CYP2E1 in Alcoholic and Non-Alcoholic Liver Injury. Roles of ROS, Reactive Intermediates and Lipid Overload

Riina Harjumäki 1,2*, Chris S. Pridgeon 1 and Magnus Ingelman-Sundberg 1,*

1. The Discovery of CYP2E1

Early studies of the metabolism of ethanol, independent of alcohol dehydrogenase (ADH), were performed using a mutant strain of deermouse (Peromyscus maniculatus) that is genetically deficient in low-Km ADH. Nonetheless, these mice were found to eliminate ethanol by a previously unidentified enzyme system. One of the enzyme candidates proposed was a cytochrome P450 active in ethanol oxidation in liver microsomes [1], which was also metabolized other short chain alcohols [2] and appeared to be inducible by ethanol treatment in animal models [3].

The exact mechanisms of P450-dependent ethanol oxidation were initially unclear but were associated with the production of reactive oxidative species (ROS), including hydroxyl radicals, which can indirectly oxidize ethanol [4,5]. The finding of an ethanol-dependent increase in P450-mediated oxidation [3,6] was important for the isolation of a specific form of ethanol inducible P450 and to understand the direct P450-mediated oxidation of ethanol. One such enzyme was purified from ethanol- and benzene-induced rabbits and from ethanol-treated rats [7,8]. This enzyme had the highest ethanol oxidation capacity of several different P450 forms isolated [7]. The corresponding cDNA was cloned in 1987 [9] and the enzyme was named cytochrome P450 2E1 (CYP2E1) in 1991 [10].
2. Expression, Functions and Cellular Fate of CYP2E1

CYP2E1 is well conserved across mammalian species, indicating an important physiological function. In humans, the CYP2E1 gene has nine exons located on chromosome 10 and spans 11,413 base pairs [11]. Substantial inter-ethnic polymorphisms exist in CYP2E1. However, only very rare variants causing amino acid shifts have been described [12], and despite several epidemiological studies there is no clear evidence that any polymorphic variants have any functional relevance [13,14]. One recent study suggested a link between CYP2E1-333A > T and NASH, the authors showed increased inflammation and NASH in patient biopsies with the TA allele, largely mediated by a small increase in interferon-inducible protein 10 [15]. However, because only a relatively low number of patients were studied, much credence cannot be given to these findings unless they are reproduced independently. In liver, CYP2E1 is expressed mainly in the endoplasmic reticulum (ER) of the hepatocytes, but is also found in the hepatic Kupffer cells [16–18]. Hepatic CYP2E1 is also present in the mitochondria, by translocation, following expression in the nuclear genome [19–21] and the plasma membrane [19,22–24]. However, the magnitude of induction of CYP2E1 in mitochondria is smaller than that of ER-resident CYP2E1.

CYP2E1 metabolizes a variety of small, hydrophobic substrates and drugs (reviewed in [13,14,21,25]). It is responsible for the metabolism of many toxic or carcinogenic chemicals, including chloroform and benzene, and drugs, such as paracetamol, salicylic acid and several inhalational anesthetics (e.g., isoflurane, sevoflurane and halothane). It therefore follows that conditions which elevate the expression of CYP2E1 can increase the damage caused by conversion of drugs to toxic intermediates [26]. The bioactivation of several pre-carcinogens by CYP2E1 has been discussed in relation to the development of cancers, particularly hepatocellular carcinoma (HCC) [27,28]. In addition, CYP2E1 metabolizes endogenous substances including acetone, acetal, steroids and polyunsaturated fatty acids, such as linoleic acid and arachidonic acid to generate ω-hydroxylated fatty acids [29–32]. CYP2E1 also metabolizes ethanol and other short chain alcohols, however its contribution to the overall ethanol clearance is low and P450-dependent alcohol metabolism does not influence overall ethanol clearance in rats [33]. Despite its importance as a metabolic enzyme the crystal structure and binding sites were only determined relatively recently, in 2008 [34,35].

CYP2E1 is regulated by multiple, distinct mechanisms at the transcriptional, post-transcriptional, translational, and post-translational levels (Figure 1) [36–40]. CYP2E1 expression is elevated in response to a variety of physiological and pathophysiological conditions, such as starvation and uncontrolled diabetes, and also by ethanol, aceton and several other low molecular weight substrates [7,29,41–45]. However, it is primarily regulated at the post-transcriptional and post-translational levels [46]. Substrate-induced enzyme stabilization is the most important regulatory mechanism for CYP2E1 [47,48]; substrates and other chemicals binding to the substrate binding region stabilize the enzyme and prevent degradation by the proteasome-ER complex [38,48,49], involving the UBC7/gp78 and UbcH5a/CHIP E2-E3 ubiquitin ligases [50]. Thus, ER-mediated degradation is mainly active on CYP2E1 in the absence of substrates, whereas ligand-stabilized CYP2E1 is degraded more slowly by the autophagic-lysosomal pathway [51]. Due to its important physiological functions, the expression of CYP2E1 is under tight homeostatic control and multiple endogenous factors regulate CYP2E1 mRNA stability and protein expression including hormones (such as insulin, glucagon, growth hormone, adiponectin and leptin), growth factors (such as epidermal growth factor and hepatocyte growth factor) and various cytokines (reviewed in [40]).
Figure 1. Factors that induce CYP2E1 expressions. CYP2E1 can be induced by multiple mechanisms in transcriptional, post-transcriptional, translational, and post-translational levels. Some physiological and pathophysiological conditions, such as starvation and uncontrolled diabetes, increase CYP2E1 on the transcriptional level. Many hormones and cytokines regulate CYP2E1 on the mRNA and protein expression levels. Enzyme substrates stabilize the enzyme, preventing degradation by the ER proteasome and the enzyme degradation instead takes place via the autophagosome-lysosomal pathway. Figure made using BioRender.

As previously mentioned, the CYP2E1 gene is highly conserved and no functionally important genetic variants have been described. This indicates an important endogenous role, which is supported by our findings, wherein the knockdown of human CYP2E1 expression in the in vivo relevant three-dimensional (3D) liver spheroids [52] causes dramatic cell death in hepatocytes (unpublished observations from our lab). It is clear that CYP2E1 has important effects during catabolic conditions as the gene is transcriptionally induced during starvation [41]. The hepatic production of glucose is essential under starvation conditions with the primary supply of brain glucose, and about 10% of plasma glucose originating from acetone [53]. CYP2E1 readily oxidizes acetone to acetal [36], which is subsequently converted to pyruvate and then to glucose during gluconeogenesis. In addition, during conditions of starvation energy supply from fatty acids is essential and CYP2E1 is efficient in the ω-oxidation of fatty acids.

3. Mechanisms of Action of CYP2E1; Radical Mediated Toxicity

CYP2E1 is unique in that its heme iron is constitutively in the high spin state. In the cytochrome P450 redox cycle, substrate binding is necessary in order to transfer the low spin form into a high spin form. The conversion of low spin (three electrons in the outer Fe$^{3+}$ shell with opposite spin) to a high spin (parallel spin of the three outer shell electrons) form of P450 can be determined by absorption spectra analyses where the high spin form has a peak at 390 nm. The constitutive high spin form of CYP2E1 facilitates electron transfer to dioxygen in the absence of the substrate and is the major reason for
CYP2E1 being a ‘leaky’ enzyme which generates ROS such as superoxide and hydrogen peroxide. In the presence of iron, hydrogen peroxide is split yielding hydroxyl radicals formed in an iron-catalyzed Haber-Weiss reaction. Such reactions occur spontaneously in an environment enriched in hydrogen peroxide and non-heme iron. The hydroxyl radicals generated via the action of CYP2E1 can react with the hydrogen on the α-carbon of ethanol yielding a cytotoxic ethanol radical [54], this radical is oxidized spontaneously to acetaldehyde.

The extent of contribution of non-heme Fe$^{2+}$ in the reaction cycle of CYP2E1 is unknown. The enzyme can oxidize several different substrates based on a conventional cytochrome P450 redox cycle and it is presumed that both reaction mechanisms work in parallel with regard to ethanol and other short chain aliphatic alcohols. Thus, it is difficult to distinguish ROS production by CYP2E1 from ROS production from the uncoupling of the nicotinamide adenine dinucleotide phosphate acid (NADPH)-dependent electron transport chain and cytochrome P450 reductase. Accordingly, many experiments have been conducted in the presence of EDTA-chelated iron, which causes uncoupling and nonspecific generation of ROS, including the hydroxyl radical by extracting a proton from ethanol, yielding acetaldehyde (Figure 2) [4,55]. As such, determination of the true contribution of CYP2E1-mediated ROS formation and effects on the subsequent development of alcoholic liver disease (ALD) has been based on the use of CYP2E1 specific antibodies, knockdowns and CYP2E1 transgenic animals.

![Figure 2. Mechanisms of P450-dependent ROS formation and ethanol oxidation in the presence of non-heme iron. The generated superoxide is dismutated to hydrogen peroxide (dashed arrow), which is cleaved in the presence of non-heme iron (Fe$^{2+}$) yielding hydroxyl radicals. These can extract the hydrogen on the α-carbon of ethanol creating a radical of ethanol which is spontaneously converted to acetaldehyde. Non-heme iron chelated to EDTA enhances the cleavage of hydrogen peroxide yielding the reactive hydroxyl radical. Basic mechanism as described in [54]. POR-P450 reductase.](image)

The high spin nature of the CYP2E1 heme iron also makes the enzyme unique in that it can reduce compounds. The most well-known reaction is the reduction of carbon tetrachloride into the corresponding radicals that efficiently induce lipid peroxidation [56]. This reaction is believed to be the major cause of the hepatotoxicity of carbon tetrachloride. In addition, halothane is reduced in a similar manner by CYPE1 to a reactive radical
and binding of this radical metabolite to CYP2E1 converts the enzyme into a cell surface autoantigen believed to contribute to halothane hepatitis (Figure 3) [29]. In addition to halothane, other anesthetics, such as enflurane, isoflurane and desflurane, are metabolized by CYP2E1 to trifluoroacetylated components some of which may be immunogenic [57,58]. Similar autoimmune consequences of CYP2E1 action are also found in CYP2E1-mediated formation of hydroxyethyl radicals which bind to cellular proteins causing the production of autoantibodies in alcoholics [59].

**Figure 3.** Mechanisms of CYP2E1 dependent oxidation and reduction of halothane [60,61] (A) and carbon tetrachloride [62] (B). Aerobically, halothane undergoes cytochrome P450 catalyzed oxidation to trifluoroacetic acid (TFA), bromide and a reactive intermediate that can acetylate liver proteins and produces neo-antigens which stimulate an immune reaction that mediates severe hepatic necrosis. Anaerobically, halothane is reduced to a radical that can generate the metabolites chlorotrifluoroethane (CTE) and chlorodifluoroethylene (CDE) and also covalently bind proteins generating autoimmune reactions and also induce lipid peroxidation.
4. CYP2E1 in ALD

ALD is the most frequent liver disease in Europe, causing approximately 500,000 deaths per year, HCC resulting from pathological changes in the liver contributes highly to this death rate. There are strong genetic determinants in the development of ALD, e.g., PNPLA3, TM6SF2, and only approximately 10–20% of alcoholics develop cirrhosis of the liver [63].

ALD and NAFLD have several common mechanisms; generally, fibrosis occurs in response to enhanced ROS levels, lipid mediators and pro-inflammatory cytokines. Thus, ALD and NAFLD are mechanistically similar and share histopathological features, particularly in terms of CYP2E1 induction and oxidative stress (reviewed in [64]). In addition, they share common genetic risk factors.

CYP2E1 is the most relevant CYP in ALD [65], it is highly inducible, has high catalytic activity for ethanol [25] and is prone to futile cycling in the absence of substrate to produce ROS [66]. CYP2E1 is regarded as a ‘leaky’ enzyme due to loose coupling of the CYP redox cycle, permitted by the constitutively high-spin state of the heme iron and, therefore, it has a great capacity to produce oxyradicals and initiate lipid peroxidation [67–71]. Thus, CYP2E1 may be important in mediating the effects of ethanol on ALD via increased lipid peroxidation [67].

In order to study the link between CYP2E1 and ALD, Morgan et al., generated CYP2E1 transgenic mice which exhibited increased serum ALT levels, higher histological scoring and ballooning hepatocytes with alcohol diet [72]. Using a transgenic mouse model expressing extra copies of human CYP2E1, Butura et al., showed increased liver injury and expression of stress related genes with alcohol diet [73]. Microarray analyses revealed that enhanced expression of structural genes, particularly cytokeratin 8 and 18, may be related to the observed pathology and they were suggested as biomarkers for ALD [73]. JunD, part of the transcription factor complex AP-1, was induced by CYP2E1 and alcohol, and its expression correlated with the degree of liver injury. This transcription factor complex is also linked to increased macrophage activation. Furthermore, JunD also has a role in hepatic stellate cell activation and regulates the cytokine interleukin 6 [73].

A second approach is to study CYP2E1 knockout mice in comparison to wild-type mice. Abdelmegeed et al., showed that that aged wild-type mice had increased hepatocyte vacuolation, ballooning, degeneration, and inflammatory cell infiltration compared with CYP2E1-null mice [74]. They also found that the aged wild-type mice had increased hepatocyte apoptosis, hepatic fibrosis, levels of hepatic hydrogen peroxide, lipid peroxidation, protein carbonylation, nitration and oxidative DNA damage, indicating an endogenous role for CYP2E1 for these events.

Another approach to study the influence of CYP2E1 on ALD involves the use of CYP2E1 inhibitors. Chlormethiazole is a specific CYP2E1 inhibitor [75] and has a pronounced inhibitory effect on ALD in the intra-gastric alcohol rat model [76]. Similar experiments were also conducted using diallyl sulfide and phenylethyl isothiocyanate as CYP2E1 inhibitors demonstrating a protective effect against hydroxylradical formation from ethanol and lipid peroxidation and inhibition of some pathological scores [77,78].

Together, these studies indicate a significant contribution of alcohol-dependent induction of CYP2E1 for the development of ALD [79]. The major cause is its ability to increase ROS formation after ethanol treatment, but other factors controlling the redox properties of the liver also contribute to the observed pathology.

Intestinal CYP2E1 in ALD

In addition to causing gut dysbiosis, alcohol increases CYP2E1 levels and nitroxidative stress in the intestinal epithelium similarly as in the liver [80–84]. This causes intestinal leakiness followed by increased circulating endotoxin levels [82,85–87]. Endotoxin can initiate a hepatic necroinflammatory cascade starting from increased levels of NF-κB and release of inflammatory cytokines, such as TNF-α by Kupffer cells [85,88,89]. These results suggest a role of intestinal protein nitration in mediating alcohol-induced gut leakiness and subsequent hepatic injury in a CYP2E1-dependent manner. Even though fatty acids
induce CYP2E1 in liver, as discussed later in this review, it is unclear whether this holds for intestinal CYP2E1 and increases gut leakiness playing a role in the development of NAFLD. NASH patients exhibit increased gut leakiness [90] and dysbiosis (reviewed in [91]) and gut inflammation and dysbiosis augments hepatic inflammation and fibrogenesis in mouse NASH models. However, the role of intestinal CYP2E1 and oxidative stress for gut leakiness was not tested [90,92,93]. It has been hypothesized that ethanol produced by microbiota fermenting dietary sugars could cause dysbiosis, increased CYP2E1 levels and nitroxidative stress in NAFLD patients (reviewed in [91]) and maybe endotoxin levels are thus lower in patients with alcoholic cirrhosis compared with non-alcoholic cirrhosis [87].

5. CYP2E1 in NAFLD

NAFLD has been known for over 40 years; despite this, the underlying mechanisms remain poorly understood [94]. NAFLD refers to a continuum of liver diseases beginning with non-alcoholic fatty liver (NAFL), an accumulation of hepatic lipids not explained by alcohol consumption. In some cases, this can progress towards non-alcoholic steatohepatitis (NASH), fibrosis and cirrhosis and eventually HCC. Most NAFLD patients are obese and exhibit mild systemic inflammation, which induces insulin resistance and plays a role in the mechanism of liver damage [95–98]. NASH encompasses varying degrees of liver injury and is now recognized as the hepatic component of metabolic syndrome [99–102].

Liver cirrhosis is part of alcoholic steatohepatitis (ASH) and NASH, for which the only curative solution is often liver transplantation, and can progress to HCC in 3–4% of cases. The major causes of liver fibrosis are: hepatitis C infection (33%), alcohol (30%) and NASH (23%) [103]. The individual risks for liver cirrhosis caused by NASH and alcohol are largely determined by genetic risk factors, with polymorphisms in PNPLA3 and TM6SF2 and also MBOAT7, HSD17B13 and PCKS7 being the major common genetic determinants [104].

The multiple parallel hit model aims to explain the initiation and progression of NAFLD and has, in recent years, superseded the more simplistic two-hit model [101,105]. It suggests that multiple concurrent environmental and genetic insults, such as insulin resistance, oxidative stress-induced mitochondrial dysfunction, ER stress, endotoxin-induced TLR4-dependent release of inflammatory cytokines, and free fatty acid (FFA) accumulation combine to produce cell death and damage [106]. Oxidative stress mediated by ROS likely plays a primary role as the initiator of hepatic and extrahepatic damage and can cause damage in myriad ways by peroxidation of cellular macromolecules [107]. Oxidative stress can lead to lipid accumulation both directly and indirectly, most simplistically, ROS can peroxidate cellular lipids. Presence of these peroxidated lipids increases post-translational degradation of ApoB, preventing hepatocellular lipid export and leading to lipid accumulation. Alternatively, ROS can directly peroxidate proteins such as ApoB, directly preventing their function and producing a similar effect [108].

The connection between CYP2E1 and NASH was first suggested by Weltman et al., in 1996, where elevated levels of CYP2E1 were observed in steatosis and NASH patients, particularly in the centrilobular region [109] corresponding to the site of maximal hepatocellular injury in NASH [41,110]. This connection is based on the high propensity of CYP2E1 to generate ROS, even in the absence of substrates (Figure 4). In addition, CYP2E1 levels are elevated in obesity, steatosis and NASH in both humans and rodents [111–113].
Figure 4. Functions of the CYP2E1 enzyme. CYP2E1 has a role in normal physiological homeostasis by metabolizing endogenous and exogenous compounds. Major endogenous functions are $\omega$-hydroxylation of FFA and oxidation of acetone to precursors of gluconeogenesis. Increased amounts of CYP2E1 results in increased lipid peroxidation and ROS production and is associated with the progression of NAFLD to NASH.

5.1. CYP2E1 Links Insulin Resistance and NAFLD

The increased circulating levels of ketone bodies and fatty acids observed in obesity, steatosis and NASH may induce CYP2E1 and insulin resistance [66,106,114,115]. In addition, Cyp2e1 knockout mice are protected against high-fat diet-induced obesity and insulin resistance and the production of proinflammatory cytokines in adipose tissue was prevented [116]. CYP2E1 was recently shown to be linked to insulin resistance via the anti-apoptotic protein Bax inhibitor-1, which plays an important role in the regulation of CYP2E1 [117,118]. Furthermore, the repressive effects of insulin on CYP2E1 levels are lost in insulin resistance, commonly associated with NAFLD and NASH [106,115].

5.2. The Role of CYP2E1 in Hepatic Lipid Accumulation

Experiments preventing the degradation of CYP2E1 in mice by ablation of E3 ubiquitin ligase have demonstrated that the elevation of CYP2E1 alone in this model is insufficient to induce NASH. Concurrent elevation of CYP2E1 and induction of hepatic lipid accumulation from enhanced liver fat-production or ingestion of a high fat/high carbohydrate diet is required to induce NASH-like symptoms [32]. Conversely, CYP2E1 stabilization with CHIP-knockout in mice was associated with increased functional activity together with microvesicular fat accumulation and increased lipid peroxidation by activation of the hepatic JNK-cascade [119]. This occurred even though the mice were fed with a non-fat/carbohydrate-enriched diet, suggesting that overexpression of hepatic CYP2E1 and consequent oxidative stress was sufficient for NASH development [119]. This occurred even though the mice were fed with a non-fat/carbohydrate-enriched diet, suggesting that overexpression of hepatic CYP2E1 and consequent oxidative stress was sufficient for NASH development. Further research is required to elucidate the complexities of the role of CYP2E1 in hepatic lipid accumulation.

5.3. Complementary Roles of CYP4A and CYP2E1 in Lipid Oxidation through PPARα

Animal studies have suggested complementary metabolic roles of CYP2E1 and CYP4A in lipid oxidation and the production of oxidative stress [66,120]. Besides CYP2E1, only CYP4A11 is responsible for the $\omega$-hydroxylation of medium-length chain carboxylic acids.
in humans [121,122]. Silencing of CYP2E1 in mice fed a methionine-choline deficient diet increased CYP4A levels and the animals displayed elevated lipid peroxidation and NASH-like symptoms [120]. In addition, antibodies against CYP2E1 inhibited lipid peroxidation in microsomes from wild-type mice, but antibodies against CYP4A had little effect [120]. The inverse was found with microsomes from Cyp2e1 knockout mice where antibodies against CYP4A blocked lipid peroxidation, whilst antibodies against CYP2E1 had no effect. Thus, it could be concluded that CYP4 serves as an initiator or catalyst of oxidative stress as a complimentary pathway to CYP2E1.

In humans, CYP4A11 is elevated in NAFLD patients [123]. For this reason, it is more difficult to study the role of CYP2E1 alone in oxidative stress. Structure-activity relationship studies on CYP4A11 and its orthologs and CYP2E1 have confirmed similarity in their potential to be inhibited [122]. This was further tested using a strong inhibitor of CYP4A as a new class of drug candidate in targeting CYP2E1. These drugs, 1-imidazolyl-1-dodecanol and 1-imidazolylldodecane, are competitive inhibitors of the active site of CYP2E1 and restored intracellular redox balance via reduction of ROS and lipid peroxidation in vitro as well as in rats fed with high-fat diet or alcohol. Such ω-imidazolyl-alkyl derivatives inhibiting CYP2E1 may serve as a possible new therapeutic approach to NAFLD and especially to NASH [124,125]. However, it is unclear whether dual inhibition of CYP4A and CYP2E1 or only inhibition of CYP2E1 is required for these effects.

De novo lipogenesis and fatty acid oxidation are implicated in NAFLD pathogenesis, although lipid uptake, storage and export also play a role [126,127]. Fatty acid oxidation is a cyclic process in which fatty acids are shortened, releasing acetyl-CoA after each cycle [128,129]. One of the main regulators of this process is the peroxisome proliferator-activated receptor-α (PPARα) [130]. However, other factors are also involved in fine-tuning the process. Both CYP4A and CYP2E1 enzymes appear to interact with the PPARα pathway. Thus, CYP4A genes are partly controlled by PPARα; Kroetz et al., showed that the induction of CYP4A in diabetes and under starvation conditions in rats was dependent on PPARα expression [131]. Centrilobular fat accumulation and upregulation of PPARα and PPARα-mediated pathways in Cyp2e1-null mice fed with ethanol indicates an interplay between CYP2E1 and PPARα-mediated fatty acid homeostasis [132]. Increased mitochondrial fat oxidation was speculated to be due to the PPARα mediated induction of carnitine palmitoyltransferase I. These data suggest that CYP2E1 and ethanol can regulate PPARα-mediated fatty acid homeostasis, but PPARα only becomes important when the CYP2E1 level is low in high-fat conditions. The physiological relevance of this scenario is dubious since high-fat conditions induce CYP2E1 expression.

Abdelmegeed et al., showed a significant fat-induced increase in mitochondrial CYP2E1 in both wild type and PPARα-null mice [133]. PPARα-deficient mice showed greater mitochondrial dysfunction, regardless of diet, evidenced by reduced expression of mitochondrial 3-ketoacyl-CoA thiolase. However, the resultant increase in oxidative stress was more prominent in PPARα-null mice, which exhibited higher levels of CYP2E1 than the corresponding wild-type mice. These data suggest that mitochondrial CYP2E1 might play a role, at least partially, in mediating high-fat induced NASH development in PPARα-null mice. Taken together, even though these studies show interplay between CYP2E1, CYP4A and PPARα, all of which play a role in NASH development and progression, the clinical relevance has not yet been demonstrated.

5.4. Possible Role of Mitochondrial CYP2E1 in NAFLD

Different CYP2E1 isoforms exist in several cell compartments including the ER and mitochondria as previously mentioned [82,134]. In recent years, the role of non-ER-based CYP2E1 in the development and progression of NAFLD has gathered interest. Currently, the differences, if any, between isoforms in terms of induction or substrates is not well understood. Mitochondrial CYP2E1 is expressed at approximately 30% of the level of the microsomal CYP2E1 under basal conditions in rats and is associated with elevated mitochondrial oxidative stress [135]. It is present in two forms, one highly phosphorylated
form mediated via cAMP-dependent protein kinase A, and one shortened amino terminal-truncated form \[19,20,135\]. The regulation of these modifications is unclear, however, they are hypothesized to cause conformational changes and altered interactions with molecular chaperones and signal recognition particles, directing the CYP2E1 to the mitochondria.

Differences in mitochondrial CYP2E1 from its microsomal counterpart in both alcoholic fatty liver disease (AFLD) and NAFLD have been reported. A few studies have suggested that mitochondrial CYP2E1 is a major source of alcohol and drug-induced oxidative stress \[112,136,137\]. Mitochondrial CYP2E1 may be more responsible for the damage to mitochondrial function and membrane and may contribute to the biochemical and toxicological effects which were previously ascribed to CYP2E1 in the ER. Whether CYP2E1 in both ER and mitochondria work simultaneously or sequentially, and whether mitochondrial CYP2E1 exerts more pronounced effects on mitochondrial dysfunction in AFLD and NAFLD, is unclear due to lack of specific inhibitors \[82\]. Mitochondrial CYP2E1 may have a longer half-life than CYP2E1 in the ER, possibly due to unavailable ER degradation by the ubiquitin-proteasome pathway \[138\].

5.5. Kupffer Cells and CYP2E1 in NAFLD

The progression of NAFLD to NASH is characterized by increased inflammation and fibrosis. Therefore, liver-resident immune cells, such as Kupffer cells, are implicated. Their activation is driven by several factors, including cytokines and cell death \[139\]. Macrophages may be activated when hepatocytes die as a result of ROS and lipid-induced stress, their contents are released and detected by macrophages as damage-associated molecular patterns such as HMGB1 and caspase-cleaved keratin 18 \[140\]. CYP2E1 is also inducible in Kupffer cells \[16–18\] and the lipid peroxidation product 4-hydroxynonenal upregulates transforming growth factor-β expression in macrophages, causing further inflammation \[141\]. Macrophages with stably increased CYP2E1 expression (murine RAW 264.7 macrophages transfected with \(\text{CYP2E1}\)) displayed increased levels of CD14/Toll-like receptor 4, NADPH oxidase and \(\text{H}_2\text{O}_2\), accompanied by activation of ERK1/2, p38, and NF-κB in \[142\]. Apart from mitochondria-derived ROS, NADPH oxidase 2 (NOX2) activation in liver-infiltrating macrophages has also been reported to contribute to oxidative stress-induced liver damage in NAFLD \[143\]. On the other hand, the amount of CYP2E1 detected in rat Kupffer cells was 10-fold lower than in hepatocytes \[142\] and, taking into consideration the comparative number of different liver cell types, the hepatocyte localization of CYP2E1 will be of utmost importance for causing oxidative stress.

5.6. CYP2E1-Mediated ROS Production and Lipid Peroxidation

As previously mentioned, ROS can be produced from many cell systems including the mitochondrial respiratory chain \[144\], the cytochromes P450 \[145\], and oxidative enzymes \[146\]. When compared with other cytochromes P450, CYP2E1 possesses a remarkably high NADPH oxidase activity, resulting in significant production of ROS, such as hydrogen peroxide, superoxide anion radicals and hydroxyl radicals in the presence of iron catalysts \[49,65,67,82\]. ROS production from CYP2E1 causes a free radical chain reaction with unsaturated fatty acids generating toxic lipid intermediates, a reaction magnified by the presence of free iron. Such lipid peroxidation products, e.g., the biologically active aldehydes, hydroxynonenal, 4-hydroxyhydroperoxy-2-nonenal and malondialdehyde, can also modify the integrity of cellular membranes and damage proteins and DNA \[141,147\].

ROS-mediated activation of the JNK pathway can interfere with insulin sensitivity through phosphorylation of IRS-1 and IRS-2 and impair glycogenesis via action on GSK3, leading to increased gluconeogenesis \[32,148\]. In addition, ROS increase the expression of several cytokines, including transforming growth factor-β, interleukin 8, tumor necrosis factor-α and Fas ligand \[149–152\]. Both cytokines and lipid peroxidation products may act together to trigger the diverse lesions of NASH \[153\]. By-products of ROS-induced damage, such as 4-hydroxynonenal and 3-nitrotyrosine, are significantly increased in the plasma and liver, respectively in NAFLD and NASH patients \[134,154\]. CYP2E1 is crucial in this
regard as the production of ROS when it is induced, e.g., in response to FFAs, underlies much of this process.

In vivo data on the role of CYP2E1 in lipid peroxidation comes from rodent studies [74,78] and from the observation of CYP2E1 induction in human liver in NASH [109]. Using CYP2E1 inhibitors, Morimoto et al., found a relationship between CYP2E1 and lipid peroxidation in rats with ALD [78]. Besides increased lipid peroxidation, CYP2E1 was found to increase hepatic nitroxidative stress [77]. Abdelmegeed et al., used young and aged female wild-type and Cyp2e1-null mice to show that aged wild type mice had increased hepatic fibrosis, levels of hepatic hydrogen peroxide, lipid peroxidation compared with Cyp2e1-null mice [74]. These data are consistent with a role of CYP2E1 in development of liver fibrosis. There may also be a relationship between CYP2E1 and mitochondrial dysfunction due to mitochondrial CYP2E1-mediated ROS production [137]. In addition, lower ROS detoxification and lipid peroxidation-derived reactive aldehydes play a role in mitochondrial stress associated with NAFLD development and progression [155,156]. This can trigger a vicious cycle, further increasing ROS generation through abnormal electron leakage.

Together, these studies largely agree that CYP2E1-mediated increased nitroxidative radicals, lipid peroxidation, and post-translational protein modifications are the main mechanisms by which CYP2E1 likely plays a prominent role in NAFLD development and progression [82]. Expressed simply, CYP2E1 is a major source of hepatic ROS. The action of ROS from CYP2E1 and other sources can induce lipid peroxidation and cause other non-specific damage to cellular macromolecules. Therefore, conditions that induce the expression of CYP2E1 will also increase the production of ROS and ROS-related damage.

The link between oxidative stress and the development and progression of ALD and NAFLD has led to the study of the potential of antioxidants for prevention or treatment for these diseases using either Mediterranean diet or drugs. Clinical trials have shown that vitamin E can ameliorate NAFLD by attenuating oxidative stress and inflammation (reviewed in [157,158]); however, the safety of prolonged vitamin E use is uncertain [159–161]. In addition to vitamin E, flavonoids have anti-inflammatory and antioxidant properties and reduce the expression of CYP2E1 (reviewed in [162]). For instance, quercetin and several saponins, alkaloids, terpenoids and polyphenols have been tested in vivo and in vitro and many have shown promise for NASH treatment (reviewed in [163]). Unfortunately, these effects have not persisted under clinical trial conditions thus far.

6. In Vitro Models for Examining the Role of CYP2E1 and FFA on Liver Fibrosis and Damage

The liver is composed of hepatocytes (~60%) and non-parenchymal cells (NPCs) (~40%). The main types of NPCs are stellate cells, Kupffer cells and sinusoidal endothelial cells. NAFLD and NASH are not formed solely by the action of hepatocytes but are rather the results of complex interactions between the NPCs, hepatocytes and external factors. For this reason, models with only hepatocytes or hepatocyte-like cells cannot accurately recapitulate NAFLD development and progression compared with heterocellular models. However, by comparing the monocellular and heterocellular NASH models the role of NPCs in the development and progression of NAFLD can be studied [164].

In vitro models can be divided into groups based on the cell types and culture methods employed. Many recent advances in in vitro models for NAFLD and liver toxicity in general use 3D cultures, often due to the increased physiological relevance these models can offer. There are multiple 3D culture techniques ranging from matrix-based systems to suspension cultures, both with and without liquid flow. In addition, tissue-on-a-chip models have been developed and show great promise. Some advances in modelling NAFLD were recently reviewed by Soret et al. [165].

Human primary hepatocytes (PHH) dedifferentiate in two-dimensional culture systems precluding their useful application to long-term experiments, such as the development of NAFLD. Compared with traditional monolayer cell culture models, 3D Spheroid models have been shown to more accurately mimic the in vivo environment [52]. PHH 3D
spheroids maintain tissue-like architecture, cell–cell interactions and hepatic phenotype and can be used to model the onset of NAFLD (Figure 5) [164,166–168]. Using such a 3D PHH spheroid model with NPCs, we found that NPCs and FFA induce CYP2E1 on the mRNA and protein level [164]. In some donors, heterocellular spheroids showed elevated CYP2E1 protein expression in the presence of NPCs only, without added FFA. This might be indicative of the fact that increased lipid levels during steatosis might stimulate induction of liver fibrosis by the subsequent induction of CYP2E1 causing an increase ROS. The contribution of CYP2E1 in NASH may indeed be of importance, but more studies are needed in this topic.

Figure 5. Suggested link between steatosis and liver fibrosis mediated by elevated levels of CYP2E1 caused by increase production of fat. (A) Exposure to FFA in human 3D liver spheroids causes elevated levels of lipids and increase expression of CYP2E1 (From [164]). (B) Proposed involvement of CYP2E1 in mediating increased liver fibrosis in response to elevated levels of hepatic lipids in steatosis. Upwards arrows represent an increased number of molecules or greater expression. Figure modified from [163].

7. Conclusions

It is evident that CYP2E1 has important functions for both lipid and glucose homeostasis as well as being an important enzyme in toxicology. The enzyme is highly conserved with essentially no functionally different genetic variants, emphasizing its important endogenous functions, many of which may still be unknown. The toxicologically relevant functions are to a great extent related to the high-spin nature of the iron in the enzyme, allowing effective reduction of dioxygen and other compounds in the absence of bound substrate, as well as both reductive and oxidative radical formation by the enzyme. The enzyme action is important for generation of ALD and NASH and it is likely that the link between steatosis and NASH could to some extent be explained by the fact that excess lipids highly induce the hepatic levels of CYP2E1, thus resulting in ROS stress and increased lipid peroxidation, key events for development of NASH.

Although much has been learned, there are still many factors to consider for the future. CYP2E1 resides mainly in the ER of hepatocytes where it takes part in the metabolism of fatty acids, acetone and other endogenous compounds. The function of mitochondrial CYP2E1, if any, is unknown.
The contribution of CYP2E1 to elimination of ethanol is still controversial and conclusions differ greatly between authors. In addition, the relationship between the specific and nonspecific CYP2E1-mediated oxyradical-mediated oxidation of ethanol is unclear. CYP2E1 expression is elevated following ethanol treatment, but the rate of ethanol oxidation is very low, as compared with ADH. Furthermore, ADH is strongly induced by high ethanol levels and might represent the major component for adaptive ethanol oxidation.

The role of CYP2E1 for development of NASH is still unclear. Further experimentation is necessary to directly show the role of CYP2E1 in enhanced production of mediators activating stellate cells to produce profibrotic cytokines and, indeed, the effect of CYP2E1 on activation of liver endothelial cells has not been described. The elevation of CYP2E1 following lipid treatment may have anti-steatotic effects because of higher rates of lipid degradation. Inhibitors of CYP2E1 have been shown to be effective for the development of ALD, but must also be examined for NASH production.

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Conflicts of Interest: Magnus Ingelman-Sundberg is a co-founder and shareholder in HepaPpredict AB. The other authors have no conflict of interests to declare.

Abbreviations

| 3D | three dimensional |
|---|------------------|
| ADH | alcohol dehydrogenase |
| AFLD | alcoholic fatty liver disease |
| ALD | alcoholic liver disease |
| ASH | alcoholic steatohepatitis |
| CDE | chlorodifluoroethylene |
| CTE | chlorotrifluoroethane |
| CYP2E1 | cytochrome P450 2E1 |
| ER | endoplasmic reticulum |
| FFA | free fatty |
| HCC | hepatocellular carcinoma |
| hPSC | human pluripotent stem cell |
| NADPH | nicotinamide adenine dinucleotide phosphate acid |
| NAFL | nonalcoholic fatty liver |
| NAFLD | nonalcoholic fatty liver disease |
| NASH | nonalcoholic steatohepatitis |
| NPC | non-parenchymal cell |
| PHH | human primary hepatocyte |
| POR | P450 reductase |
| ROS | reactive oxygen species |
| TFA | trifluoroacetic acid |
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