

# Critical role of cytochrome P450 2E1 (CYP2E1) in the development of high fat-induced non-alcoholic steatohepatitis

Mohamed A. Abdelmegeed<sup>1</sup>, Atrayee Banerjee<sup>1</sup>, Seong-Ho Yoo<sup>1,2</sup>, Sehwan Jang<sup>1</sup>, Frank J. Gonzalez<sup>3</sup>, Byoung-Joon Song<sup>1,\*</sup>

<sup>1</sup>Laboratory of Membrane Biochemistry and Biophysics, National Institute on Alcohol Abuse and Alcoholism, Bethesda, MD, USA;

<sup>2</sup>Department of Forensic Medicine, Seoul National University College of Medicine, Seoul, South Korea; <sup>3</sup>Laboratory of Metabolism, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA

**Background & Aims:** Ethanol-inducible cytochrome P450 2E1 (CYP2E1) activity contributes to oxidative stress. However, CYP2E1 may have an important role in the pathogenesis of high-fat mediated non-alcoholic steatohepatitis (NASH). Thus, the role of CYP2E1 in high-fat mediated NASH development was evaluated.

**Methods:** Male wild type (WT) and *Cyp2e1*-null mice were fed a low-fat diet (LFD, 10% energy-derived) or a high-fat diet (HFD, 60% energy-derived) for 10 weeks. Liver histology and tissue homogenates were examined for various parameters of oxidative stress and inflammation.

**Results:** Liver histology showed that only WT mice fed a HFD developed NASH despite the presence of increased steatosis in both WT and *Cyp2e1*-null mice fed HFD. Markers of oxidative stress such as elevated CYP2E1 activity and protein amounts, lipid peroxidation, protein carbonylation, nitration, and glycation with increased phospho-JNK were all markedly elevated only in the livers of HFD-fed WT mice. Furthermore, while the levels of inflammation markers osteopontin and F4/80 were higher in HFD-fed WT mice, TNF $\alpha$  and MCP-1 levels were lower compared to the corresponding LFD-fed WT. Finally, only HFD-fed WT mice exhibited increased insulin resistance and impaired glucose tolerance.

**Conclusions:** These data suggest that CYP2E1 is critically important in NASH development by promoting oxidative/nitrosative stress, protein modifications, inflammation, and insulin resistance. Published by Elsevier B.V. on behalf of the European Association for the Study of the Liver.

## Introduction

Non-alcoholic fatty liver disease (NAFLD), a major health burden [1 and references within], is a chronic liver disease characterized by fat accumulation (steatosis). However, in a small number of cases, NAFLD can progress to advanced forms of liver injury such as necro-inflammation, fibrosis, cirrhosis, hepatocellular carcinoma, and liver failure. NAFLD with inflammation is called non-alcoholic steatohepatitis (NASH) [2]. The “two-hit” hypothesis [3] describes the pathophysiology of NASH. Steatosis (a primary insult) can sensitize the liver to secondary serious insults including reactive oxygen/nitrogen species (ROS/RNS), gut-derived endotoxins, pro-inflammatory cytokines like tumor necrosis factor- $\alpha$  (TNF $\alpha$ ), resulting in NASH development [1–3].

Obesity and chronic over-nutrition are the major risk factors for the development of NAFLD [4]. NAFLD is also considered the hepatic manifestation of the metabolic syndrome [5]. Although several mechanisms of NAFLD/NASH development have been proposed, its pathophysiology is still elusive. The incidence of insulin resistance (IR) or type-2 diabetes mellitus with NAFLD suggests that there is a positive association between IR and the pathogenesis of NASH [6]. However, the role of IR in the development and progression of NAFLD is unclear.

Oxidative stress has been suggested to play a role in the progression of NAFLD to NASH. Sources of oxidative stress include: cytochrome P450 2E1 (CYP2E1), lipid peroxidation, mitochondrial dysfunction, cytokine induction, NADPH oxidase, etc. [7]. CYP2E1, a member of the oxido-reductase cytochrome family, can oxidize a variety of small molecule substrates including xenobiotics, ethanol, and fatty acids [7–9]. The superoxide anion, a byproduct of the CYP2E1-mediated metabolism [7–9], is a very potent ROS, which can serve as part of the second hit to advance the severity of NAFLD. CYP2E1 expression and activity in the liver are increased in humans and animal models of NAFLD [7,10,11]. Indeed, CYP2E1

**Keywords:** Liver; CYP2E1; Null mice; High-fat diet; NAFLD; NASH; Oxidative stress; Protein modifications; Inflammation; Insulin resistance.

Received 24 February 2012; received in revised form 22 May 2012; accepted 25 May 2012; available online 2 June 2012

\* Corresponding author. Address: Laboratory of Membrane Biochemistry and Biophysics, National Institute on Alcohol Abuse and Alcoholism, 9000 Rockville Pike, Bethesda, MD 20892-9410, USA. Tel.: +1 301 496 3985; fax: +1 301 594 3113.

E-mail address: bj.song@nih.gov (B.J. Song).

**Abbreviations:** AGE, advance glycation end products; ALT, alanine aminotransferase; AST, asparagine aminotransferase; CYP2E1, cytochrome P450 2E1; GT, glucose tolerance; HAE, hydroxyalkenal; HFD, high-fat diet; HNE, 4-hydroxynonenal; IR, insulin resistance; MDA, malondialdehyde; NAFLD, non-alcoholic fatty liver diseases; NAS, NAFLD histological scoring system; NASH, non-alcoholic steatohepatitis; 3-NT, 3-nitrotyrosine; null-HFD, *Cyp2e1*-null fed HFD; null-LFD, *Cyp2e1*-null fed LFD; OPN, osteopontin; p38, p38 kinase; PBS, phosphate buffered saline; PNP, p-nitrophenol; RAGE, receptor for advanced glycation end products; RNS, reactive nitrogen species; ROS, reactive oxygen species; TG, triglyceride; TNF $\alpha$ , tumor necrosis factor- $\alpha$ ; WT, wild type; WT-HFD, wild type fed HFD; WT-LFD, wild type fed LFD.



was increased in obesity, fatty liver, and NASH in both humans and rodents, and this increase appears to correlate well with the severity of NAFLD [7,10–12]. Thus, it would be of great interest to characterize the potential role of CYP2E1 in the progression of NAFLD and the development of NASH by using *Cyp2e1*-null mice as a negative control in high-fat diet (HFD)-mediated NASH models. The aim of this study was to test this hypothesis by feeding wild type (WT) and *Cyp2e1*-null mice with a HFD for 10 weeks. Steatosis, inflammatory response, oxidative stress, systemic IR, and indicators of NAFLD and NASH manifestations were monitored in both WT and *Cyp2e1*-null mice fed a HFD or a low-fat diet (LFD).

## Materials and methods

### Materials

All chemicals used in this study were from Sigma Chemical (St. Louis, MO, USA), unless indicated otherwise. Secondary antibodies and the specific primary antibody to RAGE, JNK, and p-JNK were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies to CYP2E1, p38 MAPK (p38), and 3-NT were from Abcam Inc. (Cambridge, MA, USA). The anti-AGE antibody was purchased from Fitzgerald Industries (Acton, MA, USA). The antibody for osteopontin (OPN) was obtained from the University of Iowa (Iowa City, IA, USA).

### Animal treatment and histopathology analysis

In this study, we used age-matched *Cyp2e1*-null mice on 129/Svj background [13] and WT mice [stock number 002448 from Jackson Laboratory (Bar Harbor, ME, USA)]. Male mice were randomly assigned to four groups fed solid LFD (10% energy-derived calories) or HFD (60% energy-derived calories) (Research Diets, New Brunswick NJ, USA) for 10 weeks: (1) wild type fed LFD (WT-LFD) ( $n = 4$ ); (2) wild type fed HFD (WT-HFD) ( $n = 5$ ); (3) *Cyp2e1*-null fed LFD (null-LFD) ( $n = 4$ ); and (4) *Cyp2e1*-null fed HFD (null-HFD) ( $n = 4$ ). The weight of each mouse was recorded before the start of the LFD/HFD regimen, once every week, and at the time of euthanasia. Tissue preparation for histological examination and tissue maintenance were performed as previously described [11]. The NAFLD histological scoring system (NAS) [14] was also used to blindly evaluate the samples. NASH was defined when NAS was  $\geq 5$ . All animal experiments were performed in accordance with the National Institutes of Health guidelines and approved by the Institutional Animal Care and Use Committee.

### Tissue extraction

Total homogenates from each mouse were prepared in extraction buffer (50 mM Tris-Cl, pH 7.5, 1 mM EDTA, and 1% CHAPS), which was pre-equilibrated with nitrogen gas to remove the dissolved oxygen, and liver homogenates were prepared as previously described [15,16].

### Measurements of transaminase activities, CYP2E1 activity, MDA + HAE concentration and cytokine levels

Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were monitored in each mouse using the clinical IDEXX Vet Test chemistry analyzer system (IDEXX Laboratories, West Brook, ME, USA). CYP2E1 activity levels were measured by quantifying the oxidation rate of *p*-nitrophenol (PNP) to *p*-nitrocatechol, as described [15]. The concentration of MDA + HAE ( $\mu\text{M}$ ) was measured using commercially available kits (Oxford Biomedical Research, Oxford, MI, USA) following the manufacturer's protocol. Intrahepatic TNF $\alpha$  and MCP-1 levels (in 0.5 mg liver extracts) were determined by ELISA using the manufacturer's protocols (Thermoscientific, Rockford, IL, USA).

### Protein oxidation and glycoprotein determination

The levels of carbonylated proteins were determined with the OxyBlot protein oxidation detection kit following the manufacturer's protocol (Millipore, Billerica, MA, USA). Glycoproteins were evaluated using Pro-Q Emerald 488 Dye following the manufacturer's protocol (Molecular Probes, Eugene, OR, USA).

### Immunohistochemistry

Formalin-fixed liver samples were processed and 5- $\mu\text{m}$  thick paraffin sections were used for F4/80 immunohistochemistry. Briefly, deparaffinized liver sections were treated with 3% hydrogen peroxide followed by antigen retrieval. The sections were blocked with 10% horse serum, and incubated with the primary antibody, which was then linked to the biotinylated secondary antibody followed by the avidin-biotin complex (ABC Vectastain kit; Vector Laboratories, Burlingame, CA, USA), and diaminobenzidine, which gave a brown reaction product. The sections were then counterstained with hematoxylin. F4/80 expression levels were identified by the increased brown colored staining versus normal controls by using the NIH Image analysis software.

### IR and glucose tolerance (GT) tests

After 10 weeks of HFD feeding, IR and GT tests were evaluated in mice fasted for 6 h and subsequently injected with insulin (0.75 U/kg; Eli Lilly) or glucose (2 g/kg). GT tests were performed 3 days after the IR test. Tail blood was collected at indicated times immediately after intraperitoneal injection of insulin (IR) or glucose (GT). Blood glucose levels were determined using the Elite glucometer (Bayer).

### Data evaluation

Data represent results from at least two separate measurements, unless otherwise stated. Each point represents the mean  $\pm$  SEM ( $n = 4$  for both control groups and  $n = 5$  for both HFD groups), unless otherwise indicated. Statistical significance was evaluated using the Student's *t*-test at the 95% confidence level. Other materials and methods including immunoblot analysis not specified here were previously described [11,16].

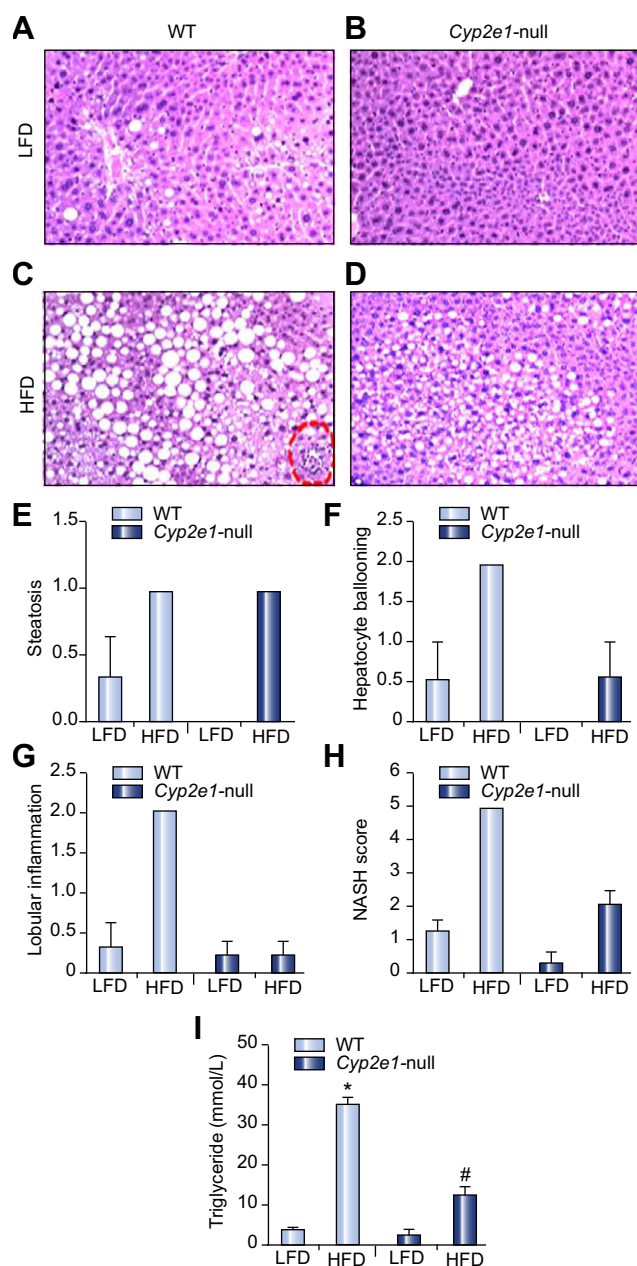
## Results

### Evaluation of NAFLD development

Livers of WT and *Cyp2e1*-null mice fed an LFD look grossly (data not shown) and histologically normal, although some occasional fat deposits were observed in the WT-LFD (Fig. 1A and B). In contrast, livers from both WT and *Cyp2e1*-null mice fed a HFD exhibited a much paler color compared with those of the corresponding LFD controls. Histological analysis (Fig. 1C and D) revealed accumulation of micro- and macro-vesicular intracellular lipid droplets in WT-HFD and to a lesser extent in *Cyp2e1*-null-HFD mice and the degrees of steatosis were markedly higher than in the corresponding LFD groups (Figs. 1A–D, and I). However, several inflammatory foci were observed only in the WT-HFD group (Fig. 1C). According to the histological scoring system for NAFLD, which reflects the sum of steatosis, hepatocytes ballooning, and lobular inflammation [14], only the WT-HFD group achieved a NAS of 5 (Fig. 1E–H). These results were supported by the analysis of hepatic triglyceride (TG) levels showing that the TG amount in HFD-WT was the highest and differed significantly from that of all the other groups (Fig. 1I).

### Effects of HFD on body weight, ratios of liver to body weights, and liver transaminases

Weight gains in both HFD-WT and HFD-*Cyp2e1*-null groups were significantly greater than in the corresponding LFD-fed groups ( $p < 0.05$ ) (Supplementary Fig. 1A). However, the ratios of liver to total body weight (liver index) were not significantly increased (Supplementary Fig. 1B) in any of the four groups, despite the fact that the histological analysis revealed that livers from both HFD-fed WT and *Cyp2e1*-null mice developed massive steatosis



**Fig. 1. Effects of LFD or HFD on hepatic steatosis and inflammation.** Photomicrographs (200 $\times$ ) following H&E staining of the indicated mouse livers are presented: (A) WT fed LFD, (B) *Cyp2e1*-null fed LFD, (C) WT fed HFD (necro-inflammatory foci indicated with circular broken lines), (D) *Cyp2e1*-null fed HFD. Individual scores of (E) hepatic steatosis, (F) hepatocyte ballooning, (G) lobular inflammation, (H) NAFLD histological scoring system, and (I) hepatic triglyceride levels were tabulated for all the four groups where the WT fed HFD was the only group to achieve a NAS of 5. \*Significantly different from all other groups. #Significantly different from the corresponding null group for panel (I). (This figure appears in colour on the web.)

(Fig. 1). Plasma ALT and AST activities did not differ between WT and *Cyp2e1*-null groups regardless of the type of diet (Supplementary Fig. 1C), as previously described [10,11].

#### Evaluation of different oxidative stress and inflammation parameters

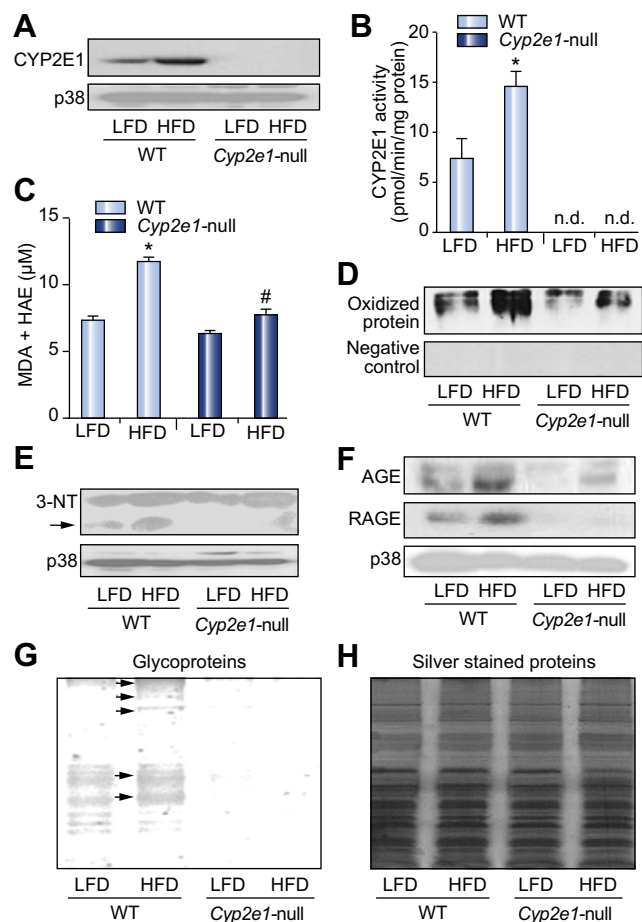
Elevated CYP2E1 is a marker of increased oxidative stress and is involved in the biotransformation of many small molecular weight xenobiotics and endogenous substrates such as fatty acids [7–9]. Furthermore, its activity and protein levels increase in response to HFD [7,10,11,17]. Thus, we evaluated whether HFD increased the activity of CYP2E1 in the four mouse groups. Indeed, both CYP2E1 activity and protein levels were significantly increased in the WT-HFD group compared to the corresponding LFD-fed group, while CYP2E1 was not detectable in *Cyp2e1*-null mice (Figs. 2A and B). p38 was used as a loading control since we observed that the levels of  $\beta$ -actin, glyceraldehyde 3-phosphate dehydrogenase, and tubulin fluctuated in response to HFD (data not shown). Staining with Coomassie blue confirmed loading of equal amounts of proteins for all the samples (data not shown).

In addition, as illustrated in Fig. 2C, MDA + HAE levels increased in both the WT and *Cyp2e1*-null groups fed a HFD compared to the corresponding LFD groups; however, the increased levels of lipid peroxidation were also significantly higher in the WT-HFD compared with the null-HFD mice (Fig. 2C). Furthermore, Oxyblot analysis demonstrated that the levels of oxidized proteins (Fig. 2D upper panel) were markedly increased in the WT-HFD groups compared to those of the other three groups. The specificity of the oxidized protein bands was confirmed by using 1 $\times$  derivatization Control Solution (Fig. 2D, lower panel) where no bands were detected. Immunoblot analysis revealed that the levels of 3-NT protein, a marker of nitrosative stress, were prominently higher in the WT-HFD group compared to those of the other three groups (Fig. 2E, upper panel), while p38 bands used as loading control (Fig. 2E, lower panel) were similar in all groups.

Advanced glycation end products (AGEs) and their receptor (RAGE) have been suggested to contribute to the pathogenesis of NASH in HFD-fed rats [18,19]. In addition, oxidative stress has been reported to increase glycated protein levels [20]. As shown in Fig. 2F, the levels of AGE and RAGE were evidently higher in the WT-HFD group compared to those of the other three groups, while p38 and Coomassie staining (data not shown) confirmed similar protein loading for all the samples. In addition, staining of glycoproteins (Fig. 2G) and silver staining (Fig. 2H) confirmed that HFD increased the levels of glycoproteins only in WT-HFD. Very few glycoproteins were detected in the *Cyp2e1*-null mouse groups, suggesting an important role of CYP2E1 in protein glycosylation/glycation.

Since the inflammatory marker OPN, the pro-inflammatory cytokine TNF $\alpha$ , and the marker of Kupffer cells F4/80, play important roles in NASH [11,21–23], the levels of these proteins were evaluated in mice fed a HFD. As shown in Fig. 3A, immunoblot analysis revealed that the levels of the cleaved (active form) OPN were only increased in the WT-HFD. Furthermore, quantitative immunohistochemistry analysis revealed that F4/80 positive cells were markedly increased in the WT-HFD compared with the other three groups (Fig. 3B–F). However, intrahepatic levels of TNF $\alpha$  and MCP-1 were lower in the WT-HFD than the corresponding WT-LFD groups, while intrahepatic MCP-1 increased in the HFD-null group compared to the corresponding control (Fig. 3G and H). We did not detect significant changes in



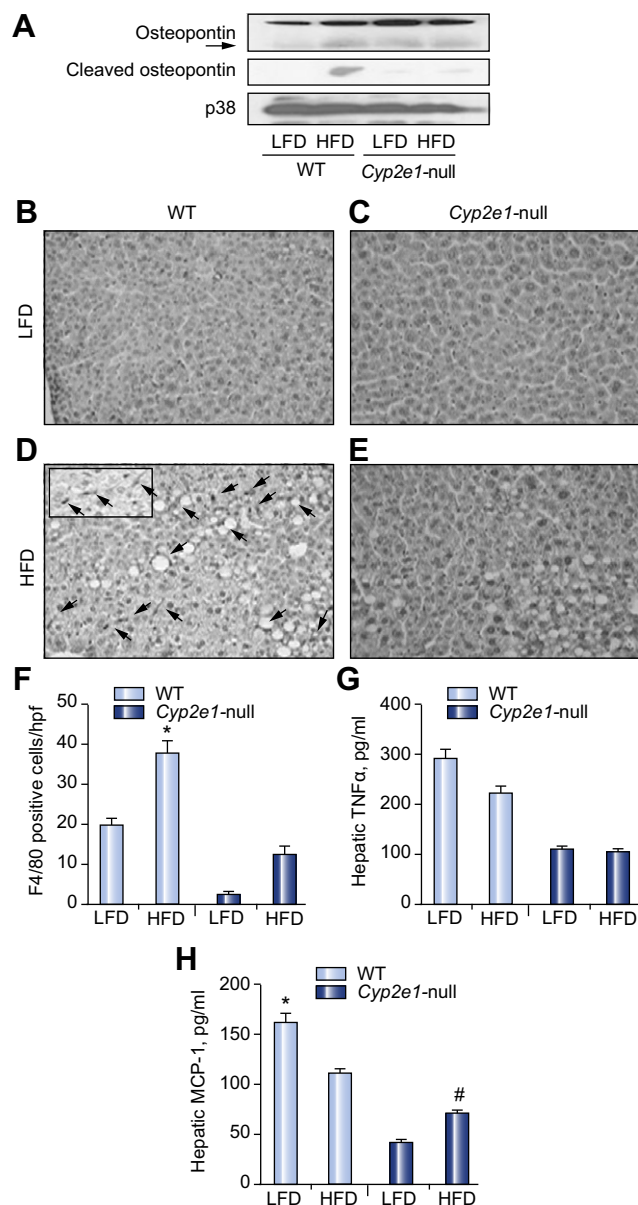


**Fig. 2. Changes in CYP2E1 protein levels, CYP2E1 activity, MDA + HAE concentration, hepatic protein oxidation, nitration, and glycosylation parameters.** (A–C) Equal amounts of whole liver lysates from different groups were used to determine: (A) CYP2E1 protein levels and p38 as loading control by immunoblot analysis; (B) CYP2E1 activity evaluated by measuring the rate of PNP oxidation to *p*-nitrocatechol, and (C) hepatic MDA + HAE as a marker of lipid peroxidation. \*Significantly different from all the other groups for panels (B and C). #Significantly different from the corresponding null group for panel (C). n.d.: not detected. (D) Protein oxidation was determined using Oxyblot analysis of total cells lysates from all the four groups or negative control. (E) Equal amounts of whole liver lysates (40 μg/well) from different groups were used to determine protein nitration using anti-3-NT antibody or p38 as loading control. (F) Total cell lysates were used to evaluate the levels of AGE, RAGE, or p38 as loading control from all the four groups using immunoblot analysis. (G) Whole liver homogenates from different groups were used to determine the levels of glycoproteins (arrows indicate increased levels of glycoproteins). (H) Equal protein loading for the samples analyzed for glycoprotein levels was confirmed by silver staining.

apoptosis (evaluated by TUNEL assay) among the groups, and in the levels of pro-apoptotic proteins such as Bax, Bcl2, and cleaved caspase 3 (data not shown).

#### IR development

Activated (phosphorylated) JNK is partly responsible for promoting IR in CYP2E1 over-expressed cells [12]. Our results revealed that WT-HFD mice with higher levels of hepatic phospho-JNK developed systemic IR and impaired GT compared to the WT-LFD group (Fig. 4A, C and E). In contrast, no significant differences were observed in JNK phosphorylation, insulin sensitivity, or GT

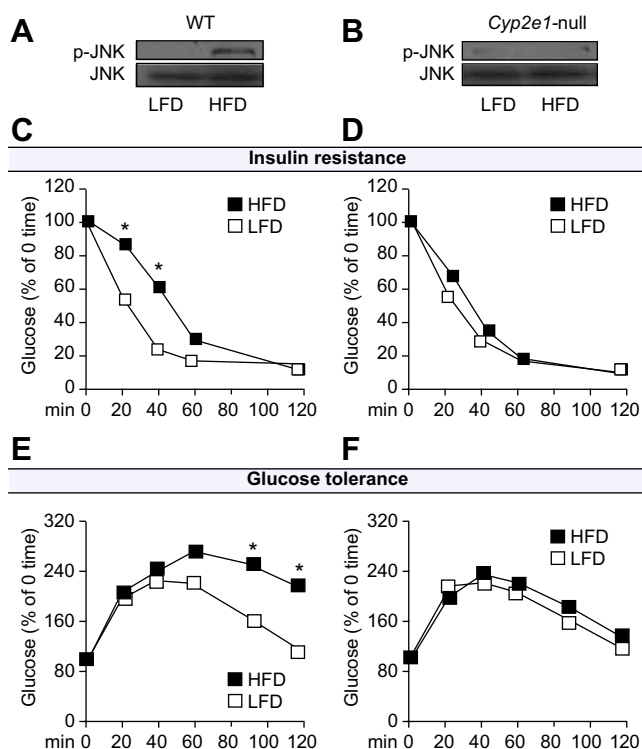


**Fig. 3. Effects of HFD on the levels of OPN, F4/80, TNFα, and MCP-1.** (A) Whole liver lysates were used to evaluate the levels of OPN, cleaved OPN, or the loading control p38 by immunoblot analyses. (B–E) F4/80 positive cells were also determined in all the four groups using immunohistochemistry. Arrows indicate F4/80 positive cells. (F) The number of F4/80 positive cells was calculated in at least five high power fields (HPF) (inset in D). Intrahepatic levels of (G) TNFα and (H) MCP-1 determined by ELISA are shown. \*Significantly different from all the other groups for panels (F and H). #Significantly different from the corresponding null group for panel (H).

in HFD-fed *Cyp2e1*-null mice compared to the corresponding LFD-exposed group (Fig. 4B, D and F).

#### Discussion

Obesity and exogenous HFD are major risk factors of NAFLD development [1,10,11]. Thus, we used solid HFD (60% energy-derived from fat) to simulate the human conditions [10].



**Fig. 4. Activation of JNK, increased IR, and impaired GT in WT-HFD.** (A and B) Whole liver lysates were used to evaluate the levels of p-JNK and JNK by immunoblot analyses. (C and D) IR was evaluated by injecting insulin (i.p., 0.75 U/kg; Eli Lilly), while GT was determined by injecting glucose (i.p., 2 g/kg) into each mouse fasted for 6 h. Tail blood from each mouse was collected at indicated times following insulin or glucose injection. Each point represents the average value (C–F). \*Significantly different from the corresponding time-course groups.

CYP2E1 produces high levels of ROS [7–9] and plays a role in the formation of peroxynitrite [16,24]. The pathological role of CYP2E1 in the development of both alcoholic steatohepatitis [25] and NASH [26,27] has been suggested, and thus, our main aim was to evaluate the hypothesis that HFD-exposed *Cyp2e1*-null mice are less susceptible to NASH compared with WT-HFD. Only the WT-HFD group achieved a NAS of 5, which is qualified as NASH [14], because of the higher levels of TG and lobular inflammation in the WT-HFD than the null-HFD. No significant differences in the ratios of liver to body weights and plasma transaminase levels were observed among all the four groups. It was previously reported that an underlying hepatic disorder can exist without concomitant elevation of liver transaminases [10,11,28]. Thus, the presence of CYP2E1 is important in NASH development in WT-HFD. This result is consistent with a previous study showing that *Cyp2e1*-null mice are less vulnerable to the alcoholic fatty liver [29].

Hepatic steatosis increases the liver susceptibility to more serious insults like inflammation and increased oxidative/nitrosative stress, especially in the presence of oxidizable unsaturated fatty acids in steatotic livers [1,11]. The oxidation of unsaturated fatty acids can increase lipid peroxidation, which can activate hepatic stellate cells thus accelerating the development of fibrosis [1,25]. Fatty acids upregulate both CYP2E1 mRNA and protein levels in cell culture models and livers of HFD-exposed rats [7,17]. Furthermore, the elevated CYP2E1 plays an important role in

potentiating obesity-related oxidative stress and liver injury in *ob/ob* mice [30]. We also witnessed that both CYP2E1 activity and protein amounts were increased remarkably only in HFD-WT mice. This result suggests the presence of higher levels of oxidative/nitrosative stress in the WT-HFD group compared to the other groups since CYP2E1 is known to increase the levels of ROS and RNS [8–10,16]. Elevated oxidative/nitrosative stress can lead to increased production of peroxynitrite, which can modify the function of various proteins [31]. Consistent with this view, we found that lipid peroxidation levels and oxidized and nitrated proteins were the highest in WT-HFD mice. Oxidative/nitrosative events are well-documented to cause DNA damage and oxidative post-translational modifications of Cys-residues of cellular proteins [15,31,32], which negatively affect functions of essential proteins prior to the observation of full-blown liver diseases [15,32]. Earlier studies revealed that the amounts of nitrated proteins, a marker of NO-dependent nitrosative stress, were increased in the livers of obese *ob/ob* mice [33], explaining a potential role of 3-NT in causing the low mitochondrial respiratory chain activity in patients suffering from NASH. Our data also showed that nitrated protein levels are remarkably increased in the WT-HFD group compared to the other three groups (Fig. 2). These results suggest that elevated levels of oxidative/nitrosative stress caused by, but may not be limited to CYP2E1, have induced protein oxidation and nitration, both of which might have contributed to the progression to NASH development following steatosis.

Protein glycation and its markers, namely AGEs and their receptor RAGE, were reported to increase in HFD-fed rats, contributing to NASH pathogenesis [18,19]. Based on this information and the fact that the liver is the main organ to clear AGEs, it was logical to evaluate protein glycation/glycosylation in the present model. Indeed, the WT-HFD group showed markedly higher levels of AGEs, RAGE, and glycoproteins compared to the other three groups (Fig. 2). These results suggest that protein glycation/glycosylation may also play a role in the faster development of NASH in WT-HFD mice and that the absence of CYP2E1 ameliorates/attenuates AGEs formation. Thus, the second hit in NASH [3] represents increased lipid peroxidation, protein oxidation, nitration, and glycation/glycosylation in the WT-HFD group (Supplementary Fig. 2).

ROS/RNS, lipid peroxidation, and protein glycation likely increase the expression of pro-inflammatory cytokines and hence increase inflammation [1,34]. Obesity and HFD are also known to induce inflammatory cytokines and increase hepatic inflammation in steatotic livers [1 and references within]. Furthermore, CYP2E1 sensitizes hepatocytes to LPS, TNF $\alpha$ , and oxidative injury in a p38- and JNK-dependent fashion [35]. However, the roles of various cytokines and chemokines in NAFLD are complex and sometimes inconsistent depending on the stimulating agents, exposure times (i.e. sample collection times), and evaluation of mRNA or protein levels, since they might give paradoxical results [36,37]. For instance, hepatic TNF $\alpha$  increased first after 1 and 2 weeks of exposure to the methionine–choline deficient diet and then actually decreased at 4 and 8 weeks despite the high levels of TNF $\alpha$  mRNA and OPN throughout the feeding periods [37]. In addition, F4/80 and high hepatic inflammatory foci have been found to be remarkably high in *Mcp-1* null mice [38]. In the current model, we observed decreased intrahepatic levels of TNF $\alpha$  and MCP-1 in the HFD-WT compared to the corresponding LFD-WT mice despite increased amounts of OPN and F4/80. These data suggest that there is a temporal change in the levels of

hepatic cytokines in response to HFD where the decreased levels of TNF $\alpha$  and MCP-1 might actually follow earlier increases, as previously reported [37]. Furthermore, this temporal response in the liver may also differ from other organs' responses (e.g. fat tissues). Thus, the temporally different responses to HFD in liver, fat tissues, and muscles for oxidative stress and inflammation development should be further investigated to understand the underlying pathophysiological process.

Obesity, NAFLD, NASH, and HFD were reported to contribute to the high incidence of IR, which may worsen the NASH condition, mainly due to increased levels of oxidative/nitrative stress and inflammation [1,7,39]. Furthermore, it was shown that overexpression of hepatic CYP2E1 both *in vitro* and *in vivo* promoted the development of hepatic IR partly through activation (phosphorylation) of JNK [12,40]. Thus, we also evaluated whether CYP2E1 deletion would lead to IR development and impaired GT. Our data revealed that only the WT-HFD group, but not the others, developed IR and impaired GT (Fig. 4), which supports the notion that increased CYP2E1 in HFD for an extended period likely promotes IR, and that the absence or blocking of CYP2E1 may exert beneficial effects. During the final preparation of this manuscript, a detailed report showed the role of CYP2E1 deletion in IR development in the same mouse strain, same diet, and very similar duration of feeding [41]. Pessin and colleagues demonstrated that, using euglycemic-hyperinsulinemic clamps, *Cyp2e1*-null mice were protected substantially against HFD-induced hepatic IR and exhibited better sensitivity of adipose tissue glucose uptake with decreased hepatic glucose output. Although we did not directly determine hepatic IR while we evaluated the development of systemic IR and GT, it is likely that the WT-HFD group in our study would also have developed earlier hepatic IR than the *Cyp2e1*-null mice, based on the massive fat accumulation, remarkably higher levels of oxidative stress, OPN, F4/80, and phospho-JNK, which eventually impair insulin signaling. Nonetheless, with the development of systemic IR, fat accumulation and inflammation will be even greater in the already-primed liver of WT-HFD mice and would accelerate NASH development, which is the main focus of this study (Supplementary Fig. 2).

In summary, CYP2E1, which appears to be involved in fat accumulation in NAFLD, plays an important role in the advancement of steatotic livers to inflammatory NASH and IR development. This can be possibly explained by the fact that upregulation of CYP2E1 leads to increased production of ROS/RNS, which likely increase lipid peroxidation, protein nitration, oxidation, glycation, with inflammation and IR development, all of which can negatively affect the vital functions of the hepatocytes and ultimately lead to NASH development.

### Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript. The underlying research reported in the study was funded by the NIH Institutes of Health.

### Financial support

This research was supported by the Intramural Research Program of National Institute on Alcohol Abuse and Alcoholism.

### Author's contributions

Study Design and concept: Mohamed A. Abdelmegeed and BJ Song.

Analysis and interpretation of data: Mohamed A. Abdelmegeed, Atrayee Banerjee, Seong-Ho Yoo, Sehwan Jang, and BJ Song.

Drafting and finalizing the manuscript: Mohamed A. Abdelmegeed, Frank J. Gonzalez, and BJ Song.

### Acknowledgements

The authors are thankful to Dr. Klaus Gawrisch for supporting this study.

### Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jhep.2012.05.019>.

### References

- [1] Begrich K, Igoudjil A, Pessayre D, Fromenty B. Mitochondrial dysfunction in NASH: causes, consequences and possible means to prevent it. *Mitochondrion* 2006;6:1–28.
- [2] Starley BQ, Calcagno CJ, Harrison SA. Non-alcoholic fatty liver disease and hepatocellular carcinoma: a weighty connection. *Hepatology* 2010;51:1820–1832.
- [3] Day CP, James OF. Steatohepatitis: a tale of two "hits"? *Gastroenterology* 1998;114:842–845.
- [4] Zelber-Sagi S, Ratzin V, Oren R. Nutrition and physical activity in NAFLD: an overview of the epidemiological evidence. *World J Gastroenterol* 2011;17:3377–3389.
- [5] Alberti KG, Zimmet P, Shaw J. The metabolic syndrome—a new worldwide definition. *Lancet* 2005;366:1059–1062.
- [6] Marchesini G, Bugianesi E, Forlani G, Cerrelli F, Lenzi M, Manini R, et al. Non-alcoholic fatty liver, steatohepatitis, and the metabolic syndrome. *Hepatology* 2003;37:917–923.
- [7] Aubert J, Begrich K, Knockaert L, Robin MA, Fromenty B. Increased expression of cytochrome P450 2E1 in Non-alcoholic fatty liver disease: mechanisms and pathophysiological role. *Clin Res Hepatol Gastroenterol* 2011;35:630–637.
- [8] Song BJ, Koop DR, Ingelman-Sundberg M, Nanji A, Cederbaum AI. Ethanol-inducible cytochrome P450 (CYP2E1): biochemistry, molecular biology and clinical relevance. 1996 update. *Alcohol Clin Exp Res* 1996;20:138A–146A.
- [9] Caro AA, Cederbaum AI. Oxidative stress, toxicology, and pharmacology of CYP2E1. *Annu Rev Pharmacol Toxicol* 2004;44:27–42.
- [10] Lieber CS, Leo MA, Mak KM, Xu Y, Cao Q, Ren C, et al. Model of non-alcoholic steatohepatitis. *Am J Clin Nutr* 2004;79:502–509.
- [11] Abdelmegeed MA, Yoo SH, Henderson LE, Gonzalez FJ, Woodcroft KJ, Song BJ. PPARalpha expression protects male mice from high fat-induced Non-alcoholic fatty liver. *J Nutr* 2011;141:603–610.
- [12] Schattenberg JM, Wang Y, Singh R, Rigoli RM, Czaja MJ. Hepatocyte CYP2E1 overexpression and steatohepatitis lead to impaired hepatic insulin signaling. *J Biol Chem* 2005;280:9887–9894.
- [13] Lee SS, Buters JT, Pineau T, Fernandez-Salguero P, Gonzalez FJ. Role of CYP2E1 in the hepatotoxicity of acetaminophen. *J Biol Chem* 1996;271:12063–12067.
- [14] Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, et al. Design and validation of a histological scoring system for non-alcoholic fatty liver disease. *Hepatology* 2005;41:1313–1321.
- [15] Abdelmegeed MA, Moon KH, Hardwick JP, Gonzalez FJ, Song BJ. Role of peroxisome proliferator-activated receptor-alpha in fasting-mediated oxidative stress. *Free Radic Biol Med* 2009;47:767–778.
- [16] Abdelmegeed MA, Moon KH, Chen C, Gonzalez FJ, Song BJ. Role of cytochrome P450 2E1 in protein nitration and ubiquitin-mediated degradation during acetaminophen toxicity. *Biochem Pharmacol* 2010;79:57–66.

## Research Article

- [17] Yun YP, Casazza JP, Sohn DH, Veech RL, Song BJ. Pretranslational activation of cytochrome P450IIE during ketosis induced by a high fat diet. *Mol Pharmacol* 1992;41:474–479.
- [18] Wu J, Zhao MY, Zheng H, Zhang H, Jiang Y. Pentoxifylline alleviates high-fat diet-induced non-alcoholic steatohepatitis and early atherosclerosis in rats by inhibiting AGE and RAGE expression. *Acta Pharmacol Sin* 2010;31:1367–1375.
- [19] Kimura Y, Hyogo H, Yamagishi S, Takeuchi M, Ishitobi T, Nabeshima Y, et al. Atorvastatin decreases serum levels of advanced glycation endproducts (AGEs) in non-alcoholic steatohepatitis (NASH) patients with dyslipidemia: clinical usefulness of AGEs as a biomarker for the attenuation of NASH. *J Gastroenterol* 2010;45:750–757.
- [20] Cai Z, Zhao B, Ratka A. Oxidative stress and beta-amyloid protein in Alzheimer's disease. *Neuromolecular Med* 2011;13:223–250.
- [21] Chapman J, Miles PD, Ofrecio JM, Neels JG, Yu JC, Resnik JL, et al. Osteopontin is required for the early onset of high fat diet-induced IR in mice. *PLoS One* 2010;5:e13959.
- [22] Stienstra R, Mandard S, Patsouris D, Maass C, Kersten S, Muller M. Peroxisome proliferator-activated receptor alpha protects against obesity-induced hepatic inflammation. *Endocrinology* 2007;148:2753–2763.
- [23] Matono T, Koda M, Tokunaga S, Kato J, Sugihara T, Ueki M, et al. Therapeutic effects of ezetimibe for non-alcoholic steatohepatitis in fatty liver shionogi-ob/ob mice. *Hepatol Res* 2011;41:1240–1248.
- [24] Gow AJ, Farkouh CR, Munson DA, Posencheg MA, Ischiropoulos H. Biological significance of nitric oxide-mediated protein modifications. *Am J Physiol Lung Cell Mol Physiol* 2004;287:L262–268.
- [25] Lieber CS. Cytochrome P-4502E1: its physiological and pathological role. *Physiol Rev* 1997;77:517–544.
- [26] Mantena SK, Vaughn DP, Andringa KK, Eccleston HB, King AL, Abrams GA, et al. High fat diet induces dysregulation of hepatic oxygen gradients and mitochondrial function in vivo. *Biochem J* 2009;417:183–193.
- [27] Raucy JL, Lasker JM, Kraner JC, Salazar DE, Lieber CS, Corcoran GB. Induction of cytochrome P450IIE1 in the obese overfed rat. *Mol Pharmacol* 1991;39:275–280.
- [28] Calvaruso V, Craxi A. Implication of normal liver enzymes in liver disease. *J Viral Hepat* 2009;16:529–536.
- [29] Lu Y, Zhuhe J, Wang X, Bai J, Cederbaum AI. Cytochrome P450 2E1 contributes to ethanol-induced fatty liver in mice. *Hepatology* 2008;47:1483–1494.
- [30] Dey A, Cederbaum AI. Induction of cytochrome P450 2E1 promotes liver injury in ob/ob mice. *Hepatology* 2007;45:1355–1365.
- [31] Radi R. Nitric oxide, oxidants, and protein tyrosine nitration. *Proc Natl Acad Sci USA* 2004;101:4003–4008.
- [32] Moon KH, Hood BL, Mukhopadhyay P, Rajesh M, Abdelmegeed MA, Kwon YI, et al. Oxidative inactivation of key mitochondrial proteins leads to dysfunction and injury in hepatic ischemia reperfusion. *Gastroenterology* 2008;135:1344–1357.
- [33] Garcia-Ruiz I, Rodriguez-Juan C, Diaz-Sanjuan T, del Hoyo P, Colina F, Munoz-Yague T, et al. Uric acid and anti-TNF antibody improve mitochondrial dysfunction in ob/ob mice. *Hepatology* 2006;44:581–591.
- [34] Yan SF, Ramasamy R, Schmidt AM. Mechanisms of disease: advanced glycation end-products and their receptor in inflammation and diabetes complications. *Nat Clin Pract Endocrinol Metab* 2008;4:285–293.
- [35] Wu D, Cederbaum A. Cytochrome P4502E1 sensitizes to tumor necrosis factor alpha-induced liver injury through activation of mitogen-activated protein kinases in mice. *Hepatology* 2008;47:1005–1017.
- [36] Brauersreuther V, Viviani GL, Mach F, Montecucco F. Role of cytokines and chemokines in non-alcoholic fatty liver disease. *World J Gastroenterol* 2012;18:727–735.
- [37] Sahai A, Malladi P, Melin-Aldana H, Green RM, Whittington PF. Upregulation of osteopontin expression is involved in the development of non-alcoholic steatohepatitis in a dietary murine model. *Am J Physiol Gastrointest Liver Physiol* 2004;287:G264–273.
- [38] Kassel KM, Guo GL, Tawfik O, Luyendyk JP. Monocyte chemoattractant protein-1 deficiency does not affect steatosis or inflammation in livers of mice fed a methionine–choline-deficient diet. *Lab Invest* 2010;90:1794–1804.
- [39] Wang Y, Millonig G, Nair J, Patsenker E, Stickel F, Mueller S, et al. Ethanol-induced cytochrome P4502E1 causes carcinogenic etheno-DNA lesions in alcoholic liver disease. *Hepatology* 2009;50:453–461.
- [40] Kathirvel E, Morgan K, French SW, Morgan TR. Overexpression of liver-specific cytochrome P4502E1 impairs hepatic insulin signaling in a transgenic mouse model of non-alcoholic fatty liver disease. *Eur J Gastroenterol Hepatol* 2009;21:973–983.
- [41] Zong H, Armoni M, Harel C, Karnieli E, Pessin JE. Cytochrome P-450 CYP2E1 knockout mice are protected against high-fat diet-induced obesity and IR. *Am J Physiol Endocrinol Metab* 2012;302:E532–539.