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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 was 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.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease in the 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.

Abbreviations: CYP2E1, cytochrome P450 2E1; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis.

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2. Materials and methods

2.1. 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).

2.2. Quantitative real-time PCR (qRT-PCR)

Primers were designed with the assistance of Primer3 (http://bioinfo.ut.ee/primer3/). Primers for CYP2E1 (Forward: 5′TGGAAGCACT CAGGAAGACC3′, Reverse: 5′AGAGGATGTCGGCTATGACG3′) and CAT (Forward: 5′CAGGACAATCAGGGTGGTG3′, Reverse: 5′CAGGGCAGAA GGCTGTTG3′). 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 0.8 μg 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 (Ct) 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: $[\text{mRNA}] = M/E^{Ct}$, where constant M is an arbitrary threshold, E is the efficiency of PCR, Ct 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:

$$[\text{mRNA}]_{\text{target}}/[\text{mRNA}]_{\text{GAPDH}} = E_{\text{GAPDH}}{}^{\text{Ct.GAPDH}}/E_{\text{target}}{}^{\text{Ct.target}}$$

2.3. 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 2 mM oleic acid, 40 mM ethanol or with DMSO (solvent for free fatty acids, as a control), respectively. After treatment for 24 h, 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 h, cells were stained with Oil Red O and counterstained with hematoxylin.

2.4.Western blot

Harvested cells were homogenized in PBS, and then boiled for 5 min in SDS-PAGE loading buffer. Samples with 40 μg 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.

2.5. 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.

3. Results

3.1. 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

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.