Chapter 5
Relevance of CYP2E1 to Non-alcoholic Fatty Liver Disease

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Abstract Non-alcoholic fatty liver disease (NAFLD) and its progression to steatohepatitis (NASH) and cirrhosis is a growing problem in most developed countries. Increased hepatic expression of CYP2E1, which carries out omega hydroxylation of fatty acids, was first shown in a mouse model of NASH and this was later also reported for human NASH, though not all studies agree with this finding and further larger studies are still needed. In view of its role in fatty acid metabolism which leads to increased levels of toxic lipid peroxides and its possible increased expression in NASH, CYP2E1 is an attractive candidate for a role as a genetic risk factor for both NAFLD generally including progression to NASH. Two studies have focused on the variant allele *CYP2E1*5*, which may be associated with increased CYP2E1 expression. Both reported increased frequencies of this allele in NASH patients, though statistical significance was not achieved because of small sample sizes. Some more indirect data also suggests a relationship between high CYP2E1 activity and progression to NASH. However, three recent genome-wide association studies on NAFLD have failed to find any evidence that single nucleotide polymorphisms in or adjacent to the *CYP2E1* gene contribute to susceptibility. Further studies are needed to investigate a possible role in disease progression in addition to susceptibility and the possibility that statistical power in the existing studies was insufficient to detect a relatively small contribution to disease susceptibility.

Keywords CYP2E1 • Gene polymorphism • NASH • NAFLD

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A. Dey (ed.), *Cytochrome P450 2E1: Its Role in Disease and Drug Metabolism*,
Subcellular Biochemistry 67, DOI 10.1007/978-94-007-5881-0_5,

5.1 Introduction

As reviewed in other chapters, the relevance of CYP2E1 to ethanol metabolism and to alcoholic liver disease is well established and has been studied for the last 40 years approximately (Lieber and DeCarli 1970). In addition to induction of CYP2E1 by ethanol, it has been known for some time that CYP2E1 is induced in diabetic and obese humans and rodents (Hong et al. 1987; Song et al. 1986, 1987). The increased expression of CYP2E1 in type II diabetes and obesity raised the possibility that CYP2E1 expression might be relevant to the pathogenesis of nonalcoholic fatty liver disease (NAFLD). NAFLD covers a disease spectrum ranging from hepatic steatosis alone though non-alcoholic steatohepatitis (NASH) to cirrhosis and is an increasingly common cause of liver dysfunction in developed countries (for review see Anstee et al. (2011a, b); de Alwis and Day (2008)). NAFLD is strongly associated with obesity, insulin resistance, type 2 diabetes and dyslipidaemia. Though most patients with these conditions develop steatosis alone, a minority progress to more advanced liver disease characterized by inflammation, fibrosis, cirrhosis and, in some cases, hepatocellular carcinoma.

Increased expression of CYP2E1 was first described in hepatocytes from rats fed a methionine-choline diet, which is an established model for human steatohepatitis (Weltman et al. 1996), followed by a study demonstrating a similar effect by immunohistochemical analysis of liver biopsies from steatohepatitis patients (Weltman et al. 1998). Both the general role of CYP2E1 in the NAFLD disease process and the possibility that either interindividual variability in levels of CYP2E1 or in ability to induce expression of this enzyme could affect susceptibility to and severity of this disease is of considerable interest. In particular, whether CYP2E1 could be a target for novel treatments and whether *CYP2E1* genotype contributes to individual risk for disease development are both questions that are of importance to understanding the pathogenesis of NAFLD.

5.2 CYP2E1 and NAFLD

Though ethanol is the best established inducer of CYP2E1 (Lieber and DeCarli 1970; Song et al. 1986), it has been known since the 1980s that CYP2E1 induction can also occur in diabetic rats (Song et al. 1987) and during starvation (Hong et al. 1987). It was subsequently demonstrated that CYP2E1 catalyzes omega hydroxylation of fatty acids (Laethem et al. 1993). The demonstration that hepatic CYP2E1 levels are elevated in a rat model of NASH (Weltman et al. 1996) and in patients with NASH (Weltman et al. 1998) suggests that fatty acids are also CYP2E1 inducers. Whether CYP2E1 induction is of direct relevance to disease pathogenesis in terms of either human NAFLD generally or NASH development remains unclear. Studies in animal models suggest it could be a factor but studies in humans have generally involved small numbers and given contradictory results.

Several different mouse models exist for both NAFLD and NASH though none of these is a true model for progression of NAFLD to NASH (for review see De Minicis and Svegliati-Baroni (2011); Hebbard and George (2011)). The most widely used models are the high fat diet (HFD) where mice given a high fat diet show steatosis, weight gain and insulin resistance and the methionine and choline-deficient (MCA) diet model where mice show some characteristics of NASH. Using the MCA diet model, a large increase in lipid peroxide formation in the liver was detected in parallel with CYP2E1 induction (Leclercq et al. 2000) but importantly, in mice lacking the CYP2E1 gene, lipid peroxides were still formed via CYP4A, which was also upregulated by the MCA diet. Lipid peroxide formation is likely to be an important contributor to NASH pathophysiology since it may result in serious toxicity within cells but CYP2E1 may have additional effects that could also contribute to NAFLD more generally. In particular, CYP2E1 overexpression in a hepatocyte cell line was demonstrated to impair insulin signalling with decreased insulin receptor substrate (IRS)-1 and IRS-2 phosphorylation and other effects including decreased glycogen synthase kinase 3 activity and glucose secretion detected in response to insulin (Schattenberg et al. 2005). Insulin resistance is an important feature of NAFLD and a possible contribution from CYP2E1 to this resistance is an interesting finding. In a more recent study using transgenic mice with CYP2E1 overexpressed specifically in hepatocytes and fed the HFD, higher fasting insulin levels and decreased insulin signalling was seen, which is consistent with the *in vitro* findings (Kathirvel et al. 2009). The converse was also demonstrated in a separate study involving CYP2E1 knockout mice fed the HFD (Zong et al. 2012). In this case, the mice did not gain weight and show insulin resistance. A further study involving the transgenic CYP2E1 overexpressing mice showed increased levels of both lipid peroxidation and protein carbonylation together with decreased activity for certain enzymes that protect against oxidative stress, possibly due to their inactivation by nitrosylation (Kathirvel et al. 2010).

Since the original observations showing increased CYP2E1 levels in NASH in human liver by immunohistochemistry (Weltman et al. 1998), the number of investigations in humans has been limited. One follow-up study compared CYP2E1 expression and activity in liver biopsies from patients with either steatosis alone or NASH (Chtioui et al. 2007) but found no difference between the two patient groups for either parameter. However, CYP2E1 activity correlated positively with both body mass index and steatosis score. Evidence for increased CYP2E1 protein levels in both steatosis and NASH was seen in a study on liver biopsies which also measured CYP2E1 enzyme activity *in vivo* using chlorzoxazone phenotyping. The phenotyping analysis showed significantly higher activity in NASH cases compared with both steatosis and controls (Varela et al. 2008). A study on adult explanted livers involving immunohistochemistry and measurement of protein and mRNA levels found that CYP2E1 levels appeared to fall with progression to NASH (Fisher et al. 2009). In a recent study on children with NAFLD where CYP2E1 protein levels in liver biopsies were determined, no increase in protein levels for either NASH cases only or NAFLD as a whole compared with samples showing normal histology was observed (Bell et al. 2011). In addition, levels of lipid peroxidation did not differ

between biopsies with and without NASH, though body mass index did correlate with levels of lipid peroxidation. It therefore appears that there is considerable inconsistency between studies on human CYP2E1 expression in NAFLD. This possibly reflects the fact that all the studies discussed above involved relatively small numbers of samples and larger studies are clearly needed.

5.3 Genetics of NAFLD Including Possible Role of CYP2E1 Polymorphisms

5.3.1 Background

There is evidence for a role for genetic factors in NAFLD from both family and inter-ethnic variation studies. In a recent study on families which included overweight children with NAFLD (Schwimmer et al. 2009), fatty liver was significantly more common in the siblings and parents of the children with NAFLD. Another study on monozygotic and dizygotic twins showed that serum alanine aminotransferase (ALT) and fasting serum insulin intrapair correlations were significantly higher in the monozygotic compared to the dizygotic twins (Makkonen et al. 2009). In a study on the offspring of participants in the Framingham Heart Study, early-onset paternal obesity was associated with elevated ALT levels in offspring, suggesting a genetic predisposition to developing elevated ALT levels and possibly NAFLD (Loomba et al. 2008). For NAFLD in adults, Struben et al. (2000) described the co-existence of NASH and/or cryptogenic cirrhosis in 7 out of 8 kindreds studied and another study (Willner et al. 2001) found that 18% of 90 patients with NASH had an affected first-degree relative. This clustering could simply be due to heritability of obesity and insulin resistance, the main risk factors for NAFLD. However, other studies examining ethnic differences in the prevalence of NAFLD suggest that susceptibility may have a specific genetic component (Browning et al. 2004a, b). It appears that African-Americans, though as prone to obesity as Americans of European or Hispanic origin, show a lower incidence of both steatosis and cryptogenic cirrhosis. This may be due to different patterns of fat accumulation and a lower incidence of insulin resistance among obese African-Americans (Guerrero et al. 2009). US Hispanics also appear more susceptible to NAFLD than US individuals of European ethnic origin (Browning et al. 2004a; Williams et al. 2010).

5.3.2 Candidate Gene Studies on NAFLD

A large number of candidate gene association studies together with three genome-wide association studies (GWAS) on susceptibility to NAFLD have now been reported (Anstee et al. 2011a; Daly et al. 2011). The candidate gene studies have focussed particularly on genes relevant to lipid metabolism and oxidative stress and have

reported a limited number of positive associations (Daly et al. 2011). The most consistent positive message from both candidate gene and genome-wide association studies is that genotype for *PNPLA3*, which codes for the enzyme patatin-like phospholipase domain-containing 3, also known as adiponutrin, modulates risk of developing NAFLD. This enzyme is a serine protease whose function is still somewhat unclear but appears to contribute to triacylglycerol hydrolysis (Huang et al. 2011). Evidence from a large number of studies including two separate GWAS suggests that for development of steatosis, there is an increased risk in those carrying one or two copies of a *PNPLA3* variant allele associated with a nonsynonymous mutation (Romeo et al. 2008; Sookoian and Pirola 2011; Speliotes et al. 2011). Further studies using candidate gene approaches indicated that *PNPLA3* genotype also predicts fibrosis severity in NAFLD (Sookoian and Pirola 2011; Valenti et al. 2010). The odds ratio for developing severe steatosis or fibrosis associated with the possession of the *PNPLA3* variant allele has been found to be approximately two in most studies. The *PNPLA3* association with NAFLD susceptibility and severity has been observed in several different ethnic groups (Li et al. 2012; Sookoian and Pirola 2011).

A number of other genes have also been reported to be associated with susceptibility to NAFLD and severity of several phenotypic features but with the exception of an association of a polymorphism in the manganese-dependent superoxide dismutase gene (*SOD2*) with development of severe fibrosis and NASH (Al-Serri et al. 2012; Namikawa et al. 2004), generally these associations are generally inconsistent with some studies showing associations but others failing to confirm them (see Daly et al. (2011) for detailed review).

5.3.3 *Candidate Gene Studies on CYP2E1 as a Genetic Risk Factor in NAFLD*

Genetic polymorphism in CYP2E1 is generally a well studied area. There is evidence of approx. 20 fold interindividual variation in expression of CYP2E1 in human livers though phenotyping studies using the muscle relaxant chlorzoxazone as probe in European populations have demonstrated only two to threefold variation in levels of activity (Kim and O'Shea 1995). A number of genetic polymorphisms in *CYP2E1* have been reported with the majority occurring in either upstream sequences or introns and mostly appearing to lack functional significance. Polymorphisms affecting coding sequences are rare. One of these, R76H encoded by *CYP2E1*2*, is associated with decreased catalytic activity and occurs at a low frequency in a Chinese population but has not been detected in other ethnic groups (Hu et al. 1997). It has been suggested that a polymorphism in the 5′-flanking region within a putative HNF-1 binding site may be of functional significance with *in vitro* studies suggesting that this allele shows approximately tenfold higher transcriptional activity than the wild-type (Hayashi et al. 1991). This variant allele (*CYP2E1*5*) occurs at a frequency of 0.27 in Japanese but only 0.02 in Europeans (Kato et al. 1992). Our overall understanding of the molecular basis of interindividual variation in CYP2E1 expression is still unclear. There is also a possibility that there is interindividual

variability in ability to induce this enzyme, as reported in a study on ethanol induction of CYP2E1 (Dupont et al. 1998), but again this is not well understood.

The possibility that CYP2E1 genotype could be a risk factor for development of NAFLD or determine disease progression has been investigated in only two small studies to date. The first of these concerned NAFLD only and found that the *CYP2E1*5* allele was more common in a group of 28 Chinese patients with "obese or diabetic" fatty liver compared with 40 controls (Piao et al. 2003). The second study concerned female steatosis (n = 18) and NASH (n = 17) patients without diabetes (Varela et al. 2008) and found that there was an apparent increase in the frequency of *CYP2E1*5* in the NASH cases compared with both healthy controls and steatosis cases but the genotype frequency differences were not statistically significant. However, when CYP2E1 protein levels in liver biopsies and enzyme activity levels *in vivo* were compared, CYP2E1 protein levels were higher in both NASH and steatosis liver biopsies and NASH patients showed higher levels of activity *in vivo* compared with steasosis cases and controls. Carriage of the *CYP2E1*5* also correlated significantly with higher *in vivo* CYP2E1activity, which is in general agreement with previous *in vitro* data suggesting that this allele shows approximately tenfold higher transcriptional activity than the wild-type (Hayashi et al. 1991). Together the two studies on CYP2E1 genotypes in NAFLD provide a suggestion that carriage of at least one *CYP2E1*5* allele might be a risk factor in NAFLD but their small size and failure to see statistical significance for actual genotypes due to small numbers is a serious limitation. As well as performing larger studies, investigating a wider range of polymorphisms and areas such as interindividual variation in ability to induce CYP2E1 in NAFLD cases would be worthwhile. *CYP2E1*5* has also been found to be a risk factor for development of alcoholic liver disease which has common features to NAFLD (Grove et al. 1998; Pirmohamed et al. 1995), though more recent studies on associations of this allele with the disease are less positive (for review see Anstee et al. (2011b)). However, the possible association with susceptibility to alcoholic liver disease is likely to relate more to the role for CYP2E1 in ethanol metabolism than fatty acid metabolism as in NAFLD. A recent report suggesting that the *CYP2E1* gene contributes to interindividual variability in alcohol response (Webb et al. 2011) is an added complication to seeking parallels with NAFLD.

5.3.4 Genome-Wide Association Studies on NAFLD

In the last 5 years, several genome-wide association studies (GWAS) on NAFLD have been performed (Chalasani et al. 2010; Romeo et al. 2008; Speliotes et al. 2011). These studies involve genotyping cases and controls for single nucleotide polymorphisms (SNPs) throughout the genome. Performing a GWAS means that a large number of possible genetic associations for a disease are being examined, not simply those that are biologically obvious, and also enables the possibility that genetic polymorphism in *CYP2E1* contributes to NAFLD susceptibility to be investigated. Table 5.1 summarizes the main features of all three studies and their findings.

Table 5.1 Genome-wide association studies on NAFLD

Reference	No. of cases and controls	No. of SNPs typed	Main findings
Romeo et al. (2008)	2,111 American individuals in cohort study who underwent hepatic fat content analysis by proton nuclear magnetic resonance analysis	12,138 nonsynonymous variant SNPs genotyped with 9,229 passing QC	Single variant in PNPLA3 (rs738409) strongly associated with hepatic fat content ($P = 5.9 \times 10^{-10}$). No other SNP showed $P < 5.4 \times 10^{-6}$ (threshold for significance based on Bonferroni correction)
Chalasani et al. (2010)	239 American females with biopsy-proven NAFLD	373,397 SNPs with 324,623 used in the final analysis	Most significant association with quantitative histologic activity score (NAS) was for rs2645424 in farnesyl diphosphate farnesyl transferase 1 (*FDFT1*) gene ($P = 8.0 \times 10^{-7}$)
Speliotes et al. (2011)	7,176 Americans with extent of hepatic steatosis measured using computed tomography studied in GWAS. Replication of findings in 592 American biopsy-proven NAFLD cases and 1,405 population controls	300,000–500,000 (meta analysis using data obtained for several cohorts)	Associations ($p < 10^{-4}$) seen for *PNPLA3*, *NCAN*, *GCKR* and *LYPLAL1* with respect to both extent of steatosis and for biopsy-proven NAFLD compared with controls

Though each of these studies suffers from a range of limitations and there is a need for further studies where the severity of NAFLD with respect to genotype in a larger group of cases with liver biopsy is analyzed, no evidence for any signal either within the *CYP2E1* gene or adjacent to it has so far emerged. It remains possible that there is insufficient statistical power in the studies described so far to detect a signal from the *CYP2E1* gene or that a genetic regulator of CYP2E1 expression but not *CYP2E1* itself affects susceptibility.

5.4 Concluding Remarks

The relevance of CYP2E1 to NAFLD and NASH is a slightly neglected area, particularly recently. Though data suggesting a role for CYP2E1 in both NAFLD and NASH from animal models has appeared generally promising, attempts to establish whether CYP2E1 expression changes in human disease during the progression of human liver to NAFLD and then further progression to NASH have been limited, probably because of the difficulties in obtaining suitable samples for analysis, especially in large numbers. Better animal and cellular models for both NAFLD and NASH would facilitate progress. In terms of CYP2E1 as a genetic risk factor for NAFLD, it is increasingly likely that as for other complex diseases with a genetic component, many different variants contribute with the overall contribution from each being quite small (Hirschhorn and Gajdos 2011). For example, in the case of type II diabetes, one of the most extensively studied complex diseases, polymorphisms in more than 30 different genes have been demonstrated to contribute to susceptibility but odds ratios for some of these genes are as low as 1.1 (Voight et al. 2010). Detecting such small effects requires in the order of 10,000 cases for statistical significance and, though NAFLD is now a very common disease worldwide, it is unlikely that DNA collections of this size are yet available. Building up such a large DNA collection from NAFLD cases is particularly challenging because of the importance of including only cases where histology has been definitively established by liver biopsy (Anstee et al. 2011c). However, given the increasing frequency of NAFLD in developed countries and the current lack of any effective treatment, performing such large studies should be given a high priority since they may lead to development of new treatments and design of more effective strategies for preventing disease progression.

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