synthase and heme oxygenase-1, the rate limiting enzymes in heme synthesis and heme breakdown (147). Both enzymes were upregulated in the HLE cells expressing CYP2E1 perhaps due to the demand for increased heme synthesis for holoCYP2E1 formation and perhaps increased availability of heme to induce heme oxygenase. Our lab has also observed an increase in heme oxygenase-1 mRNA, protein and activity in HepG2 cells expressing CYP2E1, perhaps an increase in response to CYP2E1-generated oxidant stress (148). Huan and Koop (149) established a tetracycline-controlled rabbit CYP2E1-expressing system in Hela cells in culture. This system was used to evaluate turnover of the rabbit CYP2E1, which was rapid with a half-life of 3.9 h in the absence of a stabilizing substrate or ligand. Addition of the latter, 4-methylpyrazole, decreased the degradation of CYP2E1. We observed similar results in HepG2 cells expressing CYP2E1 as the half-life of human CYP2E1 was about 3–6 h in the absence of substrate or ligand, and was elevated in the presence of various substrates and ligands (150). The CYP2E1 half-life was also elevated by an inhibitor of the proteasome complex. Recently, a comparison of mouse, rat and human CYP2E1 activities in V79 Chinese Hamster cell lines was made to study possible species differences in toxicity and metabolism of CYP2E1 substrates (151).

A HepG2 cell model expressing human CYP2E1 was established by Patten et al (152) using the vaccinia virus expression system. The oxidation of several typical substrates of CYP2E1 was evaluated in these cells and the ability of cytochrome b5 to elevate CYP2E1 activity was shown. As briefly mentioned above, an approach that our laboratory has utilized to try to understand basic effects and actions of CYP2E1 was to establish cell lines that constitutively express human CYP2E1. HepG2 cell lines, which overexpress CYP2E1, were established either by retroviral infection methods (MV2E1-9 cells, or E9 cells) or by plasmid transfection

methods (E47 cells) (145,153). Results utilizing E9 or E47 cells to study CYP2E1-generated oxidant stress have been summarized in recent reviews (154–156). We have characterized the toxicity of ethanol, polyunsaturated fatty acids (PUFA) such as arachidonic acid (AA) and iron in E9 and E47 cells. Concentrations of ethanol or AA or iron which were toxic to the CYP2E1-expressing cells had no effect on control HepG2 cells not expressing CYP2E1 or to HepG2 cells expressing a different P450, CYP3A4 (3A4 cells). Toxicity to CYP2E1-expressing cells was found when GSH was depleted by treatment with L-buthionine sulfoximine (157). Inhibitors of CYP2E1 prevented the toxicity by the above treatments. Antioxidants such as vitamin E, trolox and ascorbate also prevented toxicity found when the CYP2E1-expressing E9 HepG2 cells were treated with either ethanol or AA. The above treatment of CYP2E1-expressing cells with ethanol, AA, iron or BSO resulted in an increase in oxidative stress to the cells as reflected by increased lipid peroxidation and enhanced dichlorofluorescein fluorescence. Low concentrations of iron and AA that are not cytotoxic by themselves can act as priming or sensitizing factors for CYP2E1-dependent loss of viability in HepG2 cells or rat hepatocytes. This synergistic toxicity was associated with elevated lipid peroxidation and could be prevented by antioxidants which prevent lipid peroxidation. Damage to mitochondria by CYP2E1-derived oxidants seems to be an early event in the overall pathway of cellular injury.

Adaptation to oxidant stimuli is critical for short- and long-term survival of cells exposed to oxidative stress. We found that the levels of GSH and several antioxidant enzymes such as glutathione transferase, catalase, and heme oxygenase-1 were up-regulated in the CYP2E1-expressing cells. This upregulation was prevented by antioxidants, suggesting that ROS generated by CYP2E1 were responsible for the transcriptional activation of these antioxidant genes. Because of this activation of antioxidant genes, the CYP2E1-expressing cells were less sensitive to toxicity to H_2O_2, menadione, or HNE than control cells. We believe that the upregulation of these antioxidant genes reflect an adaptive mechanism to remove CYP2E1-derived oxidants. Recent experiments suggested that Nrf2 plays a key role in the adaptive response against the increased oxidative stress caused by CYP2E1 (158)(reviewed in 159).

A working model of CYP2E1-dependent oxidative stress and toxicity is shown in Figure 1. Ethanol increases levels of CYP2E1, largely by a posttranscriptional mechanism involving enzyme stabilization against degradation. CYP2E1, a loosely coupled enzyme, generates ROS such as $O_2^{\cdot-}$ and H_2O_2 during its catalytic cycle. In the presence of iron, which is increased after ethanol treatment, more powerful oxidants including OH, ferryl species, and 1-hydroxyethyl radical are produced. Initially, the liver cells respond to the CYP2E1-related oxidative stress by transcriptionally inducing various antioxidant enzymes via their antioxidant response elements. Ultimately, these protective mechanisms are overwhelmed and the cells become sensitive to the CYP2E1-generated oxidants. These various oxidants can promote toxicity by protein oxidation and enzyme inactivation, oxidative damage to the DNA, and disturbing cell membranes via lipid peroxidation and production of reactive lipid aldehydes, such as malondialdehyde and 4-hydroxynonenal. Mitochondria appear to be among the critical cellular organelles damaged by CYP2E1-derived oxidants. A decrease of $\Delta\Psi m$, likely due to the mitochondrial membrane permeability transition, causes release of proapoptotic factors resulting in apoptosis. A decrease in ATP levels will cause necrosis. Some CYP2E1-derived ROS, such as H_2O_2, LOOH, and HNE, are diffusible and may exit hepatocytes and enter other liver cell types such as stellate cells and stimulate these cells to produce collagen and elicit a fibrotic response (160,161). We believe that the linkage between CYP2E1-derived oxidative stress, mitochondrial injury, and GSH homeostasis contribute to the toxic actions of ethanol on the liver.

Other investigators have utilized E9 and E47 HepG2 cells expressing CYP2E1 in a variety of studies including evaluating the effects of ethanol and acetaldehyde on activation of the transcriptional factors AP-1 and NF-kB (162), in proteomic studies on ethanol-induced

oxidation of mitochondrial and cytosolic proteins (163,164), on ethanol-induced inhibition of the proteasome and cytokeratin aggresome formation (165), on comparison of gene expression patterns induced by alcohol in vivo and in vitro (166), on CYP2E1-hepatitis C virus (167) or hepatitis B virus (168) interactions, on acetaminophen alterations of the microsomal ryanodine calcium channel (169), on fatty acid ethyl ester toxicity (170), and in ethanol potentiation of TNFα cytotoxicity (171) and the role of P38 MAPK pathways in ethanol plus TNFα toxicity (172). A rather interesting HepG2 cell culture model in which CYP2E1 and alcohol dehydrogenase are both expressed has been effectively utilized to study ethanol-CYP2E1-proteasome interactions, interferon gamma induction of the proteasome and interferon gamma signaling and antigen processing (173–175). The use of these combined cell lines as a model of ethanol-elicited cytotoxicity has recently been reported (176). Thus, CYP2E1 biochemistry, oxidant stress and toxicology have been extensively studied in a variety of stable cell lines.

The CYP2E1 knockout mouse

CYP2E1 knockout mice were developed by Gonzalez and colleagues to determine the role of CYP2E1 in xenobiotic metabolism and toxicity (49,177,178). 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. For example, there was no liver pathology or elevation of transaminases induced by CCl4 in CYP2E1 knockout mice compared to wild type mice, leading to the conclusion that CYP2E1 is the major factor in CCl_4 hepatotoxicity (179). Blood acetone levels were elevated 2.5–4 times in wild type mice after 48 h fasting but elevated 28-fold in the CYP2E1 knockout mice, leading to the conclusion that CYP2E1 plays a critical role in catabolism of acetone after fasting (180). Formation of benzene metabolites such as hydroquinone, catechol and phenol were lowered more than 90% with microsomes from CYP2E1 knockout mice compared to microsomes from controls (181). The CYP2E1 knockout mice have been used to validate the important role of CYP2E1 in metabolism of thioacetamide, trichloroethylene, acrylonitrile and urethane (182–186). The half-life for urethane was 0.8 h in wild type mice expressing CYP2E1 and 22 h in CYP2E1 knockout mice (186). Interestingly, no difference in oxidation of styrene to styrene oxide was observed between microsomes from wild type mice and knockout mice (187). Since the styrene metabolite styrene oxide was comparably toxic in wild type and knockout mice, the decreased sensitivity of the CYP2E1 knockout mice to styrene likely should be due to decreased bioactivation of styrene to styrene oxide. Yet no differences in styrene metabolism were found, disconnecting the metabolism from the toxicity for unknown reasons (187). The CYP2E1 knockout mice have been used to validate that hydroxylation of p-nitrophenol may be used as a specific probe for CYP2E1 (188).

The metabolism of acetaminophen has been widely studied. CYP2E1 knockout mice were highly resistant to liver toxicity as compared to wild type mice treated with acetaminophen (49). Mice lacking both CYP2E1 and 1A2 were almost completely resistant to acetaminophen toxicity (189). The combination of ethanol plus isopentanol caused an increase in acetaminophen hepatotoxicity in CYP2E1 knockout mice which was sensitive to the CYP3A inhibitor, triacetyloleandomycin, leading to the suggestion that both CYP2E1 and CYP3A contribute to acetaminophen toxicity in ethanol plus isopentanol-treated mice (190). Recently, a CYP2E1-humanized transgenic mouse model that expresses functional and inducible human CYP2E1 was described (191). Comparisons between CYP2E1-humanized mice, CYP2E1 knockout mice and wild type mice will allow a determination whether actions of human CYP2E1 are similar to those of mouse CYP2E1 in vivo. Indeed, the CYP2E1-humanized mouse model was successfully used to characterize acetaminophen toxicity by human CYP2E1 (191).

With respect to the role of CYP2E1 in alcohol-induced liver injury, as discussed above, Bardag-Gorce et al (137) reported that ethanol-induced oxidative stress and inactivation of the proteasome complex was completely prevented in CYP2E1 knockout mice. They concluded that CYP2E1 induction by chronic ethanol treatment was responsible for the decrease in proteasome activity and accumulation of oxidized proteins in the liver. In a very interesting study, Bradford et al (139) found that ethanol treatment for four weeks led to an increase in oxidative DNA damage and induction of expression of basic excision DNA repair genes in wild type mice but not in CYP2E1 knockout mice. The increase in DNA repair genes in wild type mice was abolished by treatment with a P450 inhibitor. The induction and the DNA damage induced by ethanol was the same in wild type mice and NADPH oxidase deficient mice. The authors concluded that CYP2E1 but not NADPH oxidase is required for the ethanol induction of oxidative stress to DNA and thus CYP2E1 may play a key role in ethanol-associated hepatocarcinogenesis (139). On the other hand, as mentioned above, studies by Thurman and colleagues suggests that CYP2E1 may not play a role in alcohol-induced liver injury (135). Instead, their studies have presented powerful support for a role for endotoxin (LPS), activation of Kupffer cells and cytokines such as TNFα in the alcohol-induced liver injury found with the intragastric infusion model.

LPS/TNFα-CYP2E1 Interactions

Abnormal cytokine metabolism is a major feature of alcoholic liver disease as described in many review articles (192–195). Rats chronically fed ethanol were more sensitive to the hepatotoxic effects of administration of LPS and had higher plasma levels of TNFα than control rats (196–198). In the intragastric model of chronic ethanol administration, the development of liver injury coincided with an increase in TNFα, associated with an increase in serum LPS (195,197–199). The pioneering studies of Thurman and collaborators showed that anti-TNFα antibody prevented alcohol liver injury in rats (200) and mice lacking the TNFR1 receptor did not develop alcohol liver injury (133). Taken as a whole, these and other studies clearly implicate TNFα as a major risk factor for the development of alcoholic liver injury. 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 (201,202). Known factors which sensitize the liver to TNFα are inhibitors of mRNA or protein synthesis, which likely prevent the synthesis of protective factors, inhibition of NF-κB activation to lower synthesis of such protective factors, depletion of GSH, especially mitochondrial GSH, lowering of s-adenosyl methionine (SAM) coupled to elevation of S-adenosyl homocysteine (SAH) i.e. a decline in the SAM/SAH ratio, or inhibition of the proteasome (203–210). Of major relevance to this review, is the work by Hoek and collaborators that 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 (171). The ethanol sensitization of TNFα toxicity in E47 cells and hepatocytes from chronic ethanol fed rats also depended on P38 MAPK signaling since SB203580, a P38 MAPK inhibitor, prevented this enhanced toxicity (172). In a RALA hepatocyte cell line model, Czaja and collaborators showed that hepatocytes with increased expression of CYP2E1 were sensitized to TNFα mediated cell death (211). Toxicity was a mixture of necrosis and apoptosis, was associated with prolonged activation of JNK and phosphorylation of c-Jun, and could be prevented by a dominant negative c-Jun construct (211). These results suggest that increased oxidant stress from CYP2E1 may sensitize isolated hepatocytes to TNFα-induced toxicity.

Since CYP2E1 and LPS/TNFα are believed to be key risk factors in the development of alcoholic liver injury, we evaluated possible interactions in promoting liver injury between them in vivo (212,213). Sprague-Dawley rats were treated with 200 mg/kg body weight

pyrazole in the absence or presence of LPS (10 mg/kg) and killed at 8 h after LPS (212). C57BL/6 mice were treated with 150 mg/kg body weight pyrazole in the absence or presence of LPS (4 mg/kg) and killed at 24 h after LPS (213). The combination of LPS plus pyrazole treatment resulted in elevated ALT and AST levels in rats and mice. Liver injury was confirmed by H&E staining. LPS alone or pyrazole alone did not elevate transaminase levels and did not produce liver injury under these conditions. Increased 3-Nitrotyrosine protein adducts were observed at 8 (in rats) and 24 h (in mice) after LPS plus pyrazole treatment (212,213). Positive staining for 4-hydroxynonenal adducts was found at 24 h in the LPS plus pyrazole mice (213). The CYP2E1 inhibitor chlormethiazole (CMZ) protected against the elevation in ALT and AST in mice and the histopathology changes. CYP2E1 catalytic activity was decreased about 50% by the CMZ treatment (213). We obtained CYP2E1 knockout mice from Dr. Frank Gonzalez, NCI. These mice were treated with pyrazole plus LPS. Compared to SV/129 wild type mice, ALT and AST levels were lower in the CYP2E1 null mice, histopathology was normal and TUNEL staining was much less (213). Western blot analysis confirmed the absence of CYP2E1 in the CYP2E1 knockout mice (213). Based on such studies, we hypothesize (212,213) that increased production of ROS by CYP2E1 may prime or sensitize the liver to LPS/TNFα, and such interactions may be important in alcohol-induced liver injury.

Nonalcoholic fatty liver disease, steatohepatitis and CYP2E1

Nonalcoholic steatohepatitis (NASH) is a progressive liver disorder that occurs in patients without significant alcohol consumption. The pathogenesis of NASH is not well understood, although it has been suggested that oxidative stress and lipid peroxidation may play key roles in the pathogenesis of NASH (214–217). Elevated CYP2E1 was observed in conditions such as obesity and high fat/low carbohydrate diets (84). Weltman et al (218) reported increased liver expression of CYP2E1 in the methionine-choline deficient model of NASH. CYP2E1 activity, protein level and mRNA levels were all elevated in this experimental model of NASH, although total P450s content was decreased. Inhibitor studies further suggested that CYP2E1 was the major catalyst of lipid peroxidation in mice fed the methionine-choline deficient diet (138). However, CYP2E1 knockout mice fed with this diet still displayed elevated lipid peroxidation and NASH (138). Under these conditions, CYP4A10 and CYP4A14 but not CYP1A or CYP3A were upregulated and could replace the deficient CYP2E1 as catalysts for microsomal lipid peroxidation. Thus, while CYP2E1 contributes to the pathogenesis of NASH, it is not unique among P450 enzymes in promoting oxidant stress as some CYP4A enzymes can serve as alternative initiators of oxidant stress in the liver. Interestingly, antibody against CYP2E1 strongly inhibited lipid peroxidation by microsomes from wild type mice but antibody against CYP4A had little effect (138). The opposite was found with microsomes from CYP2E1 knockout mice as antibody against CYP4A blocked lipid peroxidation whereas antibody against CYP2E1 had no effect. Thus CYP4A can mediate lipid peroxidation as an alternative pathway when CYP2E1 is absent (219). This can partially explain the observations by Kono et al (135) that in the intragastric infusion model of alcohol-induced liver injury, injury persisted in CYP2E1 knockout mice ie, possible upregulation of CYP4A or other enzymes could replace CYP2E1 as initiators or catalysts of oxidative stress.

Hepatic CYP2E1 levels were increased in patients with NASH (220). Chalasani et al (221) measured liver CYP2E1 activity in a cohort of nondiabetic patients with NASH and controls. They found that chlorzoxazone clearance was greater in the NASH patients compared with controls and lymphocyte CYP2E1 mRNA levels were also higher in the NASH patients. Increases in CYP2E1 correlated with increases in the ketone body β-hydroxybutyrate. They suggested that although more studies were necessary, CYP2E1 is a reasonable candidate in the pathogenesis of human NASH (221). In a recent study involving obese patients with nonalcoholic liver disease, increased CYP2E1 protein content and activity correlated with the development of liver injury (222).

Since CYP2E1 is elevated in pathophysiological conditions such as obesity and diabetes, we recently evaluated the effects of CYP2E1 induction on promoting oxidative and nitrosative stress and liver injury in ob/ob mice, an experimental model of obesity (223). Ob/ob mice and lean controls were treated with pyrazole or acetone to induce CYP2E1. CYP2E1 protein and activity were elevated in acetone or pyrazole-treated obese and lean mice. Acetone or pyrazole induced distinct histological changes in liver and significantly higher aminotransferase enzymes in obese mice compared to obese controls or acetone-or pyrazole-treated lean mice (223). Increased malondialdehyde, protein carbonyls, 4-hydroxynonenal-protein adducts, elevated levels of inducible nitric oxide synthase, and higher 3-nitrotyrosine protein adducts were found in livers of pyrazole-treated obese animals, suggesting elevated oxidative and nitrosative stress (223). Liver TNFα levels were higher in pyrazole-treated animals. The CYP2E1 inhibitor CMZ and iNOS inhibitor N-(3-(amino-methyl)-benzyl) acetamidine (1400W) abrogated the elevated toxicity (transaminases, caspase 3, triglyceride) and the oxidative stress (protein carbonyl, HNE adduct formation, malondialdehyde) elicited by the induction of CYP2E1 (223). Peroxynitrite ($ONOO^-$), formed by the rapid reaction between NO and $O_2^{\cdot-}$ has been shown to nitrate free and protein-associated tyrosine residues and produce nitrotyrosine (212), therefore, either decreased NO by 1400W or declined $O_2^{\cdot-}$ by CMZ prevented 3-NT formation (223). These results show that obesity contributes to oxidative/ nitrtosative stress and liver injury and that induction of CYP2E1 may synergize with high fat in obesity to promote liver cell injury.

Future Perspectives

Alcohol-induced liver injury is probably a multifactorial process involving several mechanisms. Future studies are required to further clarify how alcohol produces oxidative stress in various tissues. Some of the major proposed systems require more detail about mechanism, e.g., how ethanol-derived NADH, itself or when reoxidized in the mitochondrial respiratory chain, produces ROS. What is the role of ethanol metabolism or ethanol metabolites like acetaldehyde in the production of ROS, and how is oxidative stress produced by ethanol in tissues with limited ethanol metabolism? What are the priming or sensitizing factors for ethanol-induced oxidant stress and cell injury? Can markers predictive of individuals particularly sensitive to ethanol-induced oxidant stress and liver injury be developed?

The role of CYP2E1 in the toxic effects of ethanol requires further study as this remains a controversial issue. This is significant not only from a mechanistic point of view but perhaps from a therapeutic treatment approach. If indeed CYP2E1-induced oxidative stress plays a central role in alcohol-induced liver damage, possible strategies for preventing this stress may be effective in attempts to minimize the hepatotoxicity of ethanol in humans. The CYP2E1 inhibitors which were partially effective in preventing ethanol-induced liver injury are not entirely selective and may be toxic, although chlormethiazole (CMZ) (126) or polyenylphosphatidylcholine (PPC) (127) may merit further consideration. YH439 is a novel synthetic compound inhibiting CYP2E1 (but also other P450s) that is being evaluated as a hepatoprotective agent (224). Actually, natural agents inhibiting CYP2E1, including dially sulfide (from garlic) mentioned above, phenylethyl isothiocyanate and sulforaphane (present in cruciferous vegetables) and bergamottin (found in the essential oils of grapefruit and certain oranges) have been proposed as possible candidates for minimizing the ethanol-induced hepatotoxicity (225). In addition, trans-1,2-dichloroethylene (DCE) was reported to be a selective inhibitor of CYP2E1 (226).

Regulation of CYP2E1 protein levels is complex, with transcriptional, translational, and posttranscriptional effects observed; more mechanistic details as to how ethanol modulates CYP2E1 levels are required to define, e.g. effects on activity of the proteasome, ubiquitination, how ethanol stabilizes CYP2E1. What are the factors which trigger the rapid turnover of

CYP2E1? Most studies on the biochemical and pharmacological actions of CYP2E1 are derived from studies with rodents and rabbits and cultured hepatocytes: extrapolation to human studies is obviously necessary. The role of polymorphic forms of CYP2E1 on CYP2E1 expression, activity, and action requires further understanding, as current literature suggests some possible relationships with certain types of cancers but not with alcohol toxicity. Are there endogenous substrates for CYP2E1? At present, acetone and some fatty acids (omega-1 hydroxylase activity) appears to be physiological substrates for CYP2E1, but further studies should be carried out because altered metabolism of such putative endogenous substrates, if any, could contribute to the cellular actions associated with CYP2E1. CYP2E1 is present, although at relatively low levels, in other tissues, e.g. kidney, lung, brain, gastrointestinal tract. Much less is known about the actions of CYP2E1 under various pathophysiological conditions or after chronic ethanol exposure in these tissues. CYP2E1-nutritional interactions require further study, especially interactions with prooxidants, such as iron; polyunsaturated fatty acids; or reagents that lower oxidant defense, e.g., lower GSH levels. There is much current interest in synergistic interactions between alcohol and hepatitis B or hepatitis C virus, especially with respect to generating oxidative stress. The role of CYP2E1 in such synergistic interactions, if any, would be important to explore in view of the many chemicals and conditions that are known to elevate CYP2E1.

The ability of alcohol to promote oxidative stress and the role of free radicals in alcohol-induced tissue injury clearly are important areas of research, particularly because such information may be of major therapeutic significance in attempts to prevent or ameliorate alcohol's toxic effects, e.g., by antioxidants, iron chelators, inhibitors of CYP2E1 or of cytokine production/actions, and GSH replenishment. As basic information continues to emerge regarding the role of oxidative stress in disease development and the mechanisms underlying ROS-related cellular toxicity, these findings will lead to more rational antioxidant therapeutic approaches. Moreover, these finding could result in the development of more effective and selective new medications capable of blocking the actions of CYP2E1 and ROS and, consequently, the toxic effects of alcohol.

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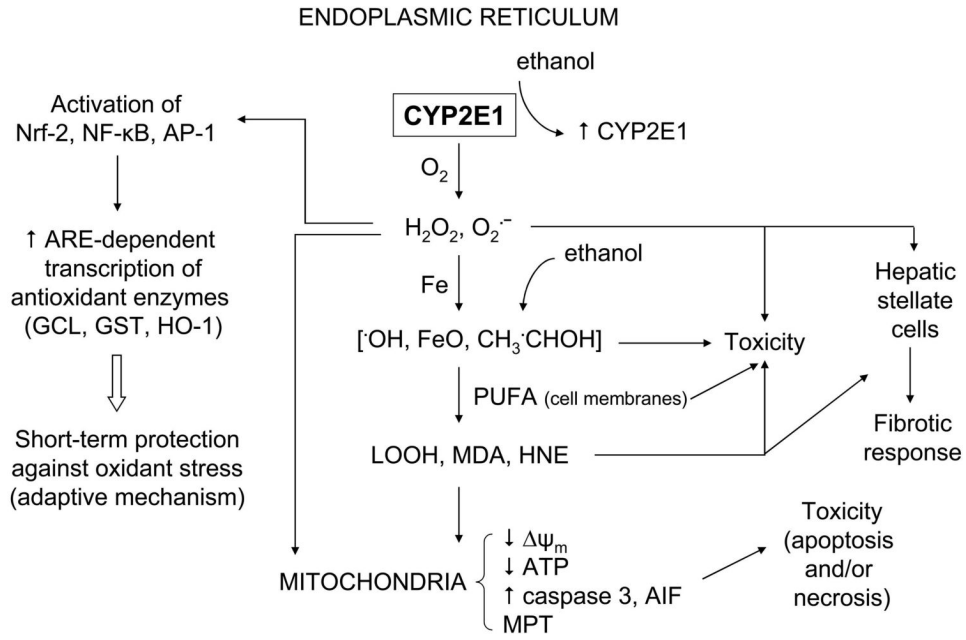

Fig. 1.

Working model of CYP2E1-dependent oxidative stress and cytotoxicity. Please see the text for discussion.