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Induction of CYP2E1 in non-alcoholic fatty liver diseases

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Abstract

Mounting evidence supports a contribution of endogenous alcohol metabolism in the pathogenesis of non-alcoholic steatohepatitis (NASH). However, it is not known whether the expression of alcohol metabolism genes is altered in the livers of simple steatosis. There is also a current debate on whether fatty acids induce CYP2E1 in fatty livers. In this study, expression of alcohol metabolizing genes in the liver biopsies of simple steatosis patients was examined by quantitative real-time PCR (qRT-PCR), in comparison to biopsies of NASH livers and normal controls. Induction of alcohol metabolizing genes was also examined in cultured HepG2 cells treated with ethanol or oleic acid, by qRT-PCR and Western blots. We found that the mRNA expression of alcohol metabolizing genes including ADH1C, ADH4, ADH6, catalase and CYP2E1 were elevated in the livers of simple steatosis, to similar levels found in NASH livers. In cultured HepG2 cells, ethanol induced the expression of CYP2E1 mRNA and protein, but not ADH4 or ADH6; oleic acid did not induce any of these genes. These results suggest that elevated alcohol metabolism may contribute to the pathogenesis of NAFLD at the stage of simple steatosis as well as more severe stages. Our in vitro data support that CYP2E1 is induced by endogenous alcohol but not by fatty acids.

Keywords

CYP2E1; NASH; NAFLD; simple steatosis

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease in US (Williams et al., 2011). In general, NAFLD is considered a comorbidity of obesity (Barshop et al., 2009) and the hepatic manifestation of metabolic syndrome (Kim and Younossi, 2008). Mild NAFLD, or simple steatosis may progress toward more severe stages such as non-alcoholic steatohepatitis (NASH), fibrosis and cirrhosis, according to the “multiple-hit” hypothesis for the pathogenesis of NAFLD (Tilg and Moschen, 2010).

Oxidative stress is a well-known feature of NAFLD livers and a potent “hit” for the pathogenesis of NAFLD (Day and James, 1998). Mounting evidence supports that the enzymatic activity of cytochrome P450 2E1 (CYP2E1) makes a significant contribution to the oxidative stress in NAFLD livers. CYP2E1, an alcohol inducible enzyme (Ingelman-Sundberg et al., 1993; Takahashi et al., 1993) known for its role in the pathogenesis of alcoholic fatty liver disease, generates reactive oxygen species (free radicals) when catalyzing the oxidation of ethanol (Lieber, 2004; Robertson et al., 2001). In 1998, increased CYP2E1 protein was observed in NASH livers (Weltman et al., 1998). Elevated CYP2E1 activity in NASH liver was also reported (Chalasani et al., 2003). In 2010, we reported that CYP2E1 was elevated in NASH livers at the mRNA level (Baker et al., 2010). These studies consistently support a role for CYP2E1 in the pathogenesis of NASH, possibly through a similar mechanism that was described for alcoholic fatty liver disease (Lieber, 2004). However, it is not known whether liver with simple steatosis also exhibits elevated CYP2E1 expression. Here we report our findings that CYP2E1 was similarly elevated in the livers of both simple steatosis and NASH, compared to normal livers.

To find the cause for induced CYP2E1, initial efforts focused on the possibility that CYP2E1 was induced by free fatty acids or ketone bodies but not by ethanol, because of the “non-alcoholic” nature of these patients. Chalasani et al. reported that hydroxyl butyrate (one type of ketone body) is correlated with the CYP2E1 activity (Chalasani et al., 2003). Soon after, it was reported that palmitate induced CYP2E1 mRNA in primary cultured human hepatocytes (Raucy et al., 2004). Similarly, CYP2E1 is also induced by fatty acids in cultured HepG2 cells (Sung et al., 2004). However, opposite results were also reported showing that fatty acid treatments down regulated CYP2E1 at mRNA and enzymatic levels in primary cultured human hepatocytes (Donato et al., 2007; Donato et al., 2006). Therefore, it remained unknown whether free fatty acid can induce CYP2E1. Here we examined the effect of free fatty acid treatment on cultured HepG2 cells and found no significant change in CYP2E1 expression.

MATERIALS AND METHODS

Patients

This study was approved by Children and Youth Institutional Review Board of the State University of New York at Buffalo. Using Kleiner’s criteria (Kleiner et al., 2005), biopsy diagnosed NAFLD patients were recruited for this study. Simple steatosis group included patients who had hepatic fatty change but no evidence for liver inflammation or fibrosis. NASH group included patients who had liver inflammation in addition to fatty change.

Required sample size was estimated with G*Power version 3.1.9.2. According to the CYP2E1 expression data in our previous study (Baker et al., 2010), the effect size f for a three-group test (healthy, simple steatosis and NASH) was 0.84 under the category of one-way ANOVA. Assuming that the gene expression is similar between simple steatosis and NASH, with an $\alpha = 0.05$ and a power = 0.8, the projected sample size is 6 for each group. A total of 6 biopsy-proven simple steatosis biopsy samples were collected from July 2011 to June 2013. Out of the pool of available biopsy-diagnosed NASH liver biopsies collected at the same time, 6 were randomly selected that are age and gender matched with the simple steatosis group. For healthy liver controls, total RNA was purchased from Admet Technologies (Durham, NC). These samples were prepared from liver grafts of pediatric healthy subjects with normal body mass index (Table 1). The healthy status of these livers was ascertained by the lower transcription levels of marker genes for inflammation and fibrosis, as reported previously (Baker et al., 2010).

Quantitative real-time PCR (qRT-PCR)

Primers were designed with the assistance of Primer3 (<http://bioinfo.ut.ee/primer3/>). Primers for CYP2E1 (Forward: 5'TGGAAGCACTCAGGAAGACC3', Reverse: 5'AGAGGATGTCTGGCTATGACG3') and CAT (Forward: 5'CAGGACAATCAGGGTGGTG3', Reverse: 5'CAGGGCAGAAGGCTGTTG3'). Primers for GAPDH, ADH1C, ADH4 and ADH6 are described previously (Baker et al., 2010). The presence of a single specific PCR product for all the primer pairs was confirmed by melting curve analysis agarose gels analysis and direct sequencing of the amplicons. Complementary DNA was synthesized from 0.8µg RNA in a volume of 20 µl, with the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). Real-time PCR was performed on an iCycler iQ real-time detection system (Bio-Rad Laboratories, Hercules, CA), using Sybergreen (iQ™ SYBR® Green Supermix; Bio-Rad Laboratories, Hercules, CA) as the monitoring fluorescein. GAPDH was run as the reference gene in parallel with the genes of interest.

Threshold cycles (C_t) for each sample were determined by Bio-rad iQ5 optical system software (Bio-Rad Laboratories). The concentration of mRNA ([mRNA]) is represented by the following equation: $[mRNA] = M/E^{C_t}$, where constant M is an arbitrary threshold, E is the efficiency of PCR, C_t is the threshold cycle. All PCR reactions had efficiencies higher than 1.9, as determined experimentally with 4-fold serial diluted samples. The relative mRNA concentration of each target gene was determined using the following equation:

$$[mRNA]_{\text{target}}/[mRNA]_{\text{GAPDH}} = E_{\text{GAPDH}}^{C_{t,\text{GAPDH}}} / E_{\text{target}}^{C_{t,\text{target}}}$$

Cell culture

HepG2 cells (the American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37 °C, under a humidified atmosphere of 5% carbon dioxide. HepG2 cells were plated on 100 mm dishes at density of 60% confluency. Next day cells were treated with 2mM oleic acid, 40mM ethanol or with DMSO (solvent for free fatty acids, as a control), respectively. After treatment for 24 hours, cells were harvested for RNA extraction and Western blotting. For

Oil Red O staining, cells were plated on cover slips placed in 6 well plates. After treatment for 24 hours, cells were stained with Oil Red O and counterstained with hematoxylin.

Western blot

Harvested cells were homogenized in PBS, and then boiled for 5 min in SDS-PAGE loading buffer. Samples with 40µg total protein each were separated on 10% SDS PAGE gels. After blotting onto nitrocellulose membranes, CYP2E1 (Abcam, Cambridge, MA) and β-actin (Clone C4, MP Biomedicals, LLP, Ohio) were probed. The results were visualized using the SuperSignal West Dura Extended Duration Substrate (Invitrogen) and recorded with ChemiDoc MP image system (Bio-Rad). The intensity of signal was analyzed using densitometry. The CYP2E1 signal was normalized to beta actin. The CYP2E1 signal in control was set as 1.

Statistical Analysis

Student's t test with a two-tailed distribution was performed to compare the means of two groups. One way ANOVA was performed to compare the means of 3 or more groups, followed by post-hoc Tukey's tests for pair-wise comparisons. P values smaller than 0.05 were considered significant.

RESULTS

Elevated expression of alcohol metabolizing genes in simple steatosis and NASH livers

This prospective pilot study evaluates the activity of alcohol metabolizing genes in the liver of simple steatosis. A total of 6 liver biopsies of simple steatosis were compared to a group of six with NASH. The NASH patients were age- and gender-matched with the steatosis patients (Table 1). The steatosis and NASH group also share similar BMI, IR-HOMA value, and AST. There was no significant difference for ALT between the steatosis and NASH groups ($P = 0.69$), which goes along with the reports that simple steatosis is associated with elevated ALT activity. (Noguchi et al., 1995; Schwimmer et al., 2005) The groups were differentiated by histology according to Kleiner's criteria (Kleiner et al., 2005). Healthy donor liver RNA samples from pediatric subjects were used as controls. The control subjects were not age- or gender-matched with the NAFLD groups because of limited availability. This is justified because of the observations that the impact of age and gender on alcohol metabolizing genes are insignificant and sometimes not detected, compared to the impact of NAFLD pathology. (Zhu et al., 2015)

Quantitative RT-PCRs were performed to examine the mRNA level for genes representative of alcohol metabolizing activities in hepatocytes. These include the alcohol dehydrogenases (ADHs), microsomal ethanol oxidizing system (MEOS) and catalase. The ADHs examined in qRT-PCR include ADH1C, ADH4 and ADH6, each belongs to a different class of ADH. The expression of these ADHs is known to be highly elevated in NASH and here we observed a similarly elevated expression in the steatosis livers as well as NASH livers, compared to the controls (Figure 1A, 1B, 1C). The fold change between steatosis and control for the expression of ADH1C, ADH4 and ADH6 were 10.6, 8.7 and 4.2, respectively. The fold change between NASH and control for the expression of ADH1C,

ADH4 and ADH6 were 9.8, 10.1 and 4.7, respectively. No difference was observed between steatosis and NASH livers for all three genes. CYP2E1 is the major alcohol metabolizing activity in MEOS, while catalase catalyzes alcohol oxidization in peroxisomes. Elevated mRNA expression of CYP2E1 and catalase was observed in both steatosis and NASH livers (Figure 1D, 1E). The fold change between steatosis and control for the expression of CYP2E1 and catalase were 3.1 and 6.0, respectively. The fold change between NASH and control for the expression of CYP2E1 and catalase were 3.0 and 5.0, respectively. Again, no difference in the expression of CYP2E1 and catalase was observed between steatosis and NASH.

Induction of CYP2E1 in cultured HepG2 cells by ethanol but not by fatty acid

Literature suggested two possible causes for elevated expression of alcohol metabolizing genes in hepatocytes: increased presence of alcohol or fatty acids. Here we compared the effect of ethanol and fatty acid treatment on the expression of CYP2E1, ADH4 and ADH6 in cultured HepG2 cells. HepG2 cells are derived from human hepatocellular carcinoma, and are often used as an *in vitro* model system for the study of human hepatocytes. HepG2 cells treated with fatty acid and control cells were first stained with Oil-red O (Figure 2A). Cells treated with fatty acid exhibited elevated intracellular lipid storage compared to the control cells. These cells and cells treated with ethanol were then subjected to qRT-PCR analysis for the expression levels of CYP2E1, ADH4 and ADH6, with GAPDH as the housekeeping gene. As expected, induction of CYP2E1 was observed in ethanol treated cells (Figure 2B). However, ethanol treatment did not induce the expression of ADH4 and ADH6 perhaps because treatment length was insufficient (Figure 2C, 2D). Fatty acid treatment did not affect the expression of CYP2E1, ADH4 or ADH6 (Figure 2B, 2C, 2D).

To confirm the above observed effects of ethanol and fatty acid on CYP2E1 mRNA expression, similarly prepared samples were analyzed for CYP2E1 protein levels in Western blot. Normalized against β -actin signal, the ethanol treated HepG2 cells exhibited higher expression of CYP2E1 protein than control cells (~3 folds increase). Fatty acid treatment did not affect CYP2E1 protein level.

DISCUSSION

Here we report that CYP2E1, as well as alcohol dehydrogenases and catalase were up-regulated in mRNA level in the livers of pediatric non-alcoholic steatosis patients. The extent of the up-regulation in these genes was similar to that in pediatric NASH patients, and no significant difference was observed between the steatosis and NASH groups. These observations suggest that elevated activity of alcohol metabolizing genes may play a role in steatosis as well as in more severe stages of NAFLD. Increased alcohol in the circulation of NAFLD patients was observed by several groups. (Michail et al., 2015; Volynets et al., 2012; Zhu et al., 2013) Evidence from animal (Cope et al., 2000) and human (Zhu et al., 2013) studies indicated that altered gut microbiome is the source of elevated blood alcohol in NAFLD.

In support of the alcohol/microbiome hypothesis for the induced alcohol metabolizing enzymes, we showed that oleic acid treatment did not induce the expression of CYP2E1

mRNA. Rather, it is likely that CYP2E1 was induced by endogenous alcohol produced in the gut. Because peroxide is a product from the reaction catalyzed by CYP2E1, the elevated CYP2E1 expression would translate into elevated reactive oxygen species and oxidative stress, and consequently contribute to the pathogenesis of NAFLD. The induction of CYP2E1 in NASH livers was first reported in 1998 (Weltman et al., 1998). This observation was confirmed with an activity analysis and the CYP2E1 protein was believed to be induced by fatty acids and ketone bodies in the circulation (Chalasani et al., 2003). However, there are conflicting data in literature regarding the induction of CYP2E1 by fatty acid.

In contrast to our results, Sung et al. reported that fatty acid treatment induced CYP2E1 protein expression in cultured HepG2 cells. (Sung et al., 2004) However, their study is compromised by several weaknesses. Firstly, they did not control loading for each sample (e.g. run an internal reference) when CYP2E1 was evaluated by Western blot. Secondly, statistical significance was not indicated for the Western blot results. Thirdly, the authors stated in the text that palmitic and oleic acid induced CYP2E1, but their data showed that palmitic acid treated sample had a CYP2E1 expression similar to that of the control. With another in vitro model, it was reported that palmitic acid treatment induced CYP2E1 mRNA expression in isolated human hepatocytes (Raucy et al., 2004). However, in a more relevant in vitro model system, the studies with hepatocytes isolated from human livers of steatosis revealed that CYP2E1 mRNA and activity are both decreased after fatty acid treatment (Donato et al., 2007; Donato et al., 2006). Conflicting data were also reported for in vivo studies. Yoo et al. reported that high fat diet induces CYP2E1 protein in 4 days in rat livers (Yoo et al., 1991). However, in an earlier study, Lieber et al. observed no induction of CYP2E1 in rat liver after high fat diet feeding for more than 4 weeks (Lieber et al., 1988).

A similar intervention study is impossible with NAFLD patients. In a cross-sectional study, it was observed that CYP2E1 activity does not correlate with obesity (Chalasani et al., 2003). This observation argues against a causal relationship between fatty acid and CYP2E1 activity in NASH liver because obesity is correlated with free fatty acid in the circulation. Nevertheless, the authors also noticed a positive correlation between CYP2E1 activity and blood ketone body β -hydroxybutyrate (Chalasani et al., 2003), which they considered evidence for indirect induction of CYP2E1 by fatty acid. However, from the long-term high fat diet fed animal study, the expected high level β -hydroxybutyrate seems not able to induce hepatic CYP2E1 expression (Lieber et al., 1988). Besides metabolism of fatty acids, alcohol (Lefevre et al., 1970) and protein is also a possible source of ketone bodies. Therefore, the interpretation for the correlation between CYP2E1 activity and β -hydroxybutyrate is a complex issue.

The strength and weakness of the current study are both with the liver biopsy samples of simple steatosis. The strength is that all the biopsy-proven simple steatosis samples we collected consistently demonstrated the elevated expression in alcohol metabolism genes. The weakness is the limited availability of the simple steatosis group and the normal control group. All available normal livers and simple steatosis livers were enrolled in this study. For ethical reasons, most of the available NAFLD liver biopsy samples are NASH. It is an effort of two years to collect 6 simple steatosis samples used in this study. Similarly, there is very limited availability of healthy donor livers from children. As such, we were not able to

match the gender ratio between normal livers and the simple steatosis livers. However, we expect that the gender bias has a limited influence in our data interpretation because the difference in alcohol metabolizing gene expression between genders is small compared to the differences between normal controls and NAFLD livers. (Zhu et al., 2015)

In summary, we showed that livers of non-alcoholic steatosis exhibited elevated expression of alcohol metabolizing genes, and that fatty acid treatment did not induce the expression of CYP2E1 in cultured HepG2 cells. Our data suggest that alcohol metabolism is one of the potent “multiple hits” (Tilg and Moschen, 2010) that drives the development and progression of NAFLD.

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Abbreviations

CYP2E1	cytochrome P450 2E1
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis

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Figure 1.

Altered gene expression in the livers of simple steatosis patients and NASH patients. The mRNA expression of ADH1C (A), ADH4 (B), ADH6 (C), catalase (D) and CYP2E1 (E) was examined in the livers of simple normal livers controls, steatosis patients, and NASH patients by qRT-PCR. Plotted relative gene expression values are mRNA copy numbers of the target gene normalized to that of the GAPDH (mean $\pm$ SEM). *, $P < 0.05$; **, $P < 0.01$.

Figure 2.

Expression of alcohol metabolizing genes in cultured HepG2 cells. (A) Elevated fat storage in HepG2 cells treated with oleic acid. Cells were stained with Oil Red O and counterstained with hematoxylin. Left, control cells; right, cells treated with fatty acid. Two typical samples are shown for each treatment. Original amplification of the objectives (20× and 40×) are indicated. The mRNA expression of CYP2E1 (B), ADH4 (C) and ADH6 (D) were examined in control HepG2 cells and HepG2 cells treated with ethanol or fatty acid by qRT-PCR. Plotted relative gene expression values are mRNA copy numbers of the target gene

normalized to that of the GAPDH (mean $\pm$ SEM). (E) Western blot analysis of CYP2E1 expression in control HepG2 cells and HepG2 cells treated with ethanol or fatty acid. β -actin blot was used for normalization purpose. (F) Average densitometry from two experiments was plotted as mean $\pm$ SEM. CYP2E1 signals were normalized with those of β -actin. *, $P < 0.05$; **, $P < 0.01$.

Table 1

Characteristics of study groups.

	Normal ¹	Steatosis	NASH
Sex (F : M) ²	F3 : M3	F1 : M5	F1 : M5
Age (years) ³	5.6 ± 2.9	15.7 ± 0.6	16.3 ± 1.0
BMI	16.0 ± 0.6	35.8 ± 3.2	34.9 ± 2.6
BMI z-score ⁴	-0.3 ± 0.5	2.3 ± 0.2	2.3 ± 0.3
ALT (U/L)	N/A	81.5 ± 25.0	96.2 ± 25.9
AST (U/L)	N/A	61.7 ± 12.1	60.4 ± 11.1
IR-HOMA	N/A	4.5 ± 1.6	5.9 ± 4.0

ALT, alanine transaminase; AST, aspartate transaminase; BMI, body mass index; IR-HOMA, insulin resistance-homeostasis model assessment; N/A, not available.

Values are mean ± standard error.

¹ Normal healthy liver donor, no liver disease reported.

² Gender not matched for limited availability of normal livers and limited availability of fatty livers without inflammation.

³ Age not matched for limited availability of normal livers and limited availability of fatty livers without inflammation.

⁴ A z-score of 1.6449 is equivalent to the 95th percentile.