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HIF-1α Expression as a Protective Strategy of HepG2 Cells Against Fatty Acid-Induced Toxicity

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ABSTRACT

Free fatty acid-induced lipotoxicity via increased endoplasmic reticulum (ER) stress and hepatocyte apoptosis is a key pathological mechanism of non-alcoholic steatohepatitis. A role of hypoxia-inducible factor 1α (HIF-1α) in this process has been suggested, but direct evidence is lacking. Here, we used HepG2 cells as a model to study whether HIF-1α can reduce palmitic acid-induced lipotoxicity and ER stress. In HepG2 cells treated with 500 μM palmitic acid, HIF-1α expression increased transiently, the decline was associated with increased cleaved caspase-3 expression. Overexpression and knockdown of HIF-1α decreased and exacerbated, respectively, palmitic acid-induced lipoapoptosis. The overexpression also blunted upregulation of the ER stress markers, C/EBP homologous protein (CHOP) and chaperone immunoglobulin heavy chain binding protein (Bip), while the knockdown increased the level of CHOP. In line with this, CHOP promoter activity decreased following HIF-1α binding to the CHOP promoter hypoxia response element. These results indicate that hepatocyte lipotoxicity is associated with decreased HIF-1α expression. It also suggests that upregulation of HIF-1α can be a possible strategy to reduce lipotoxicity in non-alcoholic fatty liver disease. J. Cell. Biochem. 115: 1147–1158, 2014.

KEY WORDS: NON-ALCOHOLIC FATTY LIVER DISEASE; HEPATIC STEATOSIS; LIPOAPOPTOSIS; ER STRESS; HIF-1α; CHOP

Non-alcoholic fatty liver disease (NAFLD) is increasingly recognized as one of the most common liver diseases. NAFLD encompasses a spectrum of liver diseases ranging from simple steatosis to non-alcoholic steatohepatitis (NASH), which may progress to cirrhosis in up to 25% of patients [Farrell and Larter, 2006]. The hallmark of NAFLD is hepatic neutral lipid accumulation, mainly triacylglycerol. This excessive lipid accumulation in hepatocytes promotes lipotoxicity-induced apoptosis, called lipoapoptosis, which is central to liver injury in NAFLD. In particular, several studies have suggested that saturated fatty acids such as palmitic acid can induce endoplasmic reticulum (ER) stress and mitochondria mediated lipoapoptosis [Pfaffenbach et al., 2010; Ibrahim et al., 2011]. In addition, enhanced hepatic lipoapoptosis is tightly coupled with infiltration of inflammatory cells and macrophages, inflammatory and fibrotic cytokine release, and fibrogenesis [Takehara et al., 2004; Hikita et al., 2009; Vick et al., 2009]. Thus, increased hepatocyte apoptosis by fatty acids plays a key role in the development and progression of NAFLD.

Hypoxia-inducible factor 1 (HIF-1) is a heterodimeric transcription factor composed of HIF-1α and HIF-1β, that acts as a master regulator of oxygen homeostasis including physiological and pathological processes [Wang et al., 1995]. Normally, HIF-1α is present at undetectable levels under normoxic conditions, because of rapid ubiquitination and proteasomal degradation, but this process is

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inhibited under hypoxic conditions [Salceda and Caro, 1997; Huang et al., 1998]. Although several studies have investigated the role of HIF-1α in liver steatosis, two studies using HIF-1α knockout mice with alcoholic fatty liver disease reported contradictory findings. Nishiyama et al. [2012] demonstrated protective roles of HIF-1α, but Nath et al. [2011] showed a causative role of HIF-1α in the development of alcoholic fatty liver. Only one in vivo study demonstrated an association between HIF-1α and NAFLD. Carabelli et al. [2011] found high fat-induced HIF-1α expression in a rat model, and that hepatic expression of HIF-1α mRNA is significantly correlated with the copy number of liver mitochondrial DNA, suggesting that increased HIF-1α expression in fatty liver may be associated with activated mitochondrial function. Although these results suggest that steatosis involves regulation of HIF-1α, the cellular and molecular mechanisms regulating lipoapoptosis in hepatocytes are poorly understood.

In addition, HIF-1α antagonizes the apoptotic machinery by activating target pro-survival genes. Thus, the HIF-1α-mediated anti-apoptotic role is critical for cells to survive under diverse conditions [Yu et al., 2004; Piret et al., 2005]. However, it is unknown whether activating HIF-1α is important cell survival signal in NAFLD. Therefore, the objective of this study was to evaluate the anti-apoptotic effect of HIF-1α and its mechanism of action in palmitic acid-mediated lipotoxicity.

MATERIALS AND METHODS

CELL CULTURE AND REAGENTS

HepG2 cells, which are human hepatocellular carcinoma cell lines, were purchased from American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium containing high glucose, penicillin/streptomycin, and 10% fetal bovine serum. All cells were plated in a cell culture plate at least 24 h before treatment. Upon reaching 70% confluence, the cultured cells were incubated with indicated concentration of palmitic acid or YC-1 (Sigma, St. Louis, MO). To induce lipotoxicity, palmitic acid was purchased from Sigma in the form of sodium salts, and conjugated to fatty acid-free bovine serum albumin by dissolving them in ethanol and mixing an aqueous BSA solution. Control cells were treated with vehicle alone [Das et al., 2008].

PLASMIDS AND TRANSIENT TRANSFECTION

The expression vector CMV7.1 human HIF-1α was kindly provided by Prof. Tae Woo Kim, Korea University, Seoul, Korea. Plasmid PBS/pU6-HIF-1alpha RNAi plasmid 1 (shHIF-1α) was kindly provided by Addgene and Dr. Connie Cepko. HepG2 cells were transfected separately with the HIF-1α or shHIF-1α plasmids. The transfection procedure was performed using electroporation according to the manufacturer's protocol (Ingenio^{TM} Electroporation System; Mirus Bio, Madison, WI).

Oil Red-O STAINING

After incubation in the presence or absence of palmitic acid, the cells were fixed in formalin (10%) for 15 min, and then stained with Oil Red-O solution for 15 min. The cells were then washed with deionized water and photographed. Stained Oil Red-O was also eluted with 100% isopropanol and quantified by measuring absorbance at 520 nm after suitable dilution.

CELL VIABILITY ASSAY

HepG2 cells were grown in 96-well plates and treated with the indicated condition. Briefly, MTT (Sigma) was dissolved in phosphate-buffered saline (PBS) at 2 mg/ml and sterilized by passage through a 0.22 μm filter (stock solution). Cells were seeded in wells of a 96-well plate containing 200 μl of culture medium, and then treated with various concentrations of palmitic acid for 24 h. After an incubation in 25 ml MTT stock solution for 3 h at 37°C, 200 μl of DMSO solution was added to all wells and mixed thoroughly to lyse the cells and dissolve the dark blue crystals. After 20 min, the absorbance was read on a microplate reader at a wavelength of 540 nm.

APOPTOSIS ANALYSIS

Apoptosis was detected using the FITC Annexin V Apoptosis Detection KIT (BD Biosciences, San Jose, CA) according to the manufacturer's instructions. In brief, cells were incubated for 24 h with different palmitic acid concentrations or at different time intervals. After the incubation, the cells were collected by trypsinization and centrifugation at 1,000 rpm for 5 min. Following resuspension in binding buffer, they were incubated with Annexin V/propidium iodide (PI) at room temperature in the dark. The analyses were performed using a FACScan and CellQuestPro software from Becton Dickinson (Fullerton, CA).

REAL-TIME QUANTITATIVE REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION

Total RNA was extracted from cells with the Hybrid-RTM (GeneAll Biotechnology, Daejeon, Korea). Equal amounts of total RNA (1 μg) were reverse transcribed using the Transcriptor First Strand cDNA Synthesis kit (Roche, Anaheim, CA). The cDNA was then amplified with a pair of forward and reverse primers for the following genes (rates normalized to the expression level of hGAPDH expression level): hCPT-1 (5′-TCAGTGGGAGCGGATGTTTA-3′ and 5′-TCCA-CAG CATCAAGAGACTGC-3′), hACC (5′-CCTGACGCCAACTG-GAAAA-3′ and 5′-TG TGCCTGGAACCTCTTTG A-3′), hSREBP-1c (5′-GGAGCCATGGATTGCACTTT-3′ and 5′-ATGTGGCAGGAGGTG-GAGAC-3′), hBip/GRP78 (5′-GCTCGACTCGA ATTCCAAAG-3′ and 5′-TTTGTCAGGGGTCTTTCACC-3′), hCHOP (5′-TTCTCT GGCTTGG-CTGACTG-3′ and 5′-CTGCGTATGTGGGATTGAGG-3′), and hGAPDH (5′-GGGGCTCTCCAGAACATCAT-3′ and 5′-AAGTGGTCGTTGAGG GCAAT-3′). Real-time PCR reactions were performed using TaqMan Universal SYBR Green Master Mix (Roche, Mannheim, Germany).

WESTERN BLOT

For each experiment, cells were rinsed twice with ice-cold PBS and 100 μl of Protein Extraction Solution RIPA (Elpis Biotech, Korea) (50 mM Tris–Cl, pH 8.0, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 0.1% sodium dodecyl sulfate, 1% Nonidet P-40, 0.5 mM EDTA) was added and incubated for 30 min on ice. The cells were then scraped and centrifuged. Protein concentrations were determined by the Coomassie Plus Protein assay (Pierce, Rockford, IL). Cell lysates

were separated by 8% or 15% SDS–polyacrylamide gel electrophoresis, transferred to a PVDF membrane (Millipore, Bedford, MA, pore size 0.45 μm), blocked with 5% non-fat dry milk for 60 min, and probed with antibodies at 4°C overnight. The blots were incubated with horseradish peroxidase-conjugated anti-IgG, followed by enhanced chemiluminescence detection (WEST-ZOL Plus, iNtRON Biotechnology). Antibodies to cleaved caspase-3 (c-caspase 3) and α-tubulin were purchased from Cell Signaling Technologies (Danvers, MA). Antibodies to HIF-1α, Bax, and Bcl-2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

CONSTRUCTION AND MUTAGENESIS OF THE HUMAN CHOP PROMOTER

The CHOP promoter construct was amplified by PCR using human genomic DNA as template and the primers: 5′-GCGGTACCCAAA-CAAGGACGGTA AGATC-3′ and 5′-GAAAGCTTGCCAGCTCATCTT-TAAC-3′. The amplified product was then cloned into the pGL3-basic vector (Promega, Madison, WI) and digested with *Kpn*I and *Hin*dIII. The resulting recombinant plasmid was called WT-CHOP pro and was characterized by sequencing. Site-specific mutations of the putative hypoxia response element (HRE) within the human CHOP promoter were performed by Muta-Direct™ Site-Directed Mutagenesis kit (iNtRON Biotechnology) using WT-CHOP pro as a template. The primer sequence used to generate the point mutation was 5′-GGGGCCAATGCCGGTTAGCCACTTTCTGAT-3′ for the mutant (Mut-CHOP pro). The mutant construct was verified by DNA sequencing.

PROMOTER REPORTER ASSAY

After transfection, HepG2 cells were seeded in 24-well plates and incubated for 24 h. The WT-CHOP pro, Mut-CHOP pro plasmid and control pGL3 basic vector were adjusted to 200 ng/μl, and beta-galactosidase was adjusted to 50 ng/μl. After a 24 h of treatment with palmitic acid in under different conditions, the cells were harvested for luciferase assay (Dual Luciferase Assay kit, Promega).

CHROMATIN IMMUNOPRECIPITATION (CHIP) ASSAYS

A ChIP kit (Millipore) was used according to the manufacturer's protocol. Briefly, HepG2 and palmitic acid-treated HepG2 cells were cross-linked using formaldehyde (1% final concentration) for 10 min at 37°C and then lysed in SDS buffer containing 1× protease inhibitor. The DNA was shared by sonication on wet ice using a Sonic Dismembrator Model 500 (Fisher Scientific, Pittsburgh, PA). This procedure generated fragments of the required length (200–1,000 bp), which were subjected to immunoprecipitation with 2 μg anti-HIF-1α antibody. The immune complexes were protein digested by with Proteinase K followed by an overnight incubation at 65°C, and then the chromatin immunoprecipitate was purified. PCR was performed on the chromatin using CHOP-specific primers (5′-TACGTC-GACCCCCT AGCGAGAG-3′ and 5′-CTCTGACCTCGGGAGCGCCTGG

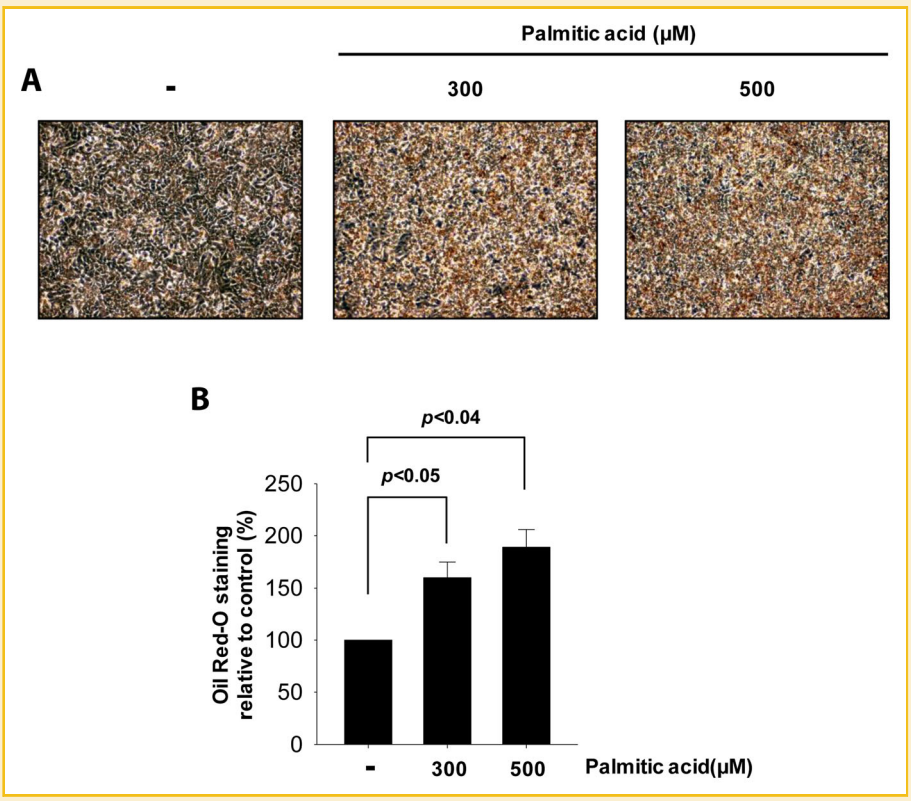

Fig. 1. Palmitic acid induces lipid accumulation in HepG2 cells. A: Representative microscopic pictures of HepG2 cells, normal or treated with various concentrations of palmitic acid for 24 h. After 24 h incubation with palmitic acid, the cells were stained with Oil Red-O. B: The Oil Red-O-stained lipids were extracted with isopropanol. The absorbance of the extracted Oil Red-O was spectrophotometrically determined at 525 nm to measure triglyceride accumulation (magnification 40×). Error bars represent mean ± SD.

C-3′). PCR products were resolved on a 2% agarose gel containing ethidium bromide and visualized under ultraviolet light.

ANIMAL EXPERIMENTS

Male C57BL/6 mice of 6 weeks of age were purchased from Orient Bio, Inc. (Seoul, Korea). The animals were housed in a temperature- and humidity-controlled room and subjected to a daily cycle of 12 h of light and 12 h of darkness. Animals had free access to chow and water until the first day of the study. The mice were fed either the methionine choline-sufficient group (control, n = 3) or methionine choline-deficient group (MCD, n = 4) for 4 weeks. Mice were sacrificed by CO_2 asphyxiation. The liver tissue was snap frozen for protein, RNA, lipid extraction, and Oil Red-O staining. The remaining fresh liver tissue was fixed in 10% formalin and embedded in paraffin for picosirius red staining to evaluate a collagen deposition. Total lipids were extracted from 200 mg of liver tissue using chloroform:methanol and solubilized in 1% triton X-100/chloroform (v/v) [Carr et al., 1993]. Hepatic triglyceride level was determined using the Triglyceride Quantification Kit (Cayman Chemical, Ann Arbor, MI) and normalized with liver tissue weight. After homogenization of liver tissue, the expression of HIF-1α mRNA and protein expression was evaluated. All animal experiments were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of Korea University School of Medicine.

STATISTICAL ANALYSIS

All data are representative of at least two separate experiments. Student's *t*-test or the two-way ANOVA test was used for statistical analysis. The statistical analysis was performed using SPSS version 12.0 software (SPSS, Chicago, IL). A *P*-value <0.05 was considered significant.

RESULTS

PALMITIC ACID INDUCES LIPID ACCUMULATION

After treating HepG2 cells with different concentrations of palmitic acid for 24 h, lipid accumulation was evaluated with Oil Red-O. As

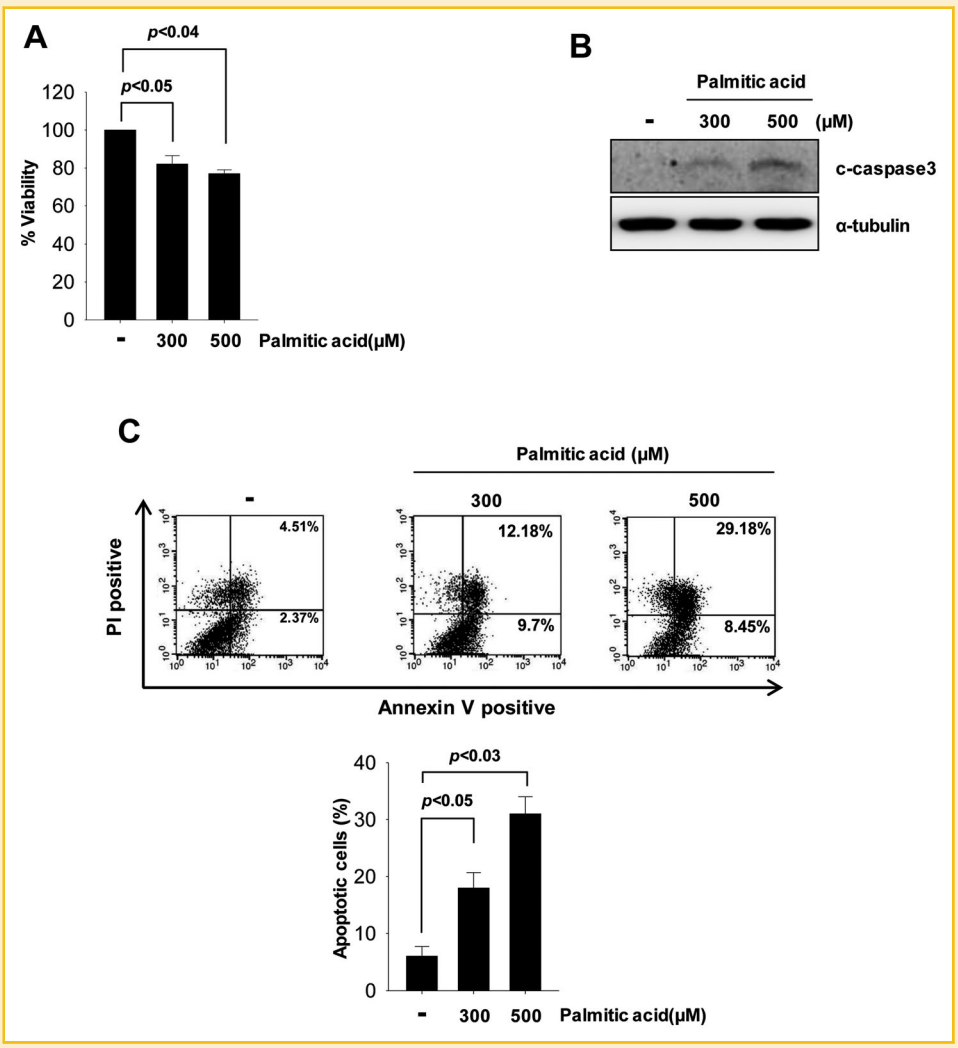

Fig. 2. Palmitic acid reduces cell viability and induces apoptosis in a dose-dependent manner. HepG2 cells were treated with palmitic acid for 24 h. A: Cell viability was measured by the MTT assay. Cell survival was represented as percentage relative absorbance compared to the control group. B: The apoptotic maker, cleaved caspase 3 (c-caspase 3), was measured by Western blot analysis. C: Apoptosis was observed by Annexin V/PI analysis. Apoptotic cells appeared as Annexin V positive. Error bars represent mean ± SD.

shown in Figure 1, palmitic acid raised the cytosolic lipid content, indicating that the palmitic acid treated HepG2 cells are a suitable in vitro model of steatosis.

PALMITIC ACID DECREASES CELL VIABILITY AND INDUCES APOPTOSIS

When the HepG2 cells treated for 24 h with increasing concentrations of palmitic acid, numbers of living cells were reduced (Fig. 2A), while c-caspase 3 (Fig. 2B) and apoptotic cell death (Fig. 2C) became activated, all in a dose-dependent fashion.

HIF-1α PROTEIN AND MRNA EXPRESSION IN HEPG2 CELLS TREATED WITH PALMITIC ACID

Next, we determined whether palmitic acid affects HIF-1α gene expression. Three hours after addition of palmitic acid (300 or 500 μM), HIF-1α mRNA levels were markedly increased, but then declined (Fig. 3A). HIF-1α protein expression was maximally induced at 24 h (300 μM palmitic acid) or 12 h (500 μM palmitic acid), and then declined (Fig. 3B); this was accompanied by increased expression of c-caspase 3 (Fig. 3B). The reciprocal relationship between levels of c-caspase 3 and HIF-1α was also seen after treating the cells with the HIF-1α inhibitor, YC-1 (Fig. S1).

ROLE OF HIF-1α IN CELL VIABILITY AND APOPTOSIS

Using gain- and loss approaches, we assessed the significance of HIF-1α for palmitic acid-induced lipoapoptosis. HepG2 cells transiently transfected with the HIF-1α gene, showed increased survival, induced Bcl-2/Bax ratio, c-caspase 3 inactivation and decreased apoptosis (Fig. 4). In contrast, transient RNA interference directed against HIF-1α enhanced the palmitic acid-induced apoptosis; was accompanied by a decrease of the Bcl-2/Bax ratio as well as increased numbers of c-caspase 3 and Annexin V/PI positive HepG2 cells (Fig. 5).

ROLE OF HIF-1α IN REGULATING ER STRESS MAKERS INDUCED BY PALMITIC ACID

Given that HIF-1α protected against palmitic acid-induced apoptosis, and that palmitic acid was previously shown to induce ER stress, we wondered whether HIF-1α was involved in palmitic acid-induced ER stress. Indeed, overexpression of HIF-1α blunted the palmitic acid-induced induction of the ER stress markers Bip and CHOP (Fig. 6A). In contrast, only CHOP increased after knockdown of HIF-1α (shHIF-1α) (Fig. 6B).

HIF-1α DIRECTLY INTERACTS WITH THE CHOP PROMOTER

To explore how HIF-1α regulates CHOP expression, we tested whether CHOP gene transcription could be modulated by HIF-1α. To this end, we transfected the HepG2 cells with a CHOP promoter-luciferase reporter constructs that did (WT-CHOP pro) or did not (Mut-CHOP pro) contain the putative HIF-1α response element (HRE) (Fig. 7A). Luciferase activity of WT-CHOP pro was more than doubled by palmitic acid, but an even greater increase was observed with Mut-CHOP pro, suggesting that HIF-1α inhibited CHOP gene expression via HRE. To gain more support for this idea, we co-transfected WT-CHOP pro or Mut-CHOP pro with HIF-1α. Indeed,

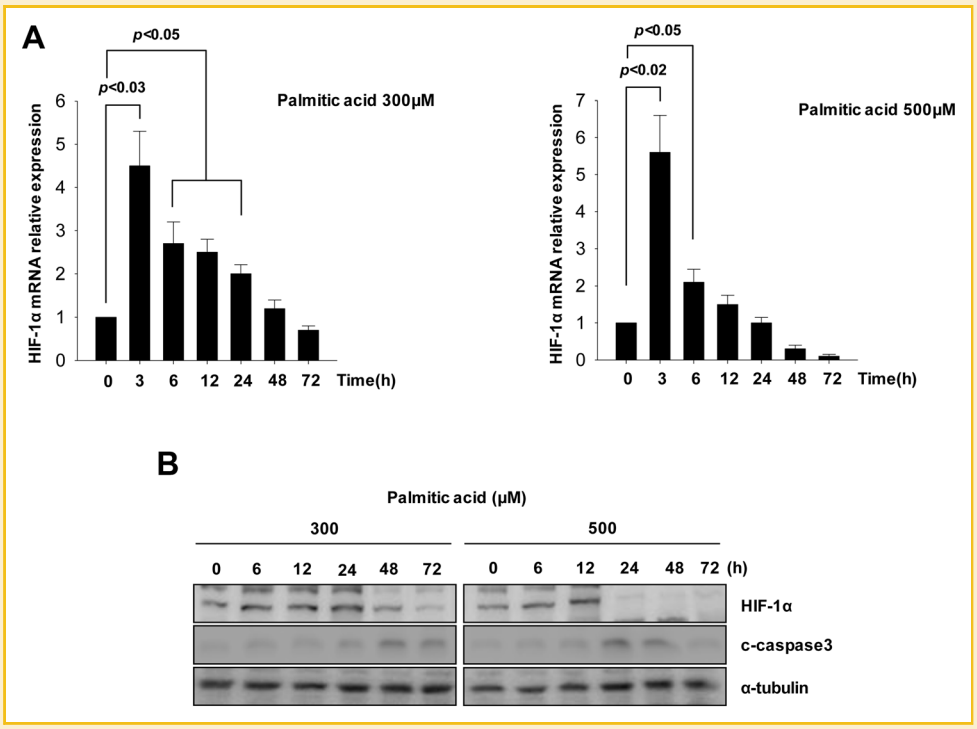

Fig. 3. Palmitic acid regulates HIF-1α protein and mRNA expression. Western blot analyses were performed to characterize the expression of HIF-1α and c-caspase 3. α-tubulin was used as an internal control. A: HepG2 cells were treated with 300 or 500 μM palmitic acid for the indicated times. B: HepG2 cells were incubated in the presence of 300 or 500 μM palmitic acid for the times indicated. HIF-1α and GAPDH were measured by real-time PCR. HIF-1α was normalized to GAPDH. Error bars represent mean ± SD.

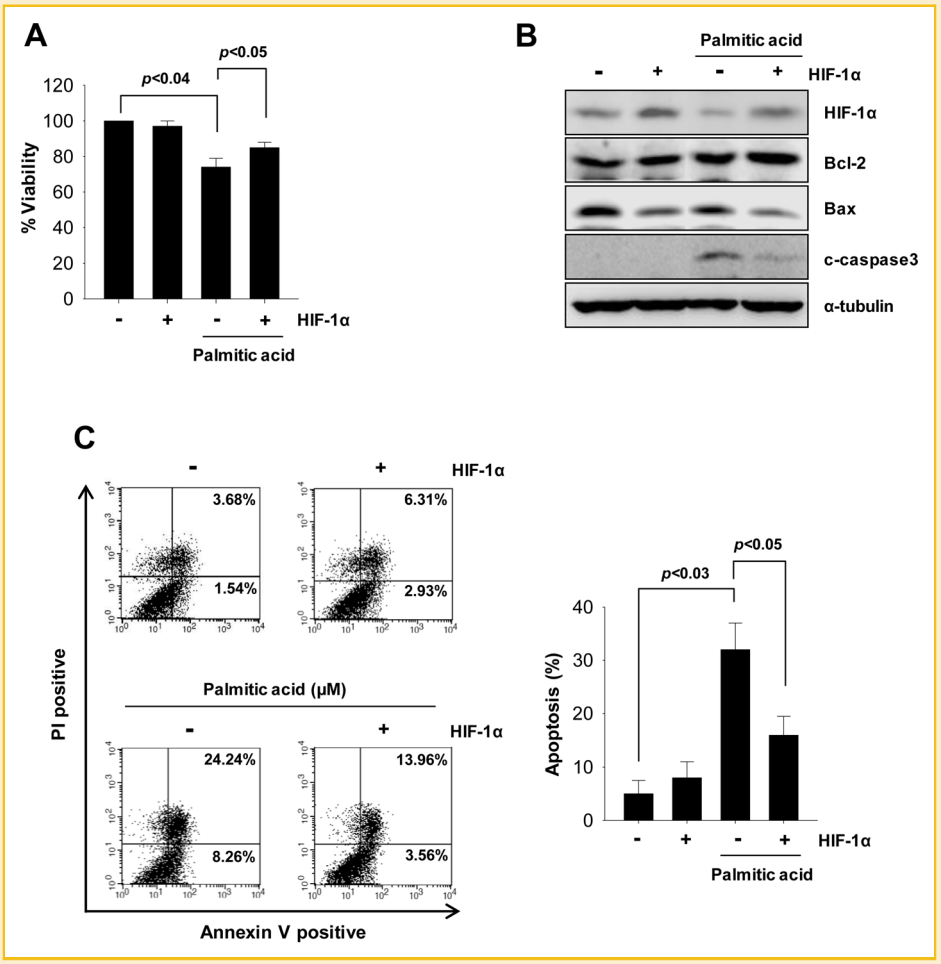

Fig. 4. HIF-1α increases cell viability and reduces cellular apoptosis. HepG2 cells were treated with palmitic acid for 24 h after transient transfection of a HIF-1α expression plasmid. A: Cell viability was measured by the MTT assay. Cell survival was represented as percentage relative absorbance compared to the control group. B: Representative Western blots of the Bcl-2, Bax, and c-caspase 3 protein levels. C: Apoptosis was observed by Annexin V/PI analysis. Apoptotic cells appeared as Annexin V positive. Error bars represent mean ± SD.

an increased level of HIF-1α inhibited the palmitic acid-induced increase of WT-CHOP pro reporter gene activity to a greater extent than the increment of Mut-CHOP pro activity (Fig. 7B).

Unexpectedly, transfection of HIF-1α decreased the luciferase expression by co-transfected Mut-CHOP pro or WT-CHOP pro to similar degrees (Fig. 7B). Perhaps this phenomenon can be explained by the existence of multiple HREs the CHOP gene promoter region, with one of them compensating for the suppressive role. Indeed, sequence analysis reveals several putative HIF-1α binding sites in the CHOP promoter region (data not shown). Finally a ChIP assay with an anti-HIF-1α antibody demonstrated that HIF-1α directly bound to the CHOP promoter (Fig. 7C).

HIF-1α EXPRESSION IS DECREASED IN MCD-FED MICE

We evaluated the expression of HIF-1α in the liver tissues of MCD-fed mice in order to examine the relationship between HIF-1α and NASH. In accordance with the previous publication [Vetelainen et al., 2007], mice fed MCD for 4 weeks exhibited a significant increase of triglyceride and collagen levels and of liver-to-body weight ratios (Fig. 8A–D). In parallel, HIF-1α mRNA and protein levels were significantly downregulated (Fig. 8E,F).

DISCUSSION

NASH, which is characterized by progressive inflammation and fibrosis, is a key histological feature of NAFLD and can progress to more severe liver injury, such as cirrhosis and cancer. Therefore, the progression from simple steatosis to NASH is a key concern in NAFLD. Lipoapoptosis, induced by excessive fat accumulation in the liver is a potential mechanism for infiltration of inflammatory cells and macrophages, inflammatory and fibrotic cytokine release, and fibrogenesis [Takehara et al., 2004; Hikita et al., 2009; Vick et al., 2009; Choi et al., 2013]. Thus, prevention or attenuation of free fatty acid-induced lipoapoptosis may halt the progression of simple steatosis to NASH. Although increased ER stress and mitochondrial injury is a putative lipotoxicity mechanism, cyto-protective pathways in a lipotoxicity model have hardly been studied. HIF-1α is a nuclear transcription factor that plays a crucial role by regulating the

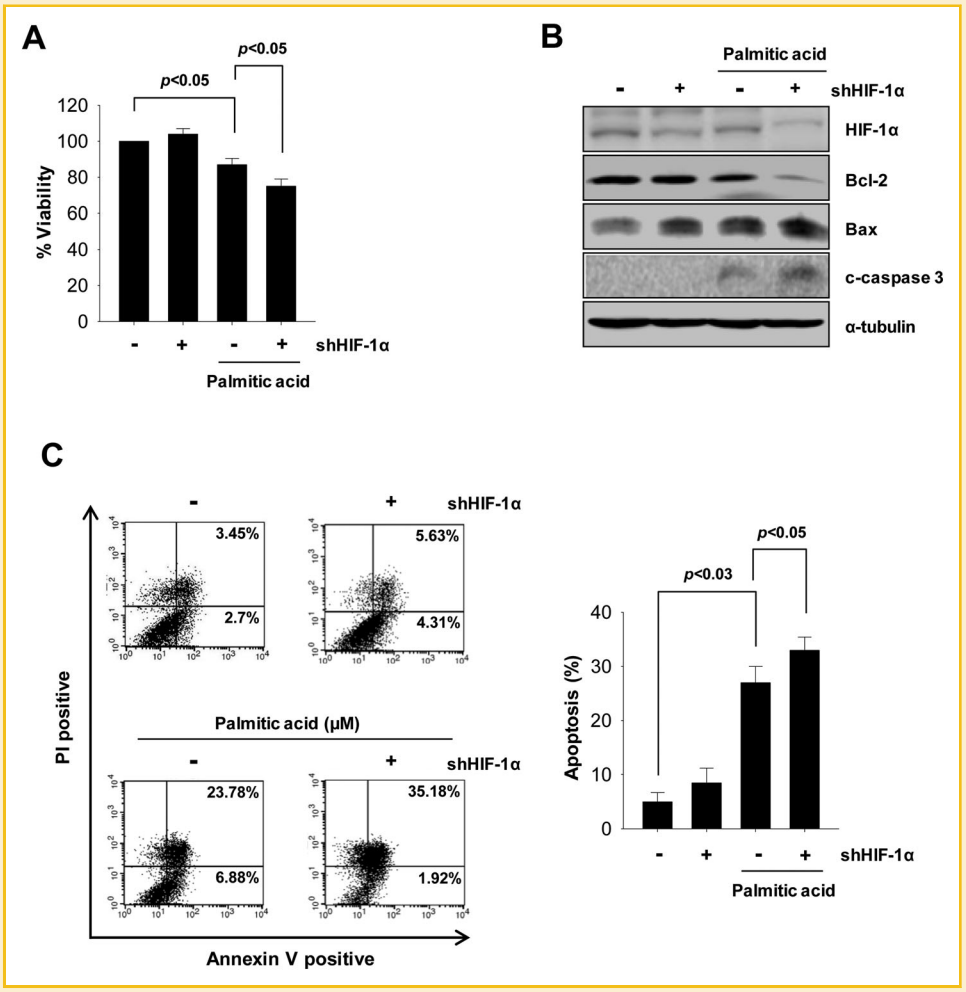

Fig. 5. Loss of HIF-1α reduces cell viability and induces cellular apoptosis. HepG2 cells were treated with palmitic acid for 24 h after transient transfection of an shHIF-1α expressing plasmid. A: Cell viability was measured by the MTT assay. Cell survival was represented as percentage relative absorbance compared to the control group. B: Representative Western blots of the Bcl-2, Bax, and c-caspase 3 protein levels. C: Apoptosis was observed by Annexin V/PI analysis. Apoptotic cells appeared as Annexin V positive. Error bars represent mean ± SD.

expression of numerous genes associated with physiological processes such as cell differentiation, proliferation, inflammation, angiogenesis, metabolic adaptation, ischemia, survival, and apoptosis. Although HIF-1α has multiple functions under diverse conditions, activating HIF-1α leads to the transcription of various genes that contribute to the cellular stress adaptation and cyto-protective effects [Yu et al., 2004; Piret et al., 2005; Loor and Schumacker, 2008]. Therefore, we wanted to investigate whether HIF-1α plays a protective role in palmitic acid-induced lipotoxicity. The present study supports such a role. Specifically, our data suggest that in the early phase of exposure to the toxic lipid, HIF-1α prevents apoptosis by inhibiting the ER stress marker, CHOP, but this strategy is abandoned upon persistent exposure to the toxic lipid.

We found increased HIF-1α expression following treatment of cells with 500 μM palmitic acid for 12 h. However, expression decreased rapidly after that time point. To verify our observation, we examined the effect of an HIF-1α inhibitor, YC-1. YC-1 inhibited the palmitic acid-mediated increase of the HIF-1α protein level and induced cleavage caspase-3 expression in HepG2 cells. HIF-1α is activated by diverse stimuli under non-hypoxic conditions such as metals, reactive oxygen species (ROS), nitric oxide, growth factors, pH, and mechanical stressors [Chandel et al., 2000; Kuschel et al., 2012]. In addition, HIF-1α is controlled by transcriptional and posttranscriptional regulation [Galban and Gorospe, 2009]. Although the mechanism of increased HIF-1α expression due to palmitic acid remains unknown, increased ROS production may be responsible [Liu et al., 2011]. In the present study, we also observed disparate levels of HIF-1α protein and mRNA at 0 and 72 h after treatment with palmitic acid (300 μM). Some previous studies attributed discrepant hypoxic HIF-1α mRNA and protein levels to increased mRNA stability and decreased protein degradation [Uchida et al., 2004; He et al., 2011]. In contrast to these studies, we treated the cells with palmitic acid to induce lipotoxicity. To our knowledge, there are no previous studies showing that HIF-1α expression was stimulated by palmitic acid. It remains unclear whether this finding is due to stimuli other than hypoxia, or due to different cell type. Nevertheless, it appears that

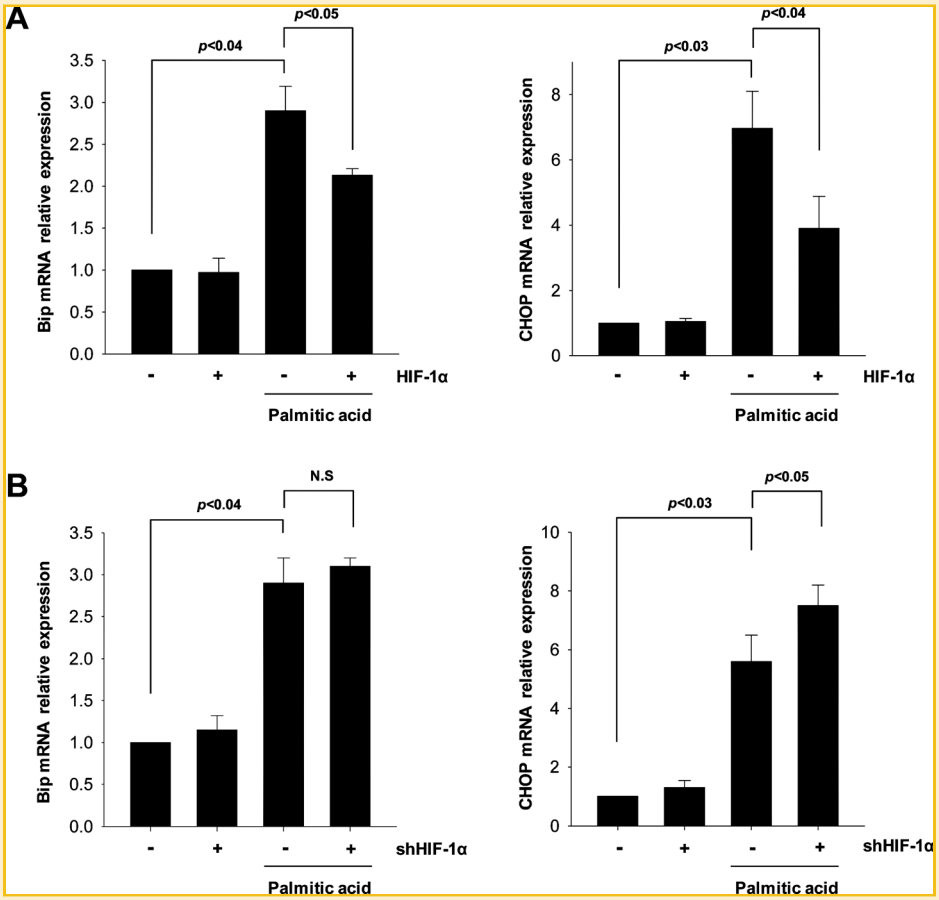

Fig. 6. Role of HIF-1α in palmitic acid-mediated ER stress. HepG2 cells transiently expressing HIF-1α (A) or shHIF-1α (B) were incubated with palmitic acid at 500 μM. Total RNA was extracted 24 h after treatments, *Bip* and *CHOP* mRNA expression was quantified by real-time PCR. Fold induction was determined after normalization to GAPDH. NS: not significant. Error bars represent mean ± SD.

several mechanisms regulating HIF-1α mRNA and protein expression may be responsible for this result.

We also observed decreased HIF-1α expression 24 h after palmitic acid treatment. Other studies also observed decreased, HIF-1α expression after initial activation in those cases due to hypoxia or mechanical stress; although those studies concentrated on increased HIF-1α expression. It is difficult to know the mechanism of the changes in HIF-1α expression when the molecule is tested under several stressful conditions. We speculate that increased HIF-1α expression is an initial defense mechanism to these toxic stimuli; but, when stress conditions persist, cells appear to switch a suicide strategy that includes downregulation of the anti-apoptotic HIF-1α. We recently described another case where the alternative choice of survival or suicide strategies could lead to contrary effects of the same environmental stimulus [Uchida et al., 2004; Kong et al., 2013]. Indeed, we found that overexpression of HIF-1α reduced apoptosis and increased cell survival in our lipoapoptosis model. Conversely, Anavi et al. [2012] demonstrated that decreased HIF-1α activation in steatotic hepatocytes was associated with significantly lower intracellular ATP levels and greater cell death. However, they also showed that HIF-1α overexpression increased cell viability by activating genes responsible for utilizing nutrients for energy. HIF-1α signaling is important in adaptation to a hypoxic environment. HIF-1α-mediated signaling drives the expression of a wide variety of genes essential to the adaptive response, including vascular endothelial growth factor, insulin growth factor-2, erythropoietin, heme oxygenase-1, and glycolytic enzymes [Lefer, 2006]. Based on these concepts, several studies have reported reduced cytotoxicity by inducing HIF-1α expression in chronic rejection of allograft transplantation, ischemia/reperfusion injury, and neovascularization model in in vitro and in vivo [Creager et al., 2011; Czibik et al., 2011; Jiang et al., 2011]. However, no studies have demonstrated a cellular protective effect of HIF-1α in a lipotoxicity model.

Interestingly, we demonstrated in our lipotoxicity model that HIF-1α directly regulated the expression of CHOP, an ER stress marker. The understanding of ER-induced apoptosis in NAFLD has improved in recent years [Pagliassotti, 2012]. In the NAFLD model, lipid accumulation induces ER stress, and this increased ER stress is responsible for lipoapoptosis [Cazanave and Gores, 2010]. ER stress-induced apoptosis pathways have been identified, including CHOP, the Bcl-2 family (Bak/Bax), caspase-12, and c-Jun N-terminal kinase [Wu and Kaufman, 2006]. Among these, CHOP activation is directly linked to apoptosis via caspase-3 activation [Cazanave et al., 2010; Pfaffenbach et al., 2010]. In agreement with our results, HIF-1α

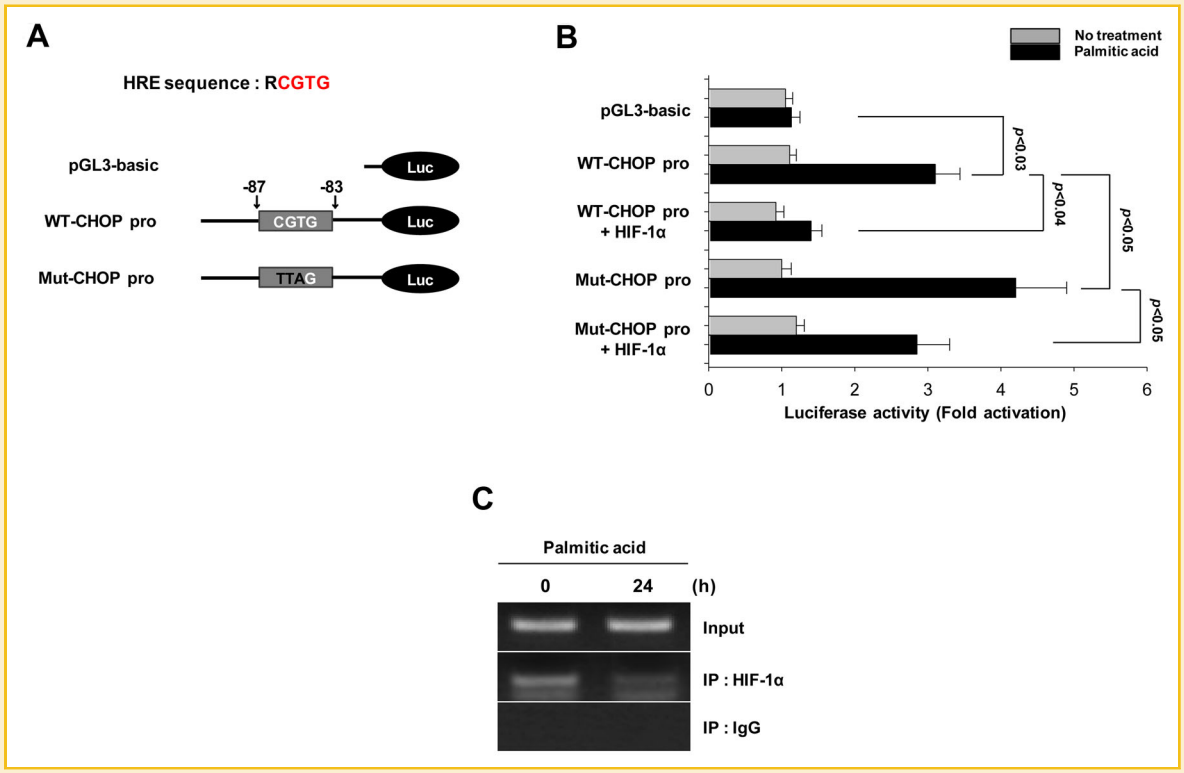

Fig. 7. HIF-1α is a transcriptional inhibitor of CHOP. A: Diagram of plasmids encoding wild-type (WT) or mutant (Mut) CHOP promoter cloned into vector pGL3 containing the luciferase gene. In the mutant plasmid, the CGT nucleotides in the putative HRE region (nt −87 to −83) have been replaced with TTA nucleotides. Luciferase assay in HepG2 cells transfected with the indicated plasmids. B: HepG2 cells were transfected with mutated CHOP promoter constructs or the wild-type CHOP promoter, and the cells were then transfected with HIF-1α or shHIF-1α. The luciferase activity was measured at 24 h after palmitic acid treatment (500 μM). The luciferase activity of the cells without palmitic acid treatment was set as 100%. The results are shown as the mean ± SD of the three experiments performed in duplicate. Error bars represent mean ± SD. C: HepG2 cells were incubated in the absence or presence of palmitic acid for 24 h. Strategy for ChIP assay, which contains the putative HRE region, of the CHOP promoter is amplified by PCR. Input was included as positive control, and IgG was included as a negative control. Error bars represent mean ± SD.

accumulation due to renal ischemic preconditioning protects the kidney against acute ischemic injury by reducing oxidative and ER stressors such as p-PERK, ATF4, and TRAF2 [Mahfoudh-Boussaid et al., 2012].

Our study also demonstrates for the first time a role for the HRE in the CHOP promoter. Normally, HIF-1α acts as a negative regulator that prevents CHOP gene transcription by directly binding to the HRE in the CHOP promoter. However, when HIF-1α expression decreases in the lipid excess state, the CHOP promoter is disinhibited, which could explain the increased apoptosis. Although the ER chaperone and signaling regulator Bip (also known as GRP78) mRNA was also induced by palmitic acid treatment, knockdown of HIF-1α expression did not increase Bip mRNA expression. CHOP and Bip, ER stress markers, are well-known apoptosis-associated proteins. Several reports have demonstrated that palmitic acid-induced ER stress and apoptosis via CHOP and Bip upregulation in several liver cell lines [Zhang et al., 2011; Cao et al., 2012], as shown in our study. Additionally, CHOP overexpression leads to cell cycle arrest and/or apoptosis in leukemia cells, pancreatic beta cells, and macrophages [Matsumoto et al., 1996; Oyadomari et al., 2001; Gotoh et al., 2002]. However, some studies have suggested that increasing Bip expression protects cells from ER stress and apoptosis [Kammoun et al., 2009; Gu et al., 2010]. In addition, Song et al. [2001] showed that HIF-1α does not affect the induction of Bip under chronic hypoxia because the Bip promoter contains no HRE motifs. Similarly, we found that Bip expression was not increased following shHIF-1α transfection, unlike CHOP induction in HepG2 cells. Although Bip has been extensively studied in ER stress, there is a still controversy about whether Bip is a causal mechanism of apoptosis in ER stress.

Unlike previous studies suggesting that HIF-1α is associated with hepatic steatosis in different alcoholic fatty liver models [Berthiaume et al., 2009; Zaouali et al., 2010], we found that lipid accumulation, and mRNA expression of SREBP-1c, ACC, and CPT-1 were independent of HIF-1α expression (data not shown). Hypoxia increases lipid accumulation by activating HIF-1α or other pathways [Krishnan et al., 2009; Na et al., 2011; Anavi et al., 2012]. It is difficult to determine the cause for this discrepancy. However, various stimuli such as alcohol, hypoxia, or FFAs may have different impacts on lipid accumulation in vitro and in vivo [Carabelli et al., 2011; Nath et al., 2011; Nishiyama et al., 2012].

Although we demonstrated a possible role for HIF-1α in reducing lipotoxicity, we used an in vitro cell line system. It would be preferable to use primary hepatocytes to evaluate the pathophysiology of NAFLD. However, primary hepatocytes show rather poor reproducibility and rapidly lose hepatocyte-specific functions. Therefore, HepG2 cell lines, which do maintain a wide variety of

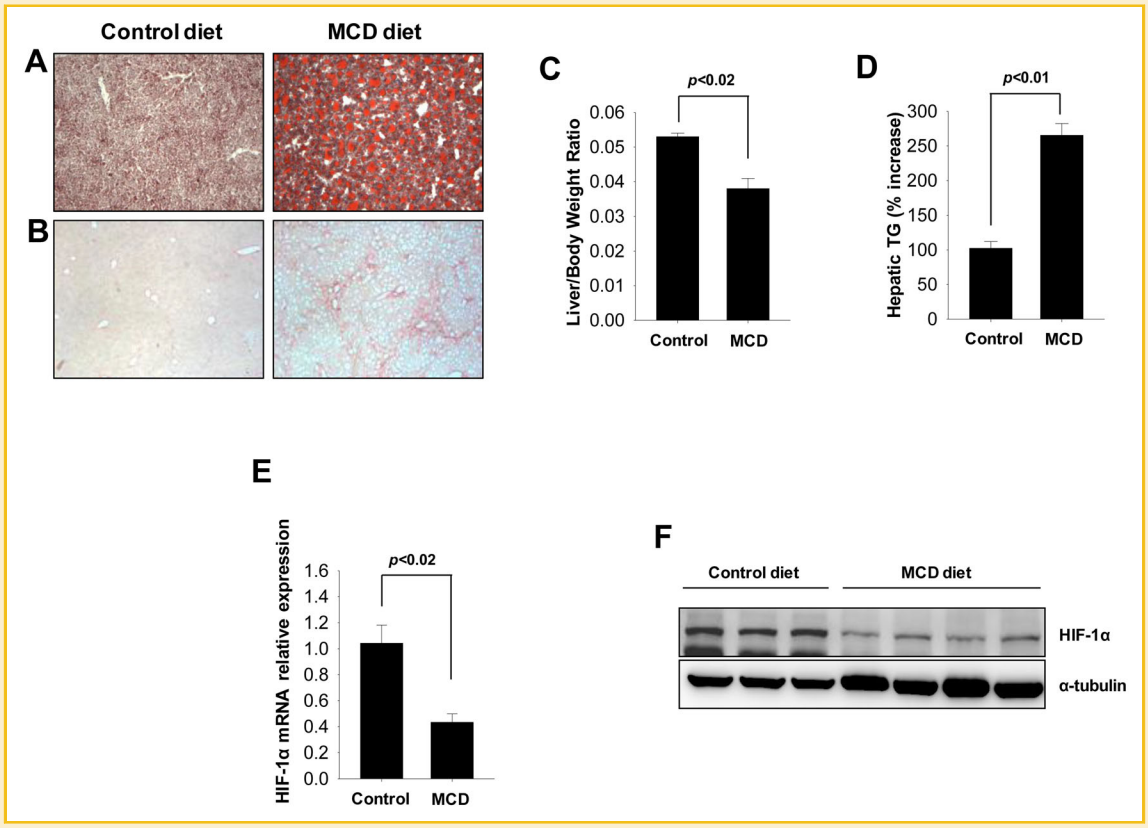

Fig. 8. Effect of methionine choline-deficient (MCD) diet on hepatic HIF-1α expression. A,B: Mice were exposed to either control diet or MCD for 4 weeks, and then liver sections were prepared and stained with Oil Red-O (A) and picosirus red (B) (magnification 100×). C,D: Relative changes of liver/body weight ratios (C) and hepatic triglyceride (TG) levels (D). E,F: Changes of liver HIF-1α mRNA (E) and protein (F) levels. Error bars represent mean ± SD, n = 3 for control diet and n = 4 for MCD-fed mice.

liver-specific metabolic functions, have been widely used in studies investigating liver metabolism and lipoapoptosis [Barreyro et al., 2007; Yang and Chan, 2009]. Nonetheless, HepG2 cells have a limited metabolic capability and show lower expression of CYP genes relative to primary cells. Therefore, we repeated the experiment with another hepatocellular carcinoma cell line, Huh-7, and obtained similar results (data not shown).

However, in accordance with the in vitro study, we demonstrated that the HIF-1α expression was decreased in MCD-fed mice, which is a well-known model of NASH. This raises the possibility that the decreased liver HIF-1α expression is involved in the pathogenesis of NASH. Nevertheless, to clarify the action of HIF-1α for preventing the progression of simple steatosis to NASH, this finding should be further verified in in vivo models.

In summary, our findings indicate that HIF-1α reduced lipoapoptosis by directly inhibiting CHOP in human liver cells. In addition, whether inducing the expression of HIF-1α could prevent the progression to NASH by reducing lipoapoptosis should be studied further.

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