

Role of Cytochrome P450 and Oxidative Stress in Alcohol-Induced Liver Injury

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ABSTRACT | Alcohol-induced liver injury is a significant global health problem. The mechanisms by which alcohol causes cell injury are still not clear. Many of the hepatic toxic effects of ethanol have been linked to its metabolism in the liver and shown to correlate with cytochrome P450 2E1 (CYP2E1) levels, lipid peroxidation, and elevated formation of reactive oxygen species (ROS). CYP2E1 is induced under a variety of pathophysiological conditions, by drugs, and by alcohol. The goal of this review is to describe and characterize the liver cytochrome P450 microsomal mixed function oxidase system including the generation of ROS. The review then discusses biochemical and toxicological properties of CYP2E1 and its contribution to ethanol-elevated oxidant stress and liver pathology.

KEYWORDS | Alcohol; CYP2E1; Cytochrome P450; Ethanol; Liver toxicity; Microsomal mixed function oxidase; Oxidative stress; Reactive oxygen species

ABBREVIATIONS | CYP, cytochrome P450; GSH, reduced glutathione; GSSG, glutathione disulfide; 4-HNE, 4-hydroxynonenal; MDA, malondialdehyde; MEOS, microsomal ethanol oxidation system; NASH, nonalcoholic steatohepatitis; ROS, reactive oxygen species; SOD, superoxide dismutase

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1. INTRODUCTION

Acute and chronic ethanol can react through many pathways to affect the liver as well as other tissues. The liver is a major target for ethanol-induced toxicity; however, the mechanisms by which ethanol causes liver damage remain unclear despite a wealth of information on how ethanol, either directly or metabolically, impacts on liver metabolism and function. Possible mechanisms by which ethanol can cause hepatic toxicity are reviewed in [1–5]. Briefly, these include redox state changes (decline in the NAD^+/NADH ratio), formation of the reactive acetaldehyde as a consequence of alcohol oxidation, solvent effects of alcohol on biological membranes, alcohol-induced hypoxia due to utilization of molecular oxygen for the oxidation of alcohol, activation of hepatic macrophages, the Kupffer cells, and release of cytokines such as tumor necrosis factor- α (TNF- α), direct immune responses and immune responses to aldehydic protein adducts formed during the metabolism of alcohol, impairment of mitochondrial function, oxidative stress, and others.

One major mechanism for ethanol-induced liver injury is the ability of ethanol to cause an increase in the production of reactive oxygen species (ROS) and to elevate oxidative stress in the liver. The role of free radicals in liver and other diseases has been reviewed [6, 7]. Ethanol-induced oxidative damage to proteins, lipids, DNA, and other cellular constituents has been demonstrated in vivo and in vitro and possible pathways by which ethanol can elevate formation of ROS and promote oxidative damage have been described. Briefly, these include decreases in antioxidant defense such as liver reduced glutathione (GSH) levels, mobilization and increases in liver iron levels, effects of acetaldehyde, the decrease in the hepatic redox ratio, release of chemoattractants and cytokines, mitochondrial injury and bioenergetic impairment, induction of the cytochrome P450 enzyme CYP2E1, and others. It is likely that many of these pathways overlap in view of the many actions of ethanol on liver function. **Table 1** summarizes some of the results which support a role for oxidative stress and ROS in ethanol-induced hepatotoxicity. One pathway that has been studied by many laboratories is the generation of ROS by the cytochrome P450 microsomal mixed function oxidase system. Ethanol elevates microsomal formation of ROS largely because it increases the content of one form of cyto-

chrome P450, CYP2E1 (reviewed below). Induction of CYP2E1 by ethanol plays a key role in the elevation of ROS in the liver by ethanol. The goal of the current review is to describe the liver microsomal cytochrome P450 mixed function oxidase system, its components and functions and generation of ROS, and then to discuss the biochemical and toxicological actions of CYP2E1 and its role in ethanol-induced oxidative stress.

2. OXIDATIVE STRESS

In biological systems, reactive oxygen radicals react with cellular macromolecules—proteins, lipids, DNA, and RNA—disrupting their structure and/or function. Because of such interactions, ROS have been implicated in many diseases including: toxicity of oxygen itself; inflammation; sepsis; carcinogenesis; cardiovascular diseases/atherosclerosis; aging (free radical theory of aging); ischemia/stroke/infarction; reperfusion/ transplantation injury; neurodegenerative diseases such as Parkinson and Alzheimer diseases; diabetes; radiation injury; smoking injury; xenobiotic and drug toxicity; iron and asbestos toxicity; vitamin and mineral deficiency (e.g., vitamins E and C, selenium, zinc); and alcohol toxicity, as described below.

In earlier terminology, oxidative stress generally referred to an imbalance between the prooxidant and antioxidant levels in cells and tissues [8–13]. Prooxidants are ROS which can cause tissue damage and whose levels may be increased by certain drugs, infection, external exposures, tissue injury, among others [14]. Antioxidants inhibit either formation of ROS or remove/scavenge the generated ROS. Oxidative stress can result from an increase in prooxidant formation, or a decrease or deficiency in antioxidant levels, or both. More recently, the role of molecular redox switches and oxygen sensing by the thiol redox proteome and by NAD^+/NADP and phosphorylation/dephosphorylation systems to define redox partners involved in redox signaling, redox control, and redox balance of a system have expanded this definition [15, 16].

The most common ROS produced in biological systems are the superoxide anion radical (a one-electron reduction product of molecular oxygen) [17] and hydrogen peroxide (H_2O_2), a two-electron reduction product of molecular oxygen [18]. Interestingly,

TABLE 1. Role of oxidant stress in ethanol-induced hepatotoxicity

Chronic alcohol increases production of ROS, lipid peroxidation, protein and DNA damage in many cellular systems in vitro and in acute and chronic models of ethanol administration.

Antioxidants usually protect against this injury: S-adenosyl-L-methionine, betaine, GSH precursors (N-acetylcysteine), SOD1/2, peroxiredoxins, thioredoxin, and ebselen, among others.

Hepatic iron levels can be elevated by ethanol administration. Iron potentiates alcohol toxicity whereas iron chelators, e.g., deferoxamine, can be protective.

Alcohol toxicity is elevated in antioxidant knockout mice, e.g., SOD1 null or TrxR null mice.

Polyunsaturated fatty acids (PUFA) and diets enriched in PUFA potentiate, while saturated fats, lower alcohol-induced liver injury.

Addition of ethanol to liver, and other hepatic cell lines elevates ROS and cell injury.

Liver cell lines expressing ADH, CYP2E1 and ADH plus CYP2E1 show ethanol-induced oxidative stress and injury.

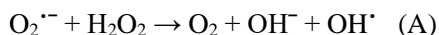
Human alcoholics show increased markers associated with lipid peroxidation, protein and DNA oxidation, isoprostanes, 4-HNE/HER adducts, and lower levels of vitamin E (α -tocopherol), selenium, and GSH.

There are correlations between hepatic fibrogenesis/necroinflammation and oxidant stress markers reported in the literature.

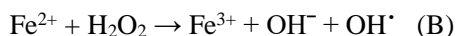
Note: ADH, alcohol dehydrogenase; HER, 1-hydroxyethyl; TrxR, thioredoxin reductase. see Abbreviations section for other abbreviations used in the table.

molecular oxygen itself is a diradical in the ground state as it has two unpaired electrons in its outer orbital, the triplet state of oxygen. Inversion of the spin of one of these electrons produces the very reactive singlet form of oxygen.

The hydroxyl radical is the most powerful of the ROS produced in biological and chemical systems and can react with any biochemical or macromolecule. It is central to the toxicity produced by ROS as it reacts and inactivates or disrupts proteins, lipids, DNA, and RNA [19, 20]. It was initially believed that hydroxyl radical was produced from a direct reaction between $O_2^{\bullet-}$ and H_2O_2 , namely, the Haber–Weiss reaction (Reaction A)

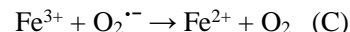


While this is thermodynamically feasible, the Haber–Weiss reaction is kinetically too slow to account for significant hydroxyl radical production. The Fenton reaction (Reaction B) is the most likely major mechanism by which OH^{\bullet} is produced in cells.



For the Fenton reaction to continue, the Fe^{3+} must be reduced back to Fe^{2+} . This is accomplished by super-

oxide which is a good reductant of metals such as iron (Reaction C).



Note, the combination of reactions B and C is the iron-catalyzed Haber–Weiss reaction (Reaction A).

Copper can also catalyze the Haber–Weiss reaction similar to iron, but iron is more plentiful and more reactive. The therapeutic significance of this is that the toxicity of ROS and its role in the above-mentioned diseases can be mitigated in part by removal or chelation of iron, which is sometimes used clinically to treat toxicity associated with ROS.

ROS are produced [21, 22] in biological systems from many enzymes, from the mitochondrial electron transport chain (e.g., complexes I and III), from heme enzymes which react with molecular oxygen (e.g., hemoglobin), from NADPH oxidases, from the cytochrome P450 mixed function oxidase electron transport chain, which oxidizes drugs and xenobiotics (discussed further below), from the autoxidation of cellular biochemicals, and from certain oxidative enzymes such as urate, glycolate, D-amino acid, and fatty acyl CoA oxidases. Monoamine oxidase uses O_2 to oxidize important neurotransmitters such as epinephrine, norepinephrine, dopamine, and serotonin,

producing H_2O_2 , and this is a major source of ROS in the brain. External sources of ROS include drugs and xenobiotics, alcohol, infection, radiation, smoke, and ozone. The mitochondrial electron transfer chain is the major source of ROS in most tissues, producing superoxide from the oxidation of reduced flavin in complex 1 and especially from the autoxidation of reduced ubiquinone. It has been estimated that about 2 or 3 percent of the oxygen reduced by the respiratory chain can be converted to superoxide radical [23].

Because of their reactivity, ROS readily interact with all cellular macromolecules [9–12, 24]. For example, ROS, especially the hydroxyl radical, oxidize the sulfhydryl (–SH) group of cysteine residues of proteins to the disulfides or to the sulfoxide (–SO) or the sulfonic acid (–SOOH). The –SCH₃ of methionine is readily oxidized to the methionine sulfoxide, interfering with functions of methionine such as methylation reactions. ROS oxidize the aromatic rings of phenylalanine and tyrosine or the indole ring of tryptophan, open up the rings to form protein carbonyls and cause loss of enzymatic activity and structure. ROS cleave the phosphodiester bonds holding bases in RNA and DNA together and break the chain structure of RNA and DNA. ROS oxidize the purine and pyrimidine bases and prevent appropriate base pairing. The carbon 8 position of purines is a very sensitive site for oxidation by ROS; formation of 8-hydroxyguanine or 8-hydroxyadenine, and 8-hydroxydeoxyguanine or 8-hydroxydeoxyadenine are footprints for ROS attack on RNA and DNA, respectively. ROS can cause deaminations, e.g., removal of the amino group from adenine and guanine to form hypoxanthine and xanthine, respectively, or removal of the amino group from cytosine to form uracil. These reactions alter correct base pairing in DNA and are major causes of mutations. Polyunsaturated fatty acids are a major target for oxidation by ROS, a process called lipid peroxidation, which disrupts normal membrane structure. Lipid radicals, lipid alkoxyl radicals, lipid peroxy radicals, and lipid hydroperoxides are formed during lipid peroxidation, and these reactive lipid species disrupt membrane structure. Fragmentation of these species also produces lipid aldehydes, with two major ones being malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE). These aldehydes are very reactive and bind to amino groups of amino acids, purines and pyrimidines, and to thiol groups of cysteines,

thus inactivating enzymes and disrupting protein, RNA, and DNA structure. Vitamin E (α -tocopherol) is the major antioxidant promoting termination of lipid peroxidation.

While large amounts of ROS cause cell damage and toxicity, ROS in low amounts are important signaling agents in biological systems [25, 26]. H_2O_2 is less reactive than superoxide and hydroxyl radicals and can catalyze “mild” oxidations which can activate or inactivate enzymes or transcription factors. In fact, several important transcription factors such as Nrf2, NF- κ B, and AP-1 are activated by H_2O_2 -dependent oxidation of thiol residues. These transcription factors subsequently activate many genes, some of which code for cellular antioxidants. Thus, low levels of ROS can protect against high levels of ROS. Besides signaling actions, there is a cell type which is designed to produce ROS to protect against infection by invading organisms and tumor cells [27, 28]. Phagocytes, a type of white blood cell, contain the enzyme NADPH oxidase, which is activated when the phagocytes come into contact with bacteria and other cells, e.g., tumor cells. This activation is called the respiratory burst, since oxygen uptake is increased because the NADPH oxidase catalyzes the following reaction: $NADPH + O_2 \rightarrow NADP^+ + O_2^{\cdot-}$. The rapid and large production of superoxide and other ROS kills the invaders. The pentose phosphate pathway is activated during the respiratory burst to generate NADPH. Individuals with failure to promote a respiratory burst, largely due to deficiencies in some of the subunits which make up the NADPH oxidase, develop chronic granulomatous disease, which makes them very sensitive to infection and is often fatal because of sepsis.

How do cells mitigate against the havoc produced by ROS? Clearly, cells have developed enzymes and biochemicals which prevent the production of large amounts of ROS or have the capacity to scavenge and remove the generated ROS [29–31].

Superoxide dismutase (SOD) catalyzes the removal of 2 moles of $O_2^{\cdot-}$ via a dismutation reaction: $O_2^{\cdot-} + O_2^{\cdot-} + 2 H^+ \rightarrow O_2 + H_2O_2$. One mole of superoxide is oxidized to O_2 while the second mole is reduced to H_2O_2 . Mammals contain two intracellular SODs, a copper/zinc SOD in the cytosol and the space between the outer and inner mitochondrial membranes, and a manganese SOD in the matrix space of the mitochondria. A mitochondrial SOD is logical since mitochondria are a major source of superoxide pro-

duction. Bacteria contain an iron-dependent SOD. Metals such as zinc and manganese are considered to be antioxidants largely because they are needed for SOD activity. Numerous experiments have shown that chemical inhibition of SOD or siRNA against SOD increases the sensitivity of cells to ROS.

Several cellular enzymes are effective in removal of H_2O_2 . Catalase, present in the peroxisomal fraction of the cell (as are many oxidase enzymes which produce H_2O_2), catalyzes removal of two moles of H_2O_2 : $2 \text{H}_2\text{O}_2 \rightarrow 2 \text{H}_2\text{O} + \text{O}_2$. Catalase, a heme-containing enzyme, has a relatively high K_m for H_2O_2 and a very high V_{\max} . Catalase can also remove H_2O_2 by its peroxidatic activity in which a hydrogen donor is oxidized as H_2O_2 is reduced. Simple alcohols such as ethanol and methanol are good substrates for the peroxidatic activity of catalase with the subsequent formations of acetaldehyde and formaldehyde, respectively. Indeed, catalase can serve as a minor ethanol oxidizing system, limited by the capacity of formation of H_2O_2 : $\text{H}_2\text{O}_2 + \text{CH}_3\text{CH}_2\text{OH} \rightarrow \text{CH}_3\text{CHO} + 2 \text{H}_2\text{O}$.

The glutathione peroxidase system is found in the cytosol fraction and in the mitochondria, and functions in conjunction with GSH, NADPH, and glutathione reductase to remove H_2O_2 : $2 \text{GSH} + \text{H}_2\text{O}_2 \rightarrow \text{GSSG} + 2 \text{H}_2\text{O}$. To regenerate GSH, GSSG (glutathione disulfide) is reduced by NADPH and glutathione reductase: $\text{GSSG} + \text{NADPH} \rightarrow 2 \text{GSH} + \text{NADP}^+$. GSH is present at very high levels in all cells (about 5–10 mM) and is likely the most important antioxidant in cells as a cofactor for glutathione peroxidase and glutathione transferases as well as a direct acting antioxidant.

There are other peroxidases in the cell that can remove H_2O_2 , such as the enzymes thioredoxin and thioredoxin reductase (which function similarly to GSH and glutathione reductase). Peroxiredoxins have potent H_2O_2 removal activity. There are six peroxiredoxins in cells, some in the cytosol and some in the mitochondria. Heme oxygenase breaks down heme; this prevents ROS formation. Vitamins E and C terminate the lipid peroxidation process. Carotenoids, such as β -carotene, are potent singlet oxygen scavengers. Other cellular antioxidant biochemicals which react with different ROS include melatonin, uric acid, taurine, and bilirubin. Importantly, there is little free iron available in cells, as most iron is stored in ferritin. Iron stored in ferritin does not catalyze the Haber–Weiss or Fenton reactions.

3. MICROSOMAL MIXED FUNCTION OXIDASE AND CYTOCHROME P450 METABOLISM

For a more detailed recent review of cytochrome P450 metabolism please refer to Ref. [32]. The microsomal cytochrome P450 mixed function oxidase system is important for the metabolism of endogenous substrates such as for the synthesis of cholesterol, bile acids, steroid hormones, androgens and estrogens, and for the metabolism of vitamin D₃, fatty acid omega hydroxylation, and the biotransformation of exogenous xenobiotics [33, 34]. Microsomal cytochrome P450s present in the liver, skin, and lungs in particular are important in converting lipophilic xenobiotics including drugs, insecticides, carcinogens, food additives, and environmental pollutants to more polar compounds which are easier to excrete. While cytochrome P450-dependent metabolism was considered to be important as a cellular detoxification system, with certain compounds, metabolism by the P450 system can generate reactive intermediates from the parent drug which are highly toxic.

Cytochrome P450 was found to function as the oxygen-activating oxidase associated with microsomal oxygenation reactions [35, 36]. In vivo administration of certain drugs increased the activity of the microsomal mixed function oxidase system due to induction of cytochrome P450 [37]. In mammals, P450, while present at highest levels in microsomes from the liver (where it plays a major role in detoxification reactions), is also present in microsomes from the kidney, small intestine, lungs, adrenal cortex, skin, brain, testis, placenta, and other tissues. Mitochondria, especially from the liver and endocrine tissue, contain P450. The nuclear envelope and plasma membrane contain low levels of P450.

Reducing equivalents from either NADH or NADPH are required for P450 to activate molecular oxygen for subsequent mixed function oxidation. The 11 β -hydroxylase system of adrenal cortex mitochondria [38] was separated into a P450-containing membrane fraction and a soluble NADPH-P450 reductase activity; the latter was further resolved into an iron-sulfur protein and a flavoprotein reductase and the sequence of “NADPH \rightarrow flavoprotein \rightarrow iron sulfur protein \rightarrow P450” was established [38]. A similar electron transfer pathway that used NADH in place of NADPH was found for the camphor hy-

droxylase system of the bacterium *Pseudomonas putida* [39]. Iron sulfur proteins, while required for mitochondrial P450 electron reduction are not involved in microsomal P450 electron reduction. The microsomal drug metabolism system contains two major components, the P450 and its reductant, the NADPH cytochrome P450 reductase [40]. The mammalian microsomal cytochrome P450 reductase contains 2 moles of flavin, one FAD and one FMN, per mole of reductase enzyme: $\text{NADPH} \rightarrow \text{FAD} \rightarrow \text{FMN} \rightarrow \text{P450}$.

Cytochrome P450s use molecular oxygen and reducing equivalents to catalyze the monooxygenation of a variety of substrates (RH) by the following general reaction: $\text{RH} + \text{NAD(P)H} + \text{O}_2 \rightarrow \text{ROH} + \text{NAD(P)}^+ + \text{H}_2\text{O}$. This reaction is referred to as a mixed function oxidase reaction since one atom of oxygen is incorporated into the substrate (hydroxylation) while the other is reduced to water. P450s cannot directly accept electrons from NADH or NADPH; therefore, a cytochrome P450 reductase containing FMN and FAD is necessary to reduce the heme of microsomal P450. For mitochondrial P450 reduction, an FAD containing reductase and an iron-sulfur protein are necessary. For microsomal P450 reduction, NADPH rather than NADH is the preferred reductant for transferring reducing equivalents to the NADPH-P450 reductase and then to cytochrome P450. NADH transfers electrons to the FAD containing flavoprotein NADH-cytochrome b_5 reductase which reduces cytochrome (Cyt.) b_5 : $\text{NADH} \rightarrow \text{FAD} \rightarrow \text{Cyt. } b_5 \rightarrow \text{P450}$ or desaturase. This system is important in fatty acid desaturation reactions.

Experiments with inhibitors and antibodies, and reconstitution experiments with purified enzymes have provided clear evidence for the major role of the NADPH-P450 reductase in reducing P450. The reduced b_5 may, to some extent, reduce P450. In some cases, NADH may further increase NADPH-dependent P450 catalytic activity by providing the second electron required for the P450 catalytic cycle.

There is tremendous diversity in the reactions catalyzed by cytochrome P450, which may be one of the most versatile catalysts known [41–45]. The reactions catalyzed by P450 may be broadly categorized as: metabolism of endogenous constituents including steroids, cholesterol, bile acids, vitamins, fatty acids, and eicosanoids; conversion of lipophilic exogenous xenobiotics into more polar products which can be conjugated to more soluble products for de-

toxification and removal from the body; and metabolism of certain xenobiotics such as CCl_4 , acetaminophen, benzene, halothane, and nitrosamines into more reactive products that are toxic or carcinogenic. Conversion of drugs into toxic products as a consequence of metabolism by the microsomal mixed function oxidase system is a major reason for failure of many drugs in pre- and post-clinical trials [45–47]. Oxidative reactions that are catalyzed by P450 enzymes have been characterized and discussed by Guengerich and colleagues [45–48]. P450 can also catalyze reductive reactions under anaerobic or hypoxic conditions, e.g., reductive dehalogenation of CCl_4 to the trichloromethyl radical. In general, some of these reactions may be carried out by one form of P450 while others may be carried out by many forms. Some substrates may be oxidized by one form of P450 at a low concentration but by another form at a high concentration. The structural features necessary for a substrate to be oxidized by a particular P450 are still not clear.

It is now recognized that more than 150 separate forms of P450 exist. The human genome project has identified 57 human genes coding for the various P450 enzymes (refer to the websites for details: <http://drnelson.utmem.edu/CytochromeP450.html> or <http://drnelson.uthsc.edu/human.P450.table.html>; accessed on July 4, 2017).

Numerous names were given to these different forms as they were purified or identified. A systematic nomenclature is now used which assigns proteins into families and subfamilies based on their amino acid similarities. Proteins within a family exhibit greater than 40% homology while those with at least 55% homology are in the same subfamily. The written notation is an Arabic number designating the gene family followed by a letter for the subfamily and an Arabic number for individual genes within a subfamily. Thus, CYP1A1 and CYP1A2 are two individual P450s in family 1 subfamily A. The approximate percent of specific P450 forms present in the human liver is: CYP1A2, 5%; CYP2A6, 2%; CYP2B6, 2–4%; CYP2C8/9, 11%; CYP2C19, 5%; CYP2D6, 20–30%; CYP2E1, 2–4%; and CYP3A4/5, 40–45%. The liver generally contains the greatest amount of most CYPs. One way of broadly categorizing the multiple forms of human CYPs is by the substrates they oxidize [47]. About one half of the human CYPs metabolize endogenous substrates such as steroids, bile acids, fatty acids, eicosanoids, and

vitamins. The other half function to metabolize xenobiotics. In general, members of the CYPs 1, 2, and 3 families are the most important in catalyzing oxidation of exogenous drugs and are generally present in highest amounts in the human liver. In the P450 reactions with drugs, 90–95% of clinical drugs are metabolized by 5 of the 57 human CYPs: 1A2 (10–12%), 2C8/9 (15%), 2C19 (7–9%), 2D6 (30%), and 3A4 (35–40%) [49]. Together, CYPs 2A6, 2B6, and 2E1 oxidize less than 10% of clinical drugs. Rendic and Guengerich [50] reported on the contributions of human P450 enzymes in carcinogen metabolism. They showed the fraction of P450 activation reactions for carcinogens attributed to individual human P450 enzymes were as follows: CYP1A1, 20%; CYP1A2, 17%; CYP1B1, 11%; CYP2A6, 8%; CYP2E1, 11%; CYP3A4, 10%; and others, 23%. Members of the CYP4 family are active in metabolizing fatty acids, especially arachidonic acid while CYPs 7, 11, 17, and 19 are most important in steroid metabolism. CYPs 24A1, 27B1, and 26B1 play a role in vitamin D and retinoic acid metabolism, respectively. CYPs 2A6, 2C9, 2C19, and 2D6 in particular display polymorphisms [51–53]. A listing of many cytochrome P450 genes and their polymorphisms can be found in Table 1 of Ref. [52] and in the website (<http://www.imm.ki.se/CYPalleles/>; accessed on July 4, 2017). The effect of the polymorphism may include formation of an inactive CYP enzyme, CYP gene deletion, formation of an unstable CYP which rapidly degrades, formation of a CYP with lower affinity for the cytochrome P450 reductase, formation of a CYP with altered substrate specificity or altered substrate affinity, or even increased CYP activity due to a gene duplication [54].

The ability of certain xenobiotics to elevate their own metabolism has long been known and the mechanism proposed was that the agent increased the content of the P450 responsible for its metabolism. Not all forms of P450 are inducible especially the steroid metabolizing P450s characteristic of the 11, 17, 19, 21, and 26 families and not all P450s in a particular family are inducible. Induction of most (but not all) P450s involves activation of the respective gene and increased *de novo* protein synthesis. In some cases, specific cell receptors which interact with the inducing agent have been identified. Induction of CYPs may be complex. For example, the CYP2E1 gene is under transcriptional regulation during development and in rats is activated immediately after birth [55].

Following fasting or induced diabetes, CYP2E1 mRNA is increased several fold due to post-transcriptional mRNA stability [56]. The elevation of CYP2E1 by many low molecular weight chemicals including ethanol, acetone, and pyrazole is largely due to protein stabilization and increases in protein half-life [55, 57, 58]. Ethanol, at very high levels can also increase CYP2E1 by a transcriptional mechanism and increased mRNA synthesis [59]. Thus, multiple mechanisms can exist by which a cytochrome P450 such as CYP2E1 can be induced.

4. REACTIVE OXYGEN SPECIES FORMATION AND ALCOHOL-INDUCED LIVER DAMAGE

Small amounts of ROS such as superoxide radical anion and H_2O_2 are produced during the P450 catalytic cycle, and cytochrome P450 enzymes can be a significant source of ROS in biological systems, especially in tissues like the liver where P450 is present in high amounts. Decay of oxygenated cytochrome P450 appears to be responsible for most of the superoxide produced during the P450 catalytic cycle while decay of peroxy cytochrome P450 is responsible for the formation of H_2O_2 in addition to the dismutation of superoxide. There are many early reports that liver microsomes produce H_2O_2 during NADPH oxidation [60–62]. Formation of superoxide during liver microsomal NADPH oxidation was reported using assays of chemiluminescence or epinephrine autoxidation [63, 64]. Kuthan et al. [65] showed superoxide formation using succinylated cytochrome c reduction in a reconstituted mixed function oxidase system. Zanger et al. [66] discussed how in eukaryotic monooxygenases a significant portion of the activated oxygen in eukaryotic mixed function oxidase systems was released from the P450 in the absence of a substrate or without metabolism or modification of the bound substrate: this was due to either the one-electron reduction of molecular oxygen and release of superoxide, or the two-electron reduction of oxygen and release of peroxide or the ultimate four-electron reduction of oxygen with the formation of two moles of water from molecular oxygen. This consumption of NADPH and oxygen in the absence of substrate reflects uncoupling of the eukaryotic cytochrome P450 monooxygenase system which is in part determined by the form of P450. For

example, Gorsky et al. [67] reported that for purified rabbit P450, the rates of oxygen uptake in the absence of a substrate were $CYP2E1 > CYP2C3 > CYP1A2 > CYP2B4$. Because of this uncoupling, rates of oxygen uptake and NADPH consumption during mixed function oxidase activity may not be or may be only weakly dependent on the presence of substrates and this can thereby contribute to cellular production of ROS, influencing cellular function, viability, and signaling [66].

Of interest with respect to the mechanism of the microsomal ethanol oxidation system (MEOS), were the studies that MEOS might reflect the presence of catalase in isolated liver microsomes (perhaps as a contaminant), which in the presence of H_2O_2 generated by mixed function oxidase activity can oxidize ethanol to acetaldehyde via catalase peroxidatic activity [61]. Lieber and colleagues subsequently demonstrated that MEOS involves oxidation of ethanol as a direct substrate for cytochrome P450 [68, 69] and can be disassociated from a catalase-hydrogen peroxide mechanism.

Most studies on ROS generation by mixed function oxidase systems have employed isolated microsomes or reconstituted systems containing the reductase and purified forms of P450. More recently, there are studies in which cell lines have been generated to express a P450 or overexpress a form of P450 and shown to display elevated ROS formation as compared to control cell lines either not expressing or having lower levels of the P450. For example, Zanger et al. [70] expressed CYP3A4 in HepG2 cells which normally contain little or no CYP3A4 and showed enhanced production of ROS by the cells expressing CYP3A4. The enhanced ROS affected secretion of proteins by the cells and autocrine and paracrine signaling. Our laboratory, and others, engineered HepG2 cells to express CYP2E1 and characterized the elevated ROS formation, oxidative stress and toxicity in these cells [71–74]. However, as a caveat, using isoprostane formation as an *in vivo* index of oxidative damage, Dostalek et al. [75, 76] reported that only in rats treated with phenobarbital, an inducer of CYP2B, was there an increase in the production of liver and urinary isoprostanes. No such increases were found with inducers of CYPs 1A, 2E, 3A, and 4A, and the authors concluded that most P450s are not involved in large scale production of ROS *in vivo*, at least in the absence of highly uncoupling substrates [75, 76]. However, there are many *in vivo*

examples of elevated formation of ROS after chronic ethanol treatment, e.g., the formation of 4-hydroxynonenal adducts, malondialdehyde adducts, hybrid 4-hydroxynonenal-malondialdehyde adducts, 1-hydroxyethyl adducts, and 3-nitrotyrosine-protein adducts, among others [77–81]. More research on *in vivo* formation of ROS by P450 systems and more specific biomarkers are clearly needed.

5. CYP2E1-ROS AND ETHANOL-INDUCED LIVER INJURY

As discussed in the Introduction, alcohol acts through numerous pathways to affect the liver and lead to the development of alcoholic liver disease. One factor playing a central role in many pathways of alcohol-induced damage is the excessive generation of free radicals and promotion of oxidative damage. Some suggestions for a role for oxidative stress in ethanol hepatotoxicity were discussed in the Introduction. CYP2E1 is a major contributor to ethanol-elevated ROS production as discussed below. CYP2E1 is a member of the cytochrome P450 superfamily of enzymes and is of special interest for several reasons when investigating alcohol-induced oxidative stress because its activity and level increase after acute and chronic alcohol exposure and because CYP2E1 itself also metabolizes alcohol. Besides ethanol, CYP2E1 metabolizes a variety of small, hydrophobic substrates and drugs [68, 69, 82–87]. Possible physiological substrates are acetone and fatty acids such as linoleic and arachidonic acid [86, 88]. 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 [83, 86]. 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 [89]. CYP2E1 is one of the most important P450s from a toxicological point of view.

Molecular oxygen itself is likely to be a most important substrate for CYP2E1. Relative to several other P450 enzymes, CYP2E1 displays high NADPH oxidase activity as it appears to be poorly coupled

with NADPH-cytochrome P450 reductase [67, 90]. 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 [90, 91]. Microsomes isolated from rats fed ethanol chronically were about twofold to threefold more reactive in generating superoxide radical, H_2O_2 , and hydroxyl radical, and undergoing lipid peroxidation compared to microsomes from pair-fed controls [92]. CYP2E1 levels were elevated about threefold to fivefold in these liver microsomes after feeding rats the ethanol Lieber–DeCarli diet for four weeks. The enhanced effectiveness of microsomes isolated from the ethanol-fed rats in generating ROS 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.

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 [68, 69]. A variety of heterocyclic compounds such as imidazole, pyrazole, 4-methylpyrazole, thiazole, and isoniazid have been shown to elevate CYP2E1 levels as do solvents such as dimethylsulfoxide, various alcohols, benzene, and acetone [57, 58]. 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 [68, 69]. Diabetes has been reported to increase the expression of CYP2E1 mRNA and protein levels several fold, largely by stabilizing CYP2E1 mRNA [56, 93, 94]. This may be related to actions of insulin. The carbohydrate content of the diet influences CYP2E1 levels as a low carbohydrate diet increased the induction of CYP2E1 by ethanol and high fat/low carbohydrate diets resulted in the highest levels of CYP2E1 induced by ethanol [95]. Nonalcoholic steatohepatitis (NASH) is a condition characterized by hepatomegaly, elevated serum aminotransferase levels, and a histologic picture similar to alcoholic hepatitis. Oxidative stress and lipid peroxidation are among the critical factors involved in the genesis and probably the progression of NASH [96]. In a mouse model of NASH, hepatic CYP2E1 was upregulated, and this was associated with a dramatic increase in

total lipid peroxide levels that were substantially inhibited by anti-CYP2E1 IgG [97]. The induction of CYP 2E1 by low molecular weight chemicals mentioned above, including pyrazole and ethanol, is largely due to a posttranscriptional mechanism in which the inducer stabilizes CYP2E1 against proteasome-mediated degradation [55, 57, 58, 98, 99]. The half-life of CYP2E1 has been shown to increase from less than 5–7 hours in the absence of inducer to more than 24 hours in the presence of inducers such as acetone [57] or pyrazole [99]. **Table 2** provides a brief summary of some of the important properties associated with CYP2E1.

Since CYP2E1 can generate ROS during its catalytic cycle 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 even though it is only a minor pathway of alcohol oxidation as alcohol dehydrogenase is the major pathway. In the intragastric model of ethanol feeding, prominent induction of CYP2E1 occurs as does significant liver injury [100–102]. In this model, 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 [100, 101]. Experimentally, a decrease in CYP2E1 induction was found to be associated with a reduction in alcohol-induced liver injury [100]. CYP2E1 inhibitors such as diallyldisulfide, phenethyl isothiocyanate, and chlormethiazole [102] blocked the lipid peroxidation and ameliorated the pathologic changes in ethanol-fed rats. A CYP2E1 transgenic mouse model was developed that overexpressed CYP2E1 [103, 104]. When treated with ethanol, the CYP2E1 overexpressing mice displayed higher transaminase levels and histological features of liver injury compared with the control mice. Infection of HepG2 cells with an adenoviral vector which expresses human CYP2E1 potentiated acetaminophen toxicity as compared to HepG2 cells infected with a LacZ expressing adenovirus. Administration of the 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 [105]. Chronic ethanol-induced oxidative stress and fat accumulation in the liver were decreased in CYP2E1 knockout mice as compared to wild type mice and these effects of ethanol were restored in

TABLE 2. Characterization of cytochrome P4502E1

Induction and mechanism	Induced by ethanol, solvents, drugs, heterocyclic compounds, and halogenated compounds.
	Induced under pathophysiological conditions such as obesity, high fat diet, low carbohydrate diet, diabetes, and fasting.
	Induced under conditions causing non-alcoholic fatty liver disease and non-alcoholic steatohepatitis.
	Mechanism of induction is complex and may be associated with enhanced transcription (e.g., at birth, very high blood ethanol levels), increased translation of CYP2E1 mRNA (pyridine), mRNA stabilization (diabetes, acetone), and by protein stabilization (e.g., ethanol, pyrazole, methylpyrazole, other low molecular weight ligands).
	Minor pathway of ethanol metabolism becomes more significant at high levels of ethanol (high Km for ethanol) and after induction by chronic ethanol consumption.
	Produces reactive metabolites such as acetaldehyde and 1-hydroxyethyl radical.
	Plays a role in alcohol-drug interactions, e.g., competition for oxidation.
	Activates toxins to reactive intermediates: acetaminophen, benzene, CCl ₄ , halogenated hydrocarbons, and halothane.
	Activates procarcinogens such as nitrosamines and azo-compounds to reactive intermediates.
	Activates O ₂ to produce ROS: O ₂ ^{•-} and H ₂ O ₂ .

CYP2E1 knockin mice [81, 106]. Ethanol induction of oxidative stress to DNA and mutagenesis was blunted in CYP2E1 knockout mice but not in NADPH oxidase knockout mice suggesting that CYP2E1 was the primary source of ROS for damage to DNA [107]. On the contrary, ethanol-induced liver injury was attenuated in the NADPH oxidase knockout mice but not in the CYP2E1 knockout mice [107]. Thus, depending on the toxicity parameter being evaluated, either CYP2E1 or NADPH oxidase could be the primary enzyme responsible for the developing oxidant stress and injury. Clearly, further studies are necessary to resolve the above discrepancies and the roles of CYP2E1 and NADPH oxidase in the actions of ethanol. 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.

HepG2 cell lines expressing the human CYP2E1 were established by retroviral infection or plasmid transfection methods [71, 72]. Ethanol, or hepatotoxins such as acetaminophen and CCl₄, or a polyunsaturated fatty acid prone to lipid peroxidation such as arachidonic acid caused a loss of cell viability in the CYP2E1-expressing HepG2 cells but not to HepG2 cells which do not express CYP2E1 (reviewed in Refs. 3–5). Lowering of cellular levels of

GSH, the most important antioxidant in cells, caused a loss in cell viability only in the CYP2E1 expressing HepG2 cells. There was an increase in the production of ROS when the CYP2E1-expressing cells were treated with ethanol and other prooxidants. The cellular toxicity and elevated oxidative stress were blunted when antioxidants were added to the CYP2E1-expressing HepG2 cells. Other cell lines expressing CYP2E1 have been developed and also shown to display elevated ROS and damage due to the expression of CYP2E1 [74, 108–110]. Thus, CYP2E1 is one form of cytochrome P450 which is an active generator of ROS and which contributes to the biochemical and toxicological actions of ethanol.

Briefly, in other studies, CYP2E1 was shown to play a key role in ethanol-induced mitochondrial dysfunction and post-translational modifications of mitochondrial proteins [111] and in ethanol-induced DNA damage and formation of etheno-DNA adducts [112]. Ethanol-induced gut permeabilization and subsequent bacterial translocation followed by Kupffer cell activation and cytokine formation play a major role in sensitizing the liver to ethanol toxicity: Intestinal CYP2E1 was shown to mediate this alcohol-gut interaction-induced leakiness [113, 114]. CYP2E1/ROS plays a role in innate immune effects of ethanol [115] and CYP2E1 autoantibodies have been found in several models of liver disease inclu-

TABLE 3. Evidence for a role for CYP2E1 in the toxic effects of ethanol

Various CYP2E1 inhibitors, e.g., chlormethiazole, diallyldisulfide, lowered fat accumulation in wild type mice fed ethanol chronically.

Similarly, chlormethiazole and diallyldisulfide blunted ethanol-induced oxidative stress in wild type mice fed ethanol chronically.

Ethanol-induced steatosis and the elevated ROS production were blunted in CYP2E1 knockout mice but restored in CYP2E1 knockin mice.

The decline in hepatic GSH levels and the increase in malondialdehyde and 3-nitrotyrosine staining produced by chronic ethanol was lowered in CYP2E1 knockout mice and restored in CYP2E1 knockin mice.

Significant liver injury was produced by ethanol in CYP2E1 knockin mice with elevated levels of CYP2E1 compared to wild type mice.

Ethanol elevated ROS production and increased fat accumulation in HepG2 cells expressing CYP2E1 thus providing an in vitro model to evaluate molecular mechanisms by which CYP2E1 promotes ethanol-induced steatosis.

CYP2E1 sensitizes the liver to toxicity of iron, TNF- α and lipopolysaccharide and to obesity and high fat diets.

CYP2E1/ROS plays a key role in ethanol-induced hypoxia and activation of HIF-1 α .

CYP2E1/ROS plays an important role in ethanol-induced gut leakiness.

CYP2E1/ROS plays a role in ethanol-induced mitochondrial dysfunction.

CYP2E1/ROS plays a role in effects of ethanol on the immune system.

ding alcoholic liver disease [116]. CYP2E1 plays a role in alcohol-induced hypoxia and activation of hypoxia-inducible factor-1 α [117, 118]. Many of these and other actions of CYP2E1 have been reviewed in a recent volume [119]. **Table 3** summarizes some of the results which suggest a role for CYP2E1 in the toxic actions of ethanol.

6. CONCLUSIONS AND PERSPECTIVES

This review discusses cytochrome P450 metabolism with an emphasis on the generation of ROS and on CYP2E1. Because of many of the above described factors modulating levels of CYPs as well as polymorphisms of many CYPs, there is a large variability in the content of the xenobiotic metabolizing CYPs in families 1, 2, and 3 in human livers, which likely accounts for the large variability in drug oxidation by humans. Because of this variability in levels of a specific P450, the presence of multiple CYPs with overlapping substrate specificity and the ability of a CYP to metabolize many structurally distinct substrates, there is extensive overlapping substrate specificities which likely contributes to drug-drug interactions and adverse drug reactions. Generation

of ROS, as a consequence of uncoupling of the P450 catalytic cycle or metabolism of certain drugs by P450 to reactive intermediates, is a major reason for adverse drug reactions and plays a key role in the toxicity associated with certain diseases such as inflammation, iron toxicity, hepatocellular carcinoma and hepatitis, and as discussed above, alcohol-induced liver damage.

From a general point of view, future studies as to how ethanol-induced oxidative stress and ROS formation may be modulated by external factors such as diet, smoking (most alcoholics also smoke), insulin resistance, viral infection (HCV, HCB), and possible gender influence (women are more sensitive to toxicity of ethanol) would be important to define. While most studies have helped to define the role of ethanol metabolism by alcohol dehydrogenase and CYP2E1 in the production of ROS and oxidant stress, how significant and does ethanol produce ROS in tissues with limited ethanol metabolism and with low levels of alcohol dehydrogenase and CYP2E1, e.g., the heart and brain? Are there priming or sensitizing factors for ethanol-induced ROS formation? Are there metabolic adaptations to ethanol-induced oxidative stress and ROS generation, e.g., Nrf2 signaling? Can markers predictive of individuals especially sensitive

to ethanol-induced oxidative stress and ROS generation and tissue injury be developed? What are effective therapeutic interventions against ethanol-induced ROS formation and tissue damage? Obviously, there is a need for considerable more research in this research front.

Understanding the biochemical and toxicological properties of CYP2E1 is important for many reasons, even besides its role in contributing to alcohol-induced liver injury, since CYP2E1 is induced under a variety of pathophysiological conditions such as fasting, diabetes, obesity and high fat diet, and by drugs and in nonalcohol-induced steatohepatitis. There are many questions remaining to further our understanding of the role of CYP2E1 and of ROS in ethanol-induced liver damage. For example, it is still not clear whether ethanol needs to be metabolized to cause fat accumulation and liver injury, and if so, what the exact contributions of CYP2E1 or alcohol dehydrogenase are to these actions, and clearly further research is warranted on the direct metabolic effects and indirect effects of ethanol in the actions of ethanol. Elevated oxidative stress is central to this potentiated hepatotoxicity; the possible role of endoplasmic reticulum stress needs to be further defined. Mitochondria are a target for ethanol actions, and ethanol is known to decrease mitochondrial function [120]. It would be important to evaluate whether CYP2E1 plays a role in ethanol-induced toxicity to mitochondrial functions. In this respect, although CYP2E1 is mainly located in the endoplasmic reticulum, it is also present in the mitochondria [121–124]. There have been few studies as to whether mitochondrial CYP2E1 contributes to alcoholic liver disease and oxidative stress, and if it does, what are the contributions of mitochondrial versus microsomal CYP2E1 to these actions [124]. The biochemical function and significance of microsomal and mitochondrial CYP2E1 require further study. Ultimate questions are whether metabolic adaptations or effective therapeutic interventions against ethanol/CYP2E1-induced oxidative stress and tissue injury can be developed.

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