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ERp57 Is Up-Regulated in Free Fatty Acids-Induced Steatotic L-02 Cells and Human Nonalcoholic Fatty Livers

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ABSTRACT

Pathogenesis of nonalcoholic fatty liver disease (NAFLD) is not clear. In this study we aimed to identify proteins involved in NAFLD development in free fatty acids (FFA)-induced hepatosteatotic cells and in human liver biopsies. Steatosis was induced by incubating a normal human hepatocyte-derived cell line L-02 with FFA. Differentially expressed proteins in the steatotic cells were analyzed by two-dimensional gel electrophoresis-based proteomics. Involvement of one of the up-regulated proteins in steatosis was characterized using the RNA interference approach with the steatotic cells. Protein expression levels in liver biopsies of patients with NAFLD were assessed by immunohistochemistry. Proteomic analysis of L-02 steatotic cells revealed the up-regulation of ERp57, a condition not previously implicated in NAFLD. Knockdown of ERp57 expression with siRNA significantly reduced fat accumulation in the steatotic cells. ERp57 expression was detected in 16 out of 17 patient biopsies and correlated with inflammation grades or fibrosis stages, while in 5 normal biopsies ERp57 expression was not detectable in hepatocytes. In conclusion, ERp57 was up-regulated in FFA-induced steatotic hepatic cells and in NAFLD patient livers and demonstrated steatotic properties in cultured cells. Further investigations are warranted to verify the involvement of ERp57 in NAFLD development. J. Cell. Biochem. 110: 1447–1456, 2010.

KEY WORDS: ERp57; L-02 CELLS; LIVER BIOPSIES; NONALCOHOLIC FATTY LIVER DISEASE; PROTEOMICS

Nonalcoholic fatty liver disease (NAFLD) includes a series of progressive liver abnormalities ranging from simple triglyceride accumulation to inflammation, fibrosis, and cirrhosis [Dixon et al., 2001]. Along with increasing incidences of obesity and diabetes, NAFLD is becoming the most common form of chronic liver disease and a recognized cause of liver-related morbidity and mortality. Epidemiological studies showed that NAFLD affects 25–30% of the general population and up to 80% of obese and diabetes individuals [Gentile and Pagliassotti, 2008b]. The pathogenesis of NAFLD has not been fully elucidated, although the "two-hit" hypothesis is generally accepted. According to this hypothesis, the first hit is the development of steatosis and the second is an undefined hit which is a source of oxidative stress capable of initiating significant lipid peroxidation [Day and James, 1998]. One important aspect of the "two-hit" hypothesis is that steatosis per se is not causal in the progression of NAFLD. A growing body of evidence, however, shows that fat deposition in the liver, involving especially saturated fatty acids, may directly damage hepatocytes and precipitate the development of nonalcoholic steatohepatitis [Gentile and Pagliassotti, 2008b].

Increased fatty acids, particularly long chain saturated fatty acids, induce endoplasmic reticulum (ER) stress and activate the unfolded protein response (UPR) in hepatocytes. Notably ER stress in response to increased hepatic lipids appears to decrease the ability of the liver to secrete triglycerides by limiting apoB secretion, potentially worsening steatosis. These findings suggest that UPR-mediated signaling contributes to the development of steatosis, which in turn induces ER stress and subsequent complications such as cell dysfunction and death [Gentile and Pagliassotti, 2008a]. The ER protein ERp57 (ER 60, ER-60, Grp58), a member of the protein

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disulfide isomerase (PDI) family, has been implicated in a number of cellular functions related to thiol-dependent protein disulfide isomerase, chaperone, protease, and MARRS (membrane associated rapid response steroid binding) receptor [Khanal and Nemere, 2007]. It has been reported that expression of ERp57 was down-regulated in livers of ER stress-induced steatosis mice [Rutkowski et al., 2008] and differentially expressed in male and female rats with experimental alcoholic steatohepatitis [Banerjee et al., 2008]. However, the role of ERp57 in NAFLD has not been clearly defined. In this study, using two-dimensional gel electrophoresis (2-DE)-based proteomic analysis we found that ERp57 was up-regulated in free fatty acids (FFA)-induced steatotic cells. Reducing the expression of ERp57 significantly inhibited lipid accumulation in the steatotic cells. Furthermore, ERp57 was found to be expressed in livers of NAFLD patients. These findings suggest that further investigation is needed and should be done to determine whether ERp57 plays a role in the development of NAFLD.

MATERIALS AND METHODS

CELL CULTURE

L-02 (Institute of Biochemistry and Cell Biology, Shanghai Institute for Biological Sciences, Shanghai, China) cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C in an atmosphere containing 5% CO_2. When FFA mixture (sodium salts of oleate and palmitate, Sigma, Malaysia) was added, bovine serum albumin was added to a final concentration of 1% in the culture medium. Cell cultures were used for experiments when they reached 75% confluence.

NILE RED STAINING AND FLOW CYTOMETRIC ANALYSIS OF INTRACELLULAR LIPID CONTENTS

Cells were treated with FFA for 24 h. Photocolorimetric measurement of intracellular lipid contents in Nile Red stained cells was performed as described [McMillian et al., 2001]. Flow cytometric analysis of intracellular lipid accumulation was conducted according to a previous method [Yip et al., 2005].

MEASUREMENT OF CELLULAR TRIGLYCERIDE CONTENT

Cellular triglyceride content was measured using an enzymatic kit (Zhongsheng Beikong Biotechnology and Science, Inc., China) following manufacturer's instructions. Triglyceride content was expressed in micrograms of triglycerides per microgram of protein. Protein concentration was determined by Bio-Rad Protein Assay (Bio-Rad, USA).

APOPTOSIS ANALYSIS

Early and late phase apoptotic cells were monitored with AnnexinV-FITC Apoptosis Detection Kit I (BD Bioscience, CA). Cells were washed twice with cold PBS, resuspended in binding buffer, and incubated with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) staining solution following manufacturer's instructions. Samples of 10,000 stained cells were analyzed using a flow cytometer (BD Bioscience).

PHASE-CONTRAST AND FLUORESCENT MICROSCOPE IMAGING

Cells were washed with PBS, stained with 1 μM Nile Red for 15 min and then examined under phase-contrast (Leica, Germany) and fluorescence (Nikon, Japan) microscopes.

PROTEOMIC SAMPLE PREPARATION AND TWO-DIMENSIONAL ELECTROPHORESIS

Cells in 100 mm culture dishes were harvested by trypsinization, washed three times with isotonic buffer (10 mM Tris, 250 mM sucrose, pH 7.2) and lysed in a lysis buffer (8 M urea, 4% CHAPS, 40 mM dithiothreitol and 0.5% pH 3–10 NL IPG buffer) by gentle shaking for 60 min on ice. The extracts were centrifuged at 25,000*g* for 60 min at 4°C. Supernatants were purified with 2D Clean Up Kit (GE Healthcare, USA) following manufacturer's instructions. Protein concentration of the purified samples was determined using 2D Quant Kit (GE Healthcare) and protein samples were stored in aliquots at −80°C prior to analysis.

First-dimension separation was performed using 13 cm IPG strips (pH 3–10 NL, GE Healthcare). Samples were diluted in rehydration solution (8 M urea, 2% CHAPS, 0.5% IPG buffer, 0.002% bromophenol blue, freshly added 0.28% dithiothreitol) to reach a final protein load of 100 μg (in 250 μl) per strip. IPG strips were actively rehydrated at 30 V for 12 h then focused at 500 V for 2.5 h, 1,000 V for 0.5 h, and then the voltage was increased to 8,000 V gradually over the next 3 h and maintained at 8,000 V for 40,000 Vh. Focused strips were equilibrated for 15 min in equilibration buffer (6 M urea, 75 mM Tris–HCl pH 8.8, 29.3% glycerol, 2% SDS) with 1% (w/v) dithiothreitol (DTT, GE Healthcare) freshly added in order to reduce electroendosmotic effects. This was followed by 15 min in the same equilibration buffer containing 2.5% iodoacetamide (IAA, Amersham Biosciences, USA) in order to remove excess DTT and to prevent streaking. The second dimension separation was carried out in 10% SDS-PAGE (20 mA/gel, 10 min; 25 mA/gel, 260 min).

SILVER STAINING AND IMAGE ANALYSIS

Protein spots were visualized by silver staining as described previously [Lei et al., 2008]. The stained gels were scanned with LabScan 6.0 (GE Healthcare) and data were analyzed with Progenesis SameSpots (Nonlinear Dynamics, UK). One image was chosen and allocated as the reference gel to align all other images in the experiment. Following manual alignment, spot detection, background subtraction, and normalization were performed. Spot volume, expressed relative to total spot density, was used to identify spots that differed significantly in normalized volume between control and test samples. In all cases when statistical variance of the test/control spot intensity ratio was within 95% ($P < 0.05$) the paired spots were considered to be significantly different. Only those significantly and consistently up- or down-regulated spots (>2 folds) or spots which appeared or disappeared after treatment in three independent experiments were selected for analysis with mass spectrometry (MS).

IN-GEL DIGESTION AND MALDI-TOF-MS/MS ANALYSIS

Protein spots were excised from gels, and in-gel digestion was performed as described previously [Wu et al., 2006]. The mass spectra were obtained using the Bruker AutoflexIII MALDI-TOF/TOF

Mass Spectrometer (Bruker Daltonics, USA). Protein identification was performed automatically by searching the Swiss-Prot 55.3 database using the MASCOT 2.04 search engine (Matrix Science, UK). Database searches were carried out using the following parameters: type of search, MS/MS ion search; enzyme, trypsin; and allowance of one missed cleavage. Carbamidomethylation was selected as a fixed modification, and oxidation of methionine was allowed to be variable. The peptide and fragment mass tolerance were set at 50 ppm and 0.5 Da, respectively. The instrument was selected as MALDI-TOF-TOF. Proteins with probability-based MOWSE scores ($P < 0.05$) were considered to be positively identified.

WESTERN BLOT ANALYSIS

L-02 cells in 100 mm culture dishes were collected and proteins were extracted with RIPA lysis buffer [50 mM Tris-Cl, 1% v/v NP-40, 0.35% w/v sodium-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, pH 7.4, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM NaF, 1 mM Na_3VO_4] containing a protease inhibitor cocktail (Roche, Germany). Protein concentration was determined by the Bio-Rad Protein Assay. Twenty micrograms of individual protein samples were separated by SDS-PAGE and then electro-transferred onto the nitrocellulose membrane (Amersham Biosciences). Membranes were blocked for 30 min with 5% skim milk in TBST buffer composed of 50 mM Tris (pH 7.6), 150 mM NaCl and 0.1% Tween-20 and incubated with specific antibodies against human ERp57 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) overnight at 4°C. β-Actin was used as internal control and was detected using a polyclonal antibody (Santa Cruz Biotechnology, Inc.). After incubation with secondary antibodies, ECL detection reagents (Amersham Biosciences) were used to detect the signals. The intensity of the signal was quantified with the imageQuant software.

REAL-TIME PCR

L-02 cells in 100 mm culture dishes were collected and total RNA was isolated using Trizol reagent (Invitrogen, USA) according to manufacture's protocol. Five micrograms of RNA was used for reverse transcription by oligo-dT using the SuperScriptII Reverse Transcription Kit (Invitrogen). The real-time PCR primers were designed as follows: ERp57 (sense 5′-cctggtgtggacactgcaag-3′and anti-sense 5′-ccctcaagttgctggctgct-3′), Hsp90 (sense 5′-tccatctatta-catcactggtgaga-3′ and anti-sense 5′-gattgtcaccttctcaaccttctta- 3′), FAS (sense 5′-agaagctcgtgttgacttctcg-3′ and anti-sense 5′-ggcttgca-gacgtcctggaaga -3′). To normalize the amounts of RNA in samples, a PCR reaction was also performed with primers of β-actin (sense 5′-gactacctcatgaagatc-3′ and anti-sense 5′-gatccacatctgctggaa-3′). Real-time PCR was performed in a total volume of 20 μl, with 1× Power SYBR Green PCR Master Mixture (Applied Biosystems, UK) in the 7500 Fast Real-time PCR system (Applied Biosystems, USA). All samples were run in triplicate.

SMALL INTERFERING RNA (siRNA) TRANSFECTION

RNA interference was used to silence ERp57 gene expression. ERp57 siRNA was purchased from Santa Cruz Biotechnology, Inc. Transient gene silencing was attained by transfection of siRNA into cells using FuGENE HD Transfection Reagent (Roche) according to the manufacturer's instructions. Briefly, L-02 cells were grown in six-well plates overnight and transiently transfected with 27 nM siRNA or control siRNA (Santa Cruz Biotechnology, Inc.) by using 6 μl FuGENE in a total transfection volume of 1.5 ml of DMEM medium (FBS and antibiotic free). After incubation for 22 h at 37°C, 1.5 ml of growth medium (DMEM medium with 20% FBS) was added. Cells were incubated at 37°C for a further 24 h and then subjected to 1 mM FFA mixture treatment for 24 h. The cells were then collected for verification of ERp57 knockdown by Western blotting, analysis of apoptosis (as described above) and fat accumulation by flow cytometry.

PATIENT POPULATION AND LIVER HISTOLOGY

Seventeen patients with documented histological diagnosis of steatosis or steatohepatitis were selected for study. All patients were seen at Caritas Medical Centre, Hong Kong, China and had had liver biopsies done between 2005 and 2008. Patients with significant alcohol drinking history and/or liver diseases such as hepatitis B, hepatitis C, and autoimmune hepatitis, and/or drug-induced liver injury, were excluded. Five normal liver cases without steatosis and steatohepatitis were also included in the study. Demographic data of subjects and their liver pathology are summarized in Table I.

Pathological assessment was made on sections from formalin-fixed and paraffin-embedded liver biopsy samples stained with hematoxylin-eosin and Masson's trichrome. The degree of steatosis was graded according to the percentage of hepatocytes involved: I, 5% to <33%; II, 33–66%; III, >66%. Steatohepatitis was graded and staged according to the Brunt classification [Brunt, 2004].

ERP57 IMMUNOHISTOCHEMISTRY

Formalin-fixed human liver tissue samples were embedded in paraffin, sectioned at 4 μm and mounted on SUPERFROST® PLUS slides. Sections were deparaffinized with xylene and brought to absolute alcohol. The sections were then treated with 1% methanolic H_2O_2 for 10 min to block endogenous peroxidase activities. After blocking, the slides were placed in 20 ml pH 6.0 (0.01 M) citrate buffer in the 5-slide mailers, which were heated in boiling distilled water for 20 min. The immunohistochemical test was performed on the Ventana NexES module using Ventana Endogenous Biotin Blocking Kit and Ventana iView Detection Kit. The sections were incubated at 37°C for 32 min with the ERp57 antibody (dilution: 1:200; Santa Cruz Biotechnology, Inc.). Finally, the sections were washed with distilled water, counterstained with hematoxylin, and mounted. Negative controls were obtained by omitting the primary antibody. A human adult testis section was mounted on each slide of the test cases and stained together to serve as the positive control.

TABLE I. Demographic and Liver Pathology Data of NAFLD Patients (n = 17) Studied

Age (year)	52.4 (mean); 29–88 (range)
Sex (female/male)	9/8
Degree of steatosis (I–III)	I: 4, II: 11, III: 2
Grade of inflammation (0–III)	0: 3, I: 9, II: 3, III: 2
Stage of fibrosis (0–IV)	0: 2, I: 5, II: 2, III: 2, IV: 6

The intensity and extent of the specific staining were separately assessed. Intensity levels were labeled as 1+ to 3+, by matching with the staining intensity of the spermatogenic cell cytoplasm (3+) in the testis positive control section stained together on the same slide [Zhang et al., 2007]. The extent of positive staining was recorded as "occasional," "frequent," or "diffuse" when the percentage of stained hepatocytes was <25%, 25–75%, or >75% of the steatotic or ballooned hepatocytes, respectively.

DATA ANALYSIS

Results in cellular assays were expressed as the mean ± SEM. Differences between two groups were analyzed using the Student's *t* test. Data from immunohistochemical assays were analyzed by Spearman's rank-correlation test using SPSS 16.0 program.

RESULTS

FFA INDUCED LIPID ACCUMULATION IN L-02 CELLS IN THE ABSENCE OF APOPTOSIS

We first prepared hepatosteatotic cells using the L-02 cell line which is derived from normal human fetal hepatocytes [Yeh et al., 1980; Xu et al., 2003]. Gomez-Lechon et al. reported the use of the human hepatocarcinoma cell line HepG2 for in vitro studies of steatosis. In the HepG2 model, treatment with 1 mM FFA mixture (oleate/palmitate, 2:1) for 24 h induced similar levels of intracellular lipid accumulation as found in human steatotic livers in the absence of apoptosis [Gomez-Lechon et al., 2007]. We applied this FFA treatment regime in L-02 cells and examined lipid accumulation and apoptosis. In L-02 cells 1 mM FFA treatment increased intracellular lipid content by 5.34 ± 0.65-fold compared to FFA-untreated controls determined by Nile Red staining. Fluorescent microscopy confirmed the lipid accumulation in L-02 cells (Fig. 1A). The cellular lipid accumulation level in L-02 cells is comparable to that in human steatotic livers which is 5.5-fold over non-steatotic livers [Gomez-Lechon et al., 2007].

We also determined intracellular triglyceride levels in L-02 cells treated with 1 mM FFA. It was found that the triglyceride content was increased by about 2.5-fold from 0.370 ± 0.070 μg/μg protein in control cells to 0.923 ± 0.097 μg/μg protein in FFA-treated cells ($n = 3$, $P < 0.01$). This is similar to the results obtained from human liver samples. In human steatotic livers the triglyceride content is about 2.7-fold higher than that in non-steatosis livers [Gomez-Lechon et al., 2007].

Flow cytometric analysis of the Annexin V-FITC/PI-stained steatotic L-02 cells showed that FFA treatment did not significantly affect early or late phase apoptosis. Figure 1B shows the representative results of three independent experiments. In addition, cell viability assay showed that the FFA treatment did not produce detectable cytotoxicity determined by MTT assay (data not shown).

These data indicated that we have prepared steatotic cells by incubating L-02 cells with 1 mM FFA mixture (oleate/palmitate, 2:1) for 24 h, in which lipid accumulation reached a level similar to that found in human steatotic livers in the absence of apoptosis.

PROTEOMIC ANALYSIS OF DIFFERENTIAL PROTEIN EXPRESSION IN THE NEW L-02 MODEL

We used a proteomic approach to identify proteins involved in the development of hepatosteatosis in the steatotic cells. L-02 cells were treated with 1 mM FFA (oleate/palmitate at 2:1 ratio) for 24 h and 2-DE based proteomic studies were then performed. To ascertain reproducibility each treatment was repeated three times and each protein sample was analyzed by 2-DE three times. Figure 2A shows representative gel images. Proteins within the range of 15–225 kDa with isoelectric point (PI) between 3 and 10 were well separated. Over 800 spots were detected on each gel. Protein spots with changes of more than 2-fold in terms of volume intensity were cut from the gels, trypsin digested and analyzed by MALDI-TOF-MS/MS. Among the 14 identified proteins, three up-regulated ones related to lipid metabolism were found and are listed in Table II. They were protein disulfide-isomerase A3 precursor (ERp57), heat shock protein 90 (Hsp90), and fatty acid synthase (FAS). The MALDI-TOF MS peptide mass spectra of the three proteins are shown in the Supplementary Material online.

For the three identified proteins we also performed quantitative real-time PCR analysis. Results showed that the mRNA levels of ERp57, Hsp90 and FAS were 1.8-, 2.1-, and 4-fold higher in steatotic cells than in control L-02 cells, respectively (Fig. 2B). The expression increases of ERp57 and Hsp90 at mRNA levels were comparable to those found at protein levels; whereas the up-regulation of FAS at mRNA level was higher than that at the protein level. This observation suggests that there is a differential influence of FFA treatment on the mRNA and protein expressions of FAS. FFA treatment might have direct or indirect effects on post-transcriptional stages of FAS protein synthesis.

It has been well documented that FAS plays a central role in de novo lipogenesis in mammals [Wakil et al., 1983]. FAS is a key lipogenic enzyme catalyzing the terminal steps in the de novo biogenesis of fatty acids [Menendez and Lupu, 2007]. In NAFLD patients, expression of FAS is up-regulated [Nakamuta et al., 2005; Kohjima et al., 2007]. Thus the up-regulation of FAS in the FFA-treated L-02 cells is expected. In Figure 2A, it is obvious that ERp57 was up-regulated more than Hsp90 by FFA treatment. Therefore, we further characterized ERp57 for its role in steatosis development.

ERP57 SIRNA REDUCED LIPID ACCUMULATION IN FFA-TREATED L-02 CELLS

We next explored the possible involvement of ERp57 in the FFA-induced lipid accumulation in L-02 cells by using RNA interference strategy. Transient transfection of an ERp57-specific siRNA into FFA-treated L-02 cells reduced ERp57 protein expression by about 80% (Fig. 3A) and there was a concurrent 25.9% reduction in lipid accumulation. Control siRNA had no significant effect under the same experimental conditions (Fig. 3B–D).

Transfection of ERp57 siRNA also significantly reduced cellular triglyceride contents in the steatotic cells (0.622 ± 0.114 μg/μg protein in siRNA and FFA-treated cells vs. 0.892 ± 0.099 μg/μg protein in FFA-treated cells, $n = 3$, $P < 0.05$); at the same time, control siRNA had no significant effect on triglyceride content in the steatotic cells (0.891 ± 0.078, $n = 3$).

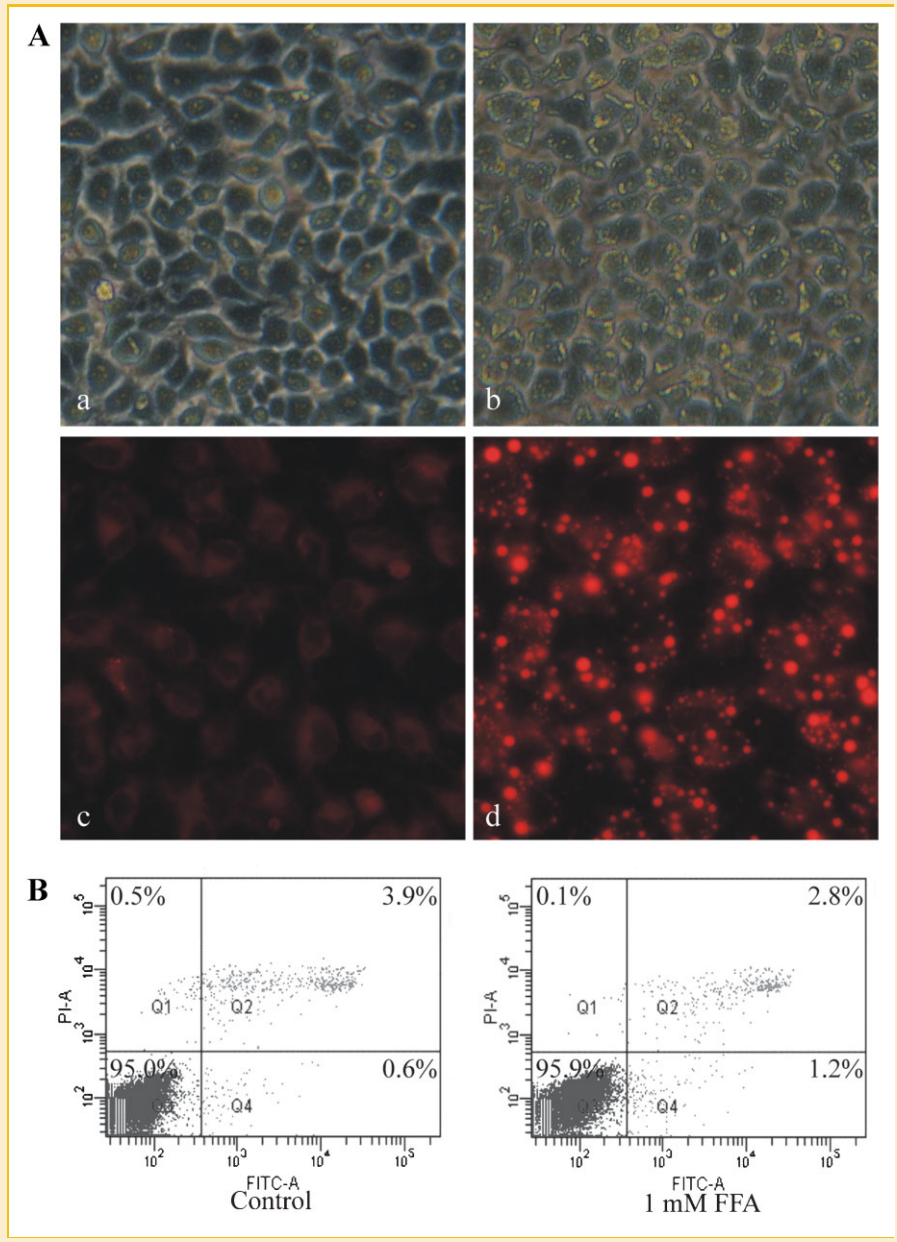

Fig. 1. FFA treatment in L-02 cells induced lipid accumulation in the absence of apoptosis. Cells were treated with 1 mM FFA mixture (oleate/palmitate, 2:1) for 24 h. A: Representative micrographs showing intracellular lipid accumulation observed by phase-contrast microscopy (a and b, 200×) and fluorescence microscopy (c and d, 400×) after Nile Red staining. B: Apoptosis monitored by flow cytometry after Annexin V-FITC/PI staining. Normal, early apoptotic, late apoptotic, necrotic cells were presented in Q3, Q4, Q2, and Q1 quadrant, respectively. The percentage of cells in each quadrant is indicated. Results are the representative of three independent experiments.

INCREASED HEPATIC ERP57 EXPRESSION IN NAFLD PATIENTS

To verify the role of ERp57 in liver steatosis, ERp57 protein expression in liver biopsies of NAFLD patients was determined by immunohistochemistry. Figure 4A shows the staining intensity of the spermatogenic cell cytoplasm (3+) in the testis section which served as a positive control. In patient samples ERp57 expression was mainly detected in cytoplasm of hepatocytes with steatotic changes or ballooning degeneration, although some unaltered hepatocytes might have been positive, depending on the disease activity (Fig. 4B–D). The detailed results are tabulated in Table III. To summarize, occasional expression was noted in steatotic hepatocytes in seven cases (five 1+, two 2+). Frequent expression was noted in seven cases (two 1+, five 2+). Diffuse positivity was noted in two cases with 3+ intensity, both with grade 3 inflammation and stage 4 fibrosis (i.e., cirrhosis). Only one case was negative. In the five normal liver control samples only one sample showed positive staining (1+ to 2+) in endothelial cells and Kupffer cells. Statistical analyses demonstrated that ERp57-expression extent and intensity correlated with the grade of inflammation and the stage of fibrosis, respectively, although did not significantly correlate with steatosis degree (Table IV). These findings suggest for the first time that increased expression of ERp57 is associated with human steatohepatitis.

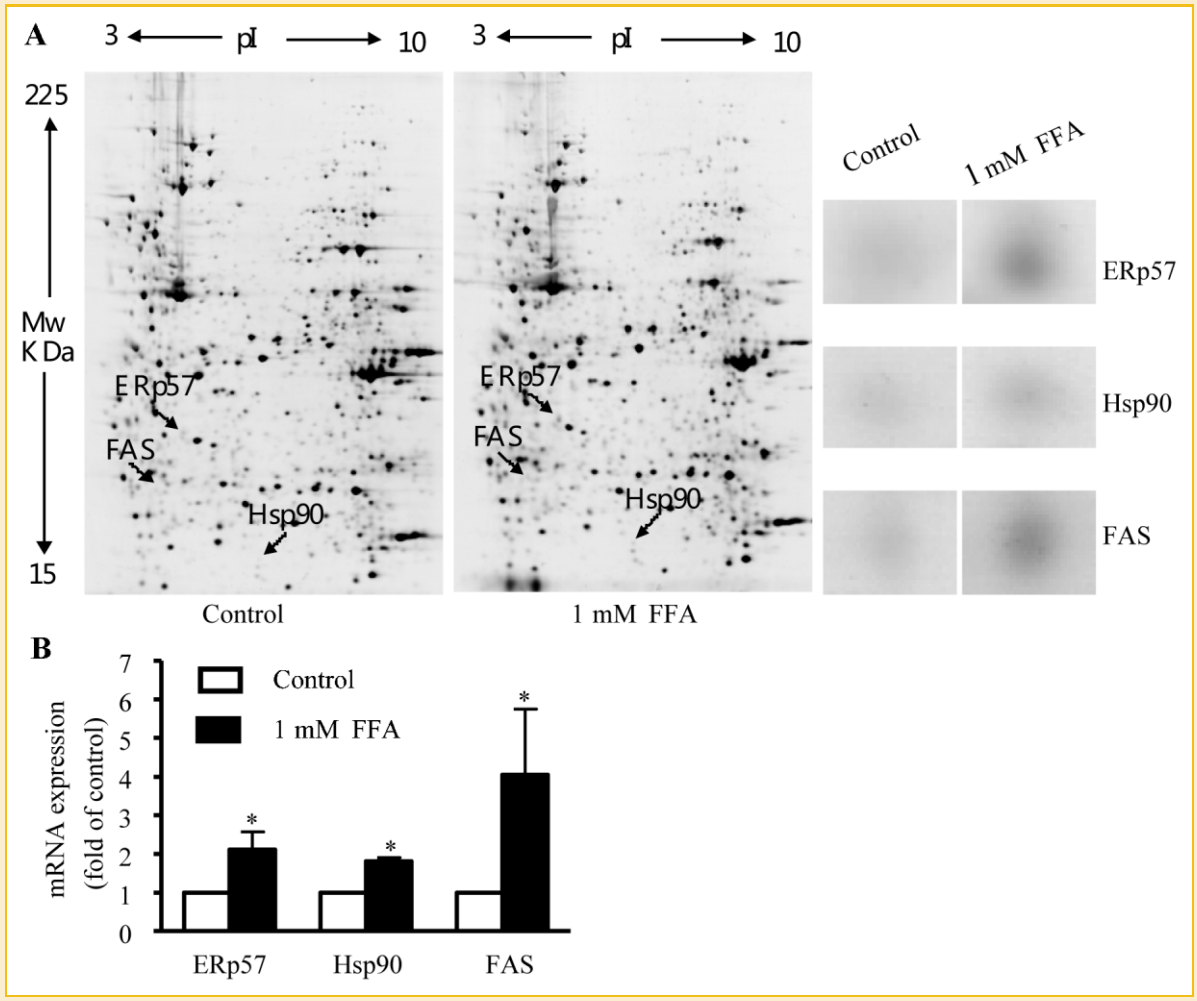

Fig. 2. Proteomic identification of differentially expressed proteins in FFA-treated L-02 cells. A: Representative silver staining images of 2-DE gels of proteins in cells treated with 1 mM FFA mixture (oleate/palmitate, 2:1) for 24 h. Three of the identified proteins are indicated on the gels and their close-up spot images are shown on the right panel. B: Real-time PCR determination of the mRNA expression levels of the three identified proteins. Data are expressed as the mean ± SEM from three independent experiments. $^{*}P < 0.05$ versus controls.

DISCUSSION

NAFLD subjects have increased lipolysis and high levels of circulating FFA [Marra et al., 2008]. The persistence of high circulating levels of FFA precipitates a series of changes in hepatic FFA metabolism that ultimately leads to hepatic steatosis [Parekh and Anania, 2007]. Therefore FFA loading has been used to induce steatosis in in vitro cell models. These models reproduce the key features of hepatic steatosis in humans [Feldstein et al., 2004; Malhi et al., 2006; Wu et al., 2008]. The human primary hepatocyte model is the closest to the human liver, but impractical for laboratory experimentation. The hepatocarcinoma HepG2 model has become popular and has been used in numerous studies [Gomez-Lechon et al., 2007]; however, the validity of cancer cell-based models

TABLE II. Lipid Metabolism Related Proteins Identified by MALDI-TOF-MS/MS

Protein name	Average normalized volumes		Fold[a,b]	*P* value[a]	Theoretical MW (Da)	Theoretical PI value	MS/MS score	Sequence coverage (%)
	Control	Treatment						
Hsp90	752122.514	1579457.279	2.1	0.022	83,554	4.97	348	8
ERp57	8386977.756	18451351.063	2.2	0.017	57,146	5.98	130	5
FAS	5956252.440	13699380.612	2.3	0.034	275,850	5.99	128	4

[a]From analyses of triplicate gels.
[b]The fold is the protein expression level in 1 mM FFA-treated cells over that in control cells.

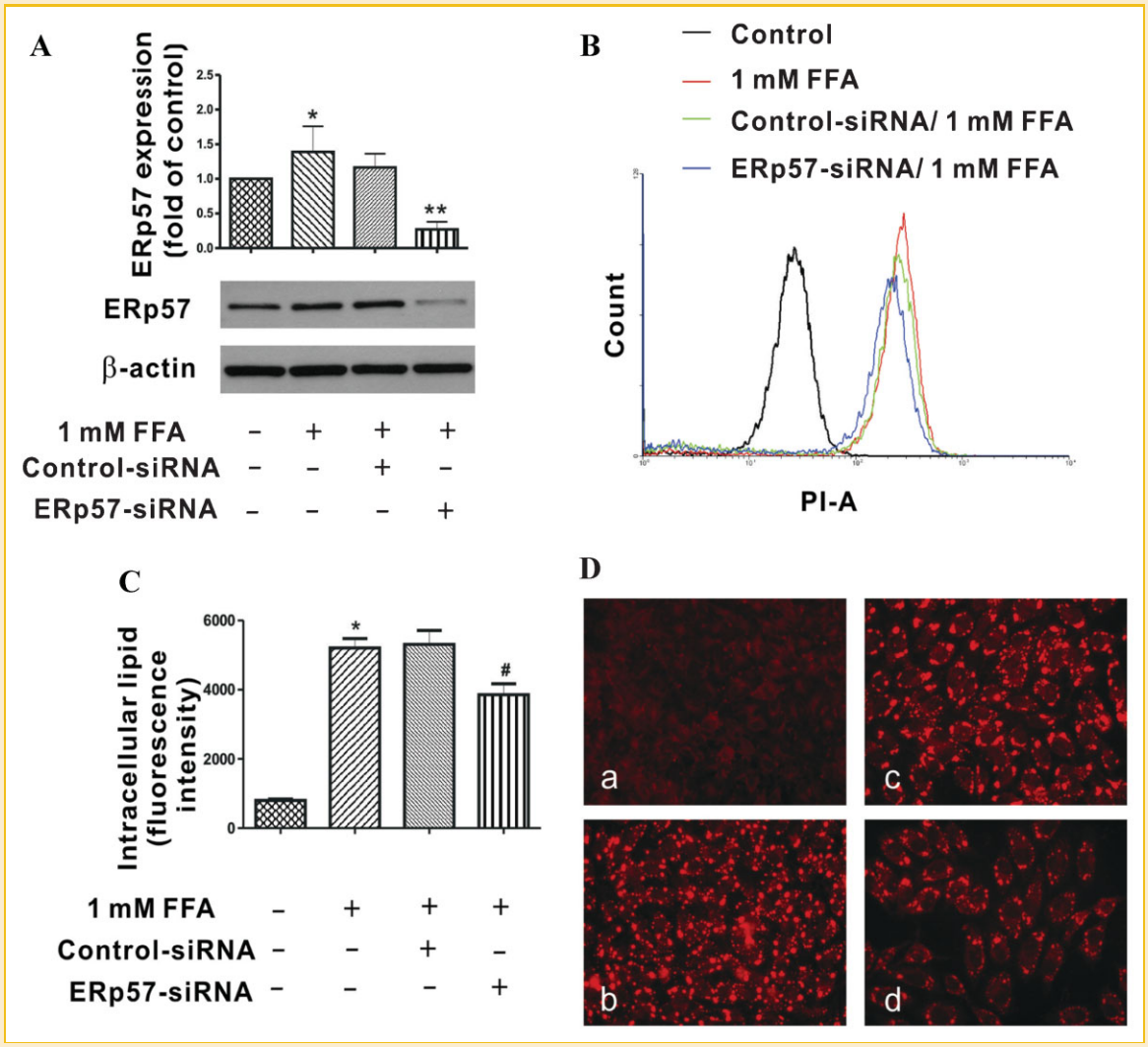

Fig. 3. Effects of ERp57 knockdown on lipid accumulation in FFA-treated L-02 cells. A: Western blot analysis of ERp57 protein expression. The upper panel shows the relative expression levels quantified by densitometry analysis of bands and normalized to β-actin protein. Data are expressed as the mean ± SEM of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ versus the control. The lower panel shows a representative Western blotting result from three independent experiments. B: Flow cytometric analysis of L-02 cell lipid content after indicated treatments and Nile Red staining. The flow cytometry diagram is a representative of three independent experiments. C: Effects of ERp57 knockdown on FFA-induced lipid accumulation. Data are expressed as the mean ± SEM of three independent experiments. $^{*}P < 0.01$ versus the control, $^{\#}P < 0.05$ versus control siRNA treatment. D: Representative micrographs of intracellular lipid accumulation in Nile Red stained L-02 cells examined by fluorescent microscopy (400×). (a) Control; (b) 1 mM FFA; (c) control-siRNA; (d) ERp57-siRNA.

has been of some concern since energy metabolism is often altered in cancer cells. For example cancer cells may carry out increased de novo fatty acid synthesis irrespective of extracellular lipid levels [Kuhajda, 2000; Menendez and Lupu, 2007]. In addition cancer cells including HepG2 cells express high levels of α-fetoprotein which is involved in lipid metabolism [Kumbla et al., 1991; Mizejewski, 2004; Shen et al., 2008]. Thus in the present study we first prepared steatotic cells using the L-02 cell line, which is derived from normal human hepatocytes. The L-02 steatotic cells behaved similarly to the human steatotic liver in two regards. Firstly, after 24-h treatment, the 1 mM FFA mixture (oleate/palmitate at 2:1 ratio) did not cause detectable cytotoxicity. Secondly, under these conditions the content of induced fat accumulation in L-02 cells was similar to that in human steatotic livers.

Using the 2-DE based proteomic technique to investigate L-02 steatotic cells we discovered three proteins that may be involved in the development of hepatic steatosis. They are lipid metabolism-related proteins, FAS, Hsp90 and ERp57, which were up-regulated in the FFA-induced steatotic cells. Real-time PCR data showed that mRNA expression levels of genes coding for the three proteins were also up-regulated. Alterations in protein and mRNA levels for Hsp90 and ERp57 were comparable, while FAS mRNA up-regulation was higher than its protein up-regulation. At the present time there is not sufficient data to clearly explain the mechanism of the differential expression of FAS mRNA and its protein. Among the three proteins, the role of FAS in the development of NAFLD has been well described, and the Hsp90 up-regulation level is lower than that of ERp57 in steatotic L-02 cells, so we sought to further characterize the role ERp57 in NAFLD.

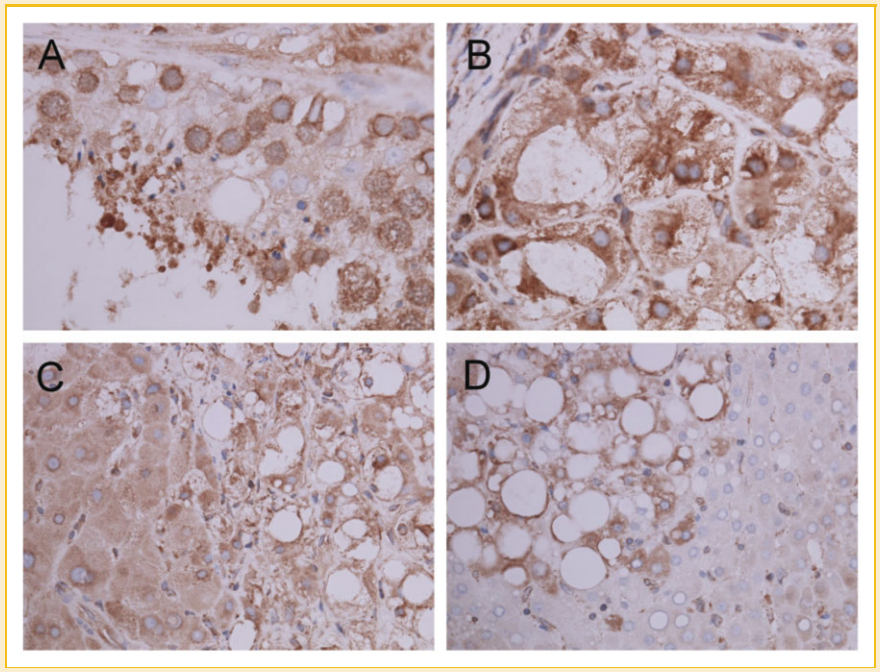

Fig. 4. Immunohistochemistical assessment of ERp57 in human liver biopsy samples. A: Intense staining (3+) in spermatogenic cells (but not Sertoli cells) of testis served as positive and matching control (600×). B: Strong expression (3+) in ballooned hepatocytes in steatohepatitis of G3S4 (600×). C: Moderate expression (2+) in ballooned and steatotic hepatocytes in G1S4 steatohepatitis, while background normal looking hepatocytes showed weak staining (400×). D: Moderate expression (2+) in a cluster of steatotic hepatocytes in G1S2 steatohepatitis, while normal hepatocytes were negative (400×).

ERp57 has been implicated in various diseases including cancer, prion diseases, Alzheimer's disease and liver injury [Khanal and Nemere, 2007]. Here we observed that knockdown of ERp57 with siRNA partially but significantly blunted FFA-induced lipid accumulation in L-02 cells, suggesting that ERp57 may be involved in FFA-induced hepatosteatosis. There are a number of plausible explanations for this observation. ERp57 has been reported to proteolytically degrade PDI [Urade et al., 1992, 1997] and apoB100 [Qiu et al., 2004] proteins. PDI [Urade et al., 1992, 1997] is an essential subunit of microsomal triglyceride transfer protein (MTP) heterodimer. MTP is involved in the transfer of triglyceride to apoB100 for the synthesis of very low density lipoprotein (VLDL).

TABLE III. Immunohistochemical Analysis of ERp57 Protein Expression in Livers of NAFLD Patients

Liver pathology	ERp57 expression	Liver pathology	ERp57 expression
ST2 G0 S0	Negative	ST2 G1 S2	Frequent, 1+
ST2 G0 S0	Occasional, 1+	ST2 G1 S1	Frequent, 2+
ST2 G1 S1	Occasional, 1+	ST1 G1 S4	Frequent, 2+
ST2 G1 S1	Occasional, 1+	ST2 G1 S4	Frequent, 2+
ST1 G1 S4	Occasional, 1+	ST2 G2 S3	Frequent, 2+
ST1 G1 S4	Occasional, 1+	ST3 G2 S3	Frequent, 2+
ST2 G1 S2	Occasional, 2+	ST1 G3 S4	Diffuse, 3+
ST2 G2 S1	Occasional, 2+	ST3 G3 S4	Diffuse, 3+
ST2 G0 S1	Frequent, 1+		

ST, steatosis; G, grade of inflammation; S, stage of fibrosis.
Occasional, frequent, and diffuse refer to stained hepatocytes at <25%, 25–75%, and >75% of steatotic or ballooned hepatocytes, respectively.

TABLE IV. Correlation Between ERp57 Expression and Steatosis, Inflammation, or Fibrosis in Livers of NAFLD Patients

	Steatosis		Inflammation		Fibrosis	
ERp57expression	r	*P*	r	*P*	r	*P*
Extent	0.154	0.554	0.557	0.020*	0.534	0.027*
Intensity	0.154	0.554	0.799	0.000*	0.534	0.027*

Data in Table III were analyzed by Spearman's rank-correlation test using SPSS 16.0 program. The extent of ERp57 expression was classified as 0, 1, 2, and 3 for negative, occasional, frequent, and diffuse, respectively; and the intensity of ERp57 expression was classified as 0, 1, 2, and 3 for negative, 1+, 2+, and 3+, respectively. n = 17.
*Significant *P* value.

Inhibiting MTP expression impedes the export of triglycerides as part of lipoproteins and consequently facilitates hepatic steatosis [Burnett and Watts, 2007]. Overexpression of ERp57 in cultured hepatocytes promotes intracellular apoB100 degradation which also impairs VLDL synthesis and triglyceride clearance [Qiu et al., 2004].

In human biopsies, it was found that ERp57 was expressed in 16 out of 17 liver samples from NAFLD patients, but was not expressed in hepatocytes of five normal liver samples. Although ERp57 has been reported to be abnormally expressed in animal models of hepatic steatosis [Banerjee et al., 2008; Rutkowski et al., 2008], its up-regulation in liver biopsies of NAFLD patients is first described in this report.

To our best understanding, this is the first effort to identify proteins involved in NAFLD development using the proteomic approach in steatotic cells prepared using a normal human hepatocyte-derived cell line. ERp57 was up-regulated in steatotic L-02 cells and NAFLD patient livers. Knockdown of ERp57 expression with siRNA significantly blunted FFA-induced fat accumulation in L-02 cells. ERp57 was expressed in liver biopsies of NAFLD patients, and its expression level significantly correlated with inflammation grades and fibrosis stages. These data suggest that further investigation is merited to determine whether ERp57 is involved in the development of NAFLD.

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