Role of X-Box Binding Protein-1 in Fructose-Induced *De Novo* Lipogenesis in HepG2 Cells

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Abstract

Background: A high consumption of fructose leads to hepatic steatosis. About 20–30% of triglycerides are synthesized via *de novo* lipogenesis. Some studies showed that endoplasmic reticulum stress (ERS) is involved in this process, while others showed that a lipotoxic environment directly influences ER homeostasis. Here, our aim was to investigate the causal relationship between ERS and fatty acid synthesis and the effect of X-box binding protein-1 (XBP-1), one marker of ERS, on hepatic lipid accumulation stimulated by high fructose.
Methods: HepG2 cells were incubated with different concentrations of fructose. Upstream regulators of *de novo* lipogenesis (i.e., carbohydrate response element-binding protein [*ChREBP*] and sterol regulatory element-binding protein 1c [*SREBP-1c*]) were measured by polymerase chain reaction and key lipogenic enzymes (acetyl-CoA carboxylase [ACC], fatty acid synthase [FAS], and stearoyl-CoA desaturase-1 [SCD-1]) by Western blotting. The same lipogenesis-associated factors were then evaluated after exposure of HepG2 cells to high fructose followed by the ERS inhibitor tauroursodeoxycholic acid (TUDCA) or the ERS inducer thapsigargin. Finally, the same lipogenesis-associated factors were evaluated in HepG2 cells after XBP-1 upregulation or downregulation through cell transfection.
Results: Exposure to high fructose increased triglyceride levels in a dose- and time-dependent manner and significantly increased mRNA levels of *SREBP-1c* and *ChREBP* and protein levels of FAS, ACC, and SCD-1, concomitant with XBP-1 conversion to an active spliced form. Lipogenesis-associated factors induced by high fructose were inhibited by TUDCA and induced by thapsigargin. Triglyceride level in XBP-1-deficient group decreased significantly compared with high-fructose group (4.41 ± 0.54 μmol/g vs. 6.52 ± 0.38 μmol/g, $P < 0.001$), as mRNA expressions of *SREBP-1c* (2.92 ± 0.46 vs. 5.08 ± 0.41, $P < 0.01$) and protein levels of FAS (0.53 ± 0.06 vs. 0.85 ± 0.05, $P = 0.01$), SCD-1 (0.65 ± 0.06 vs. 0.90 ± 0.04, $P = 0.04$), and ACC (0.38 ± 0.03 vs. 0.95 ± 0.06, $P < 0.01$) decreased. Conversely, levels of triglyceride (4.22 ± 0.54 μmol/g vs. 2.41 ± 0.35 μmol/g, $P < 0.001$), mRNA expression of *SREBP-1c* (2.70 ± 0.33 vs. 1.00 ± 0.00, $P < 0.01$), and protein expression of SCD-1 (0.93 ± 0.06 vs. 0.26 ± 0.05, $P < 0.01$), ACC (0.98 ± 0.09 vs. 0.43 ± 0.03, $P < 0.01$), and FAS (0.90 ± 0.33 vs. 0.71 ± 0.02, $P = 0.04$) in XBP-1s-upregulated group increased compared with the untransfected group.
Conclusions: ERS is associated with *de novo* lipogenesis, and XBP-1 partially mediates high-fructose-induced lipid accumulation in HepG2 cells through augmentation of *de novo* lipogenesis.

Key words: Endoplasmic Reticulum Stress; Fatty Liver; Lipogenesis; X-Box Binding Protein-1

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a common liver disease that has become a major health concern worldwide.[1] NAFLD, characterized by excessive lipid accumulation in the liver, is a hepatic manifestation of metabolic syndrome and includes liver disorders ranging from simple steatosis to nonalcoholic steatohepatitis with liver dysfunction.[2] Increased consumption of high-fructose foods, such as ice

Access this article online	
Quick Response Code:	**Website:** www.cmj.org
	DOI: 10.4103/0366-6999.241799

cream, candy, and bread, is an important pathogenic factor for hepatic steatosis.[3,4] High-fructose flux leads to enhanced hepatic triglyceride (TG) accumulation, which plays a central role in the emergence of NAFLD.[5-8] Nevertheless, the pathophysiology underlying this process is poorly understood. Some studies have shown that endoplasmic reticulum stress (ERS) signaling pathways contribute to the development of insulin resistance and hepatic steatosis in NAFLD.[9-12] X-box binding protein-1 (XBP-1) is a major transcription regulator of the unfolded protein response (UPR), mediating adaptation to ERS.[13] Here, we hypothesized that XBP-1 plays a role in the process of fructose-stimulated lipogenesis in HepG2 cells.

Hepatic steatosis has been associated with fructose, a food additive, because excessive consumption of fructose can increase hepatic lipid accumulation.[14] Fatty acids come from three sources: (1) lipolysis of TGs stored in adipose tissue, (2) uptake of dietary fatty acids from the intestinal tract, and (3) de novo lipogenesis (newly synthesized from glucose) in the liver, the last of which accounts for about 20–30% of all fatty acids in hepatocytes.[15] Previous studies in rodents have shown that fructose can facilitate de novo lipogenesis.[16-18] Feeding rats with fructose increased hepatic levels of upstream regulators of de novo lipogenesis (i.e., sterol regulatory element-binding protein 1c [SREBP-1c] and carbohydrate response element-binding protein [ChREBP]) and key lipogenic enzymes (i.e., fatty acid synthase [FAS], acetyl-CoA carboxylase [ACC], and stearoyl-CoA desaturase-1 [SCD-1]). To verify these findings at a cellular level, in the present study, we incubated HepG2 cells with fructose or palmitic acid (PA) to investigate their effects on different sources of accumulated lipid.

ERS refers to an accumulation of unfolded or misfolded proteins in the endoplasmic reticulum (ER) lumen under conditions of impaired function, leading to an adaptive signaling response originating in the ER, namely the UPR.[13,19] Three pathways are involved in ERS: (1) the inositol-requiring enzyme-1 (IRE-1)/XBP-1 pathway, (2) the protein kinase R-like ER kinase (PERK)/eukaryotic translation initiation factor-2α (eIF-2α) pathway, and (3) the activating transcription factor-6 pathway.[13] Recent studies have demonstrated that ERS is associated with the initiation and progression of many diseases, such as metabolic dyslipidemia, insulin resistance, NAFLD, cardiovascular disease, and neurodegenerative disease.[20] Some studies have reported that misfolded proteins alter ER homeostasis, creating a lipotoxic environment within hepatocytes,[21] but other studies have demonstrated that the lipotoxic environment of NAFLD directly influences ER homeostasis and ERS activation.[22-24] Thus, a link between ERS and lipid accumulation has been proposed, but the exact nature of the causal relationship between ERS and fatty acid synthesis remains unknown. In this study, we identified the factors associated with de novo lipogenesis after culturing HepG2 cells with: (1) high fructose, (2) high fructose

followed by the ERS inhibitor tauroursodeoxycholic acid (TUDCA), or (3) the ERS inducer thapsigargin.

XBP-1, also known as cAMP-response element-binding protein, belongs to a family of basic leucine zipper-containing proteins and can be found in two forms: unspliced XBP-1 (XBP-1u) and spliced XBP-1 (XBP-1s). XBP-1 is normally kept in its inactive form, but under ERS, the endoRNase domain of IRE-1 splices the mRNA of downstream sensor XBP-1, removing a 26-bp segment from the full-length XBP1 mRNA that generates a translational frameshift, leading to the expression of the active protein XBP-1s.[25-27] XBP-1s binds to intranuclear mRNA directly to regulate protein transcription, thereby affecting subsequent physiological activities.[28,29] Lee et al.'s study[30] showed for the first time that XBP-1 regulates hepatic lipid metabolism, because XBP1-knockout mice manifested hypotriglyceridemia and hypocholesterolemia. On the basis of these observations, we propose that XBP-1 serves as a key conduit for ERS-induced hepatic lipid accumulation and steatosis in response to a high-fructose stimulus. Thus, in the present study, we investigated the expression of the transcription factor XBP-1 in the human HepG2 cell line after stimulation of cells with high fructose, and the regulatory effects of XBP-1 on de novo lipogenesis in the initial stages of NAFLD by evaluating the expression of key enzymes involved in lipogenesis.

METHODS

Reagents and chemicals

Reagents: rabbit anti-SCD-1, anti-ACC, anti-IRE-1, anti-phosphorylated (p-) IRE-1, and anti-XBP-1s antibodies (Cell Signaling Technology, Beverly, MA, USA); thapsigargin (Abcam, Cambridge, UK); mouse anti-β-actin antibody (SAB Bioengineering Institute, College Park, Maryland, USA); anti-FAS antibody, goat anti-mouse secondary antibody, XBP-1 short hairpin (sh) RNA plasmid (human, sc-38627-SH) and control shRNA plasmid-A (sc-108060; Santa Cruz Biotechnology, Santa Cruz, CA, USA); and PA and fructose (Sigma Chemical, St. Louis, MO, USA). TG levels were determined using a commercially available kit (Pulilai Bioengineering Institute, Changchun, China). The ERS inhibitor TUDCA was obtained from Sichuan Hengtai Biotechnology (Sichuan, China). The plasmids pcDNA 3.1-XBP-1u and pcDNA 3.1-XBP-1s were gifts from Dr. Hao Jun (Hebei Medical University, Shijiazhuang, Hebei, China). HepG2 cells were from Bumrungrad Biomedical Technology (HUCL-0085; Jiangyin, Jiangsu, China).

Cell treatment groups

HepG2 cells were prepared with different stimulations as follows:

1. To investigate the effects of high fructose on lipid accumulation induced by fructose, HepG2 cells were stimulated with 0, 1, 5, or 20 mmol/L fructose for 12, 24, 48, or 72 h.
2. To elucidate the underlying mechanisms, HepG2 cells

were treated with 20 mmol/L fructose or 0.25 mmol/L PA for 72 h.

3. To explore the causal relationships between ERS and lipogenesis, the ERS inhibitor TUDCA (0.2 mmol/L) was added after HepG2 cells were cultured with 20 mmol/L fructose for 24 h, and other HepG2 cells were cultured with the ERS inducer thapsigargin (600 nmol/L) for 10 h (without fructose pretreatment).

4. To investigate the immediate effects of XBP-1 on lipid accumulation and whether XBP-1 mediates high-fructose-induced lipid metabolism, XBP-1 expression was downregulated using cell transfection with an shRNA targeting XBP-1, and the active form XBP-1s was upregulated using cell transfection with vector pcDNA 3.1-XBP-1s.

After the different stimulations, HepG2 cells were harvested for TG measurement and Oil Red O staining. Metabolic factors involved in lipogenesis (i.e., FAS, SCD-1, and ACC) were detected using Western blotting analysis, and gene expression of the lipogenic pathway regulators *SREBP-1c* and *ChREBP* was evaluated using polymerase chain reaction (PCR).

Transient transfection

For cell transient transfection, Lipofectamine 2000 was used. Briefly, HepG2 cells were cultured in 6-well plates. XBP-1 plasmids or empty vectors transduced into HepG2 cells. Then, cells were transfected with 0.8 g vector DNA using 2 μl Lipofectamine 2000 in 2 ml serum-free Dulbecco's modified Eagle's medium (DMEM) per well. After 8 h, the medium was replaced with 10% fetal bovine serum DMEM. Cells were harvested 48 h after transfection and subsequently harvested for determination.

Determination of triglyceride levels in HepG2 cells

After the different treatments described above, HepG2 cells were collected to wash twice with phosphate-buffered saline (PBS) and then treated with radioimmunoprecipitation (RIPA) buffer for 30 min on ice. Samples were centrifuged at 13,000 ×g for 20 min at 4°C for getting the supernatant. The concentration of protein was measured using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). TG levels were measured based on an enzymatic assay from a method adapted for 96-well plates according to the manufacturer's instructions (Pulilai Bioengineering Institute).

Oil Red O staining

Cultured cells were fixed with 4% paraformaldehyde (in PBS) for 30 min and stained with 1% Oil Red O for 2 h. The stained sections were observed with an Olympus microscope and examined by a pathologist in a blinded manner.

Real-time polymerase chain reaction

Analyses on gene transcript levels were conducted by quantitative real-time polymerase chain reaction (PCR) method. After washing HepG2 cells twice with PBS, total mRNA was extracted using a standard TRIzol RNA isolation method[7] after stimulation as described above. The concentration and purity of total RNA were measured using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). RNA was reverse transcribed using the Easy Script First-Strand cDNA Synthesis Super Mix kit (TransGen Biotech, Beijing, China). Real-time PCR was performed on an ABI PRISM 7300 PCR System (Applied Biosystems, Foster City, CA, USA) using SYBR Green I GoTaq qPCR Master Mix (Promega, Madison, WI, USA). PCRs were performed in a total volume of 25 μl as follows: 95°C for 5 min, then 40 cycles of 95°C for 15 s, 58°C for 20 s, and 72°C for 30 s in the end. Each sample's gene expression was analyzed in duplicate and normalized against that of *GAPDH* used as an internal control. Results are expressed as relative gene expression, determined using the comparative threshold cycle (CT) method and normalized by *GAPDH* as an internal control.

The primers were: *GAPDH*: forward 5'-GGATGATGTTCTGGAGAGCC-3' and reverse 5'-CATCACCATCTTCCAGGAGC-3'; *SREBP1c*: forward 5'-CTTCCGCCCTTGAGCTG-3' and reverse 5'-CTGGTGTGTCCGTGTGG-3'; and *CHREBP*: forward 5'-TGCGGGATGAGATTGAGGA-3' and reverse 5'-TCCAGTTGTGCAGCGTAC-3'.

Western blotting analysis

Proteins were extracted from HepG2 cells which were cultured in 25 cm-bottom-surface bottle. Cells were washed by 4°C PBS for 2 times firstly. Then, cells were added with 1 ml RIPA and 10 μl PMSF. The cells were collected with a cell scraper blow gently, after which in a centrifuge tube. Up steps were operated on ice. Clumps of cells were placed in 4000 ×g centrifugation at 4°C for 30 min. Supernatant of 800 μl mixture was kept at −80°C. The concentration of protein was measured using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Proteins were mixed with sodium dodecyl sulfate (SDS)-loading buffer and boiled for 10 min. Then, protein samples were separated using 10% SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride membrane, and blocked with nonfat dry milk for 2 h at 37°C. Then, membranes were probed with antibodies (primary antibodies against SCD-1, ACC, IRE-1, p-IRE-1, XBP-1s, FAS, XBP-1u, PERK, p-eIF-2α, eIF-2α, and β-actin) overnight at 4°C. Subsequently, membranes were rinsed three times with TBST, after which they were incubated with secondary antibodies at room temperature for 2 h. After incubation, membranes were washed three times with TBST and followed by detection with electrochemiluminescence method. And, β-actin was used as an internal control.

Statistical analysis

All data are represented as the mean ± standard deviation (SD) and were analyzed. Two-tailed unpaired Student's *t*-tests were used for between-group comparisons. The analysis of variance multiple comparison test (SPSS 11.0, SPSS Inc., Chicago, IL, USA) followed by Tukey's *post*

hoc test was used for comparisons among groups. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Effects of fructose on *de novo* lipogenesis in HepG2 cells

The lipotoxic effects of fructose in hepatocytes were investigated by culturing HepG2 cells in the presence of normal medium (N group) or medium containing different concentrations of fructose (1, 5, or 20 mmol/L; F1, F5, and F20 groups, respectively) for different times (12, 24, 48, or 72 h). Fructose increased TG levels in HepG2 cells in a time- and concentration-dependent manner. TG levels reached maximal value after 72 h incubation with 20 mmol/L fructose; thus, 20 mmol/L fructose was used in subsequent experiments [Figure 1a]. Oil red O staining showed an obvious increase in lipid droplets in HepG2 cells cultured with 20 mmol/L fructose compared with those cultured with other fructose concentrations [Figure 1b]. PA increased lipid accumulation in the liver by providing substrate for
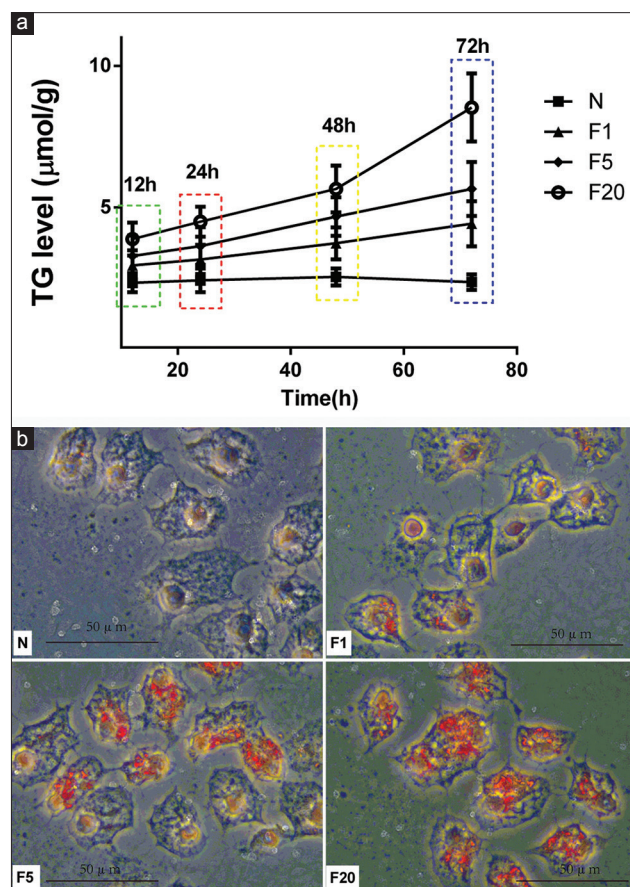
Figure 1: High fructose increased lipid accumulation in HepG2 cells. (a) TG was determined after incubated in different concentrations of fructose in different times. (b) Lipid droplets were increased in HepG2 cells cultured with 20 mmol/L fructose most obviously as shown by Oil red O staining after incubating 72 h. HepG2 cells were divided into four groups with different concentration of fructose, separately named as N (cultured with normal medium), F1 (cultured with 1 mmol/L fructose), F5 (cultured with 5 mmol/L fructose), and F20 (cultured with 20 mmol/L fructose). Scale bar = 50 μm. TG: Triglycerides.

TG synthesis. To explore the different mechanisms by which fructose induced lipid accumulation, HepG2 cells were cultured in the presence of 20 mmol/L fructose or 0.2 mmol/L PA. As expected, compared with HepG2 cells incubated with PA, high (20 mmol/L) fructose increased the mRNA expression of the two upstream transcription factors *SREBP1c* and *CHREBP* [Figure 2b, all $P < 0.01$] and the protein contents of the three downstream enzymes ACC, FAS, and SCD-1 [Figure 2c, all $P < 0.01$]. However, TG levels in HepG2 cells were not significantly different between groups [Figure 2a].

Effects of tauroursodeoxycholic acid and thapsigargin on lipogenesis

To determine the causal relationship between ERS and lipid metabolism, HepG2 cells were cultured with ordinary medium, 20 mmol/L fructose alone, 20 mmol/L fructose plus 0.2 mmol/L TUDCA, or 600 nmol/L thapsigargin alone. The fructose-induced increase in TG level and Oil Red O straining was ameliorated by TUDCA treatment of HepG2 cells ($P < 0.01$), whereas thapsigargin increased TG levels and Oil Red O straining [Figure 3a and 3c, $P < 0.01$]. Furthermore, TUDCA pretreatment decreased the fructose-induced increases in *SREBP1c*, *CHREBP* (all $P < 0.01$), and SCD-1, FAS, and ACC expression (all $P < 0.05$), whereas thapsigargin increased the expression of all factors [Figure 3b and 3d]. TUDCA pretreatment of HepG2 cells blocked fructose-induced phosphorylation of IRE-1, as well as activation of XBP-1 [Figure 3e, $P < 0.05$]. Compared with HepG2 cells incubated with normal medium, those incubated with thapsigargin showed an increase in this arm of the UPR [Figure 3e, $P < 0.05$].

Effects of silencing X-box binding protein-1 expression on lipid deposition in HepG2 cells

In this series of experiments, the level of the active form XBP-1 (XBP-1s) was decreased after transfecting cells with an shRNA targeting *XBP-1* [Figure 4a, $P < 0.01$]. Triglyceride level in XBP-1 shRNA group decreased significantly compared with high-fructose group [Figure 4b and 4c, 4.41 ± 0.54 μmol/g vs. 6.52 ± 0.38 μmol/g, $P < 0.001$]. Expression of the upstream lipogenic transcription factors *SREBP-1c* and *CHREBP* mRNA was measured by PCR. *SREBP-1c* was significantly downregulated in *XBP-1* shRNA group (2.92 ± 0.46 vs. 5.08 ± 0.41, $P < 0.01$), but *CHREBP* was not [Figure 4d]. The protein contents of the key downstream lipogenic enzymes FAS (0.53 ± 0.06 vs. 0.85 ± 0.05, $P = 0.01$), SCD-1 (0.65 ± 0.06 vs. 0.90 ± 0.04, $P = 0.04$), and ACC (0.38 ± 0.03 vs. 0.95 ± 0.06, $P < 0.01$) were significantly decreased in *XBP-1*-deficient HepG2 cells [Figure 4e].

Effects of spliced X-box binding protein-1 overexpression on lipid synthesis in HepG2 cells

To further investigate the causal relationship between XBP-1 and *de novo* lipogenesis, active XBP-1s was overexpressed by transfection with a *XBP-1s* vector. As shown by Western blotting analysis [Figure 5a], XBP-1s protein expression was induced after transfection for 8 h
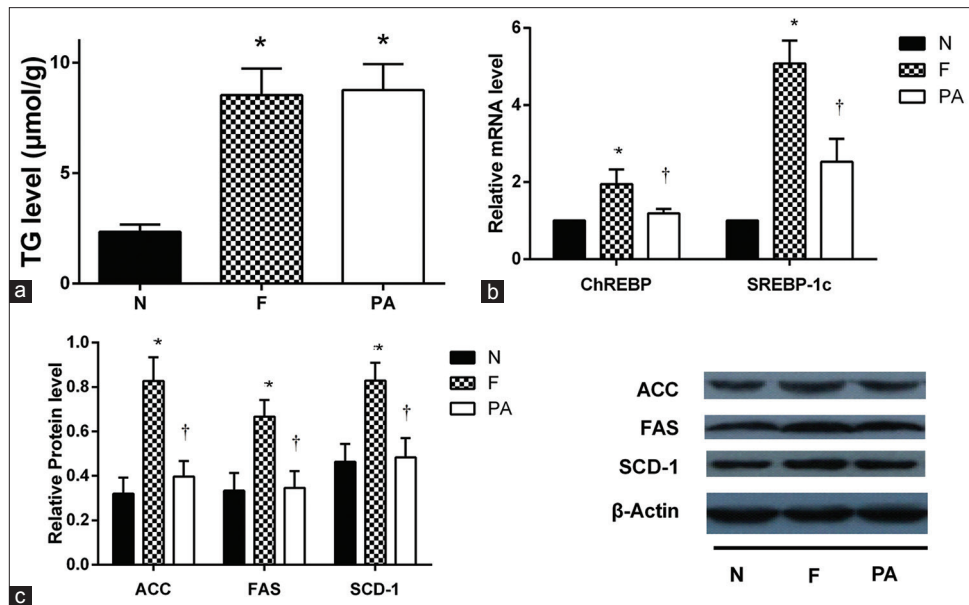
Figure 2: High fructose accelerated lipogenesis. (a) TG level in HepG2 cells of F and PA groups were significantly increased compared to N group. No change was observed between F and PA groups after incubation ($n = 3$), *$P < 0.05$ versus N group. (b) Levels of *SREBP-1c*, *ChREBP* mRNA were tested by PCR. Fold induction represents relative expression compared to that of control group. The mRNA levels of upstream transcriptional factors *SREBP-1c* and *ChREBP* in lipogenesis pathway were significantly increased in HepG2 cells in F group compared to N group, but they were lower in PA group compared to F group ($n = 6$), *$P < 0.05$ versus N group, †$P < 0.05$ versus F group. (c) Protein expression of critical enzymes (SCD-1, FAS, and ACC) in the lipogenic pathway in HepG2 cells were tested by Western blotting. β-actin expression is a loading control. Key enzymes of lipogenesis ACC, FAS, and SCD-1 protein levels significantly increased in HepG2 cells of F group compared to N group, and they were lower in PA group compared to F group ($n = 3$), *$P < 0.05$ versus N group, †$P < 0.05$ versus F group. Cells cultured with normal medium, 20 mmol/L fructose, or 0.2 mmol/L PA were assigned into N, F, or PA group respectively. Data are shown as mean ± standard deviation. TG: Triglycerides; *SREBP-1c*: Sterol regulatory element-binding protein 1c; *ChREBP*: Carbohydrate response element-binding protein; SCD-1: Stearoyl-CoA desaturase-1; FAS: Fatty acid synthase; ACC: Acetyl-CoA carboxylase; PCR: Polymerase chain reaction.

with vector pcDNA 3.1-XBP-1s, but not with vector cDNA 3.1-XBP-1u ($P < 0.01$). Cellular TG accumulation was higher in HepG2 cells overexpressing XBP-1s than in those cultured in ordinary medium or transfected with empty vector or vector pcDNA 3.1-XBP-1u [Figure 5b and 5c, 4.22 ± 0.54 μmol/g vs. 2.41 ± 0.35 μmol/g, $P < 0.001$]. Quantitative real-time PCR analysis showed a significant increase in *SREBP1c* mRNA expression in HepG2 cells transfected with XBP-1s upregulation vector (2.70 ± 0.33 vs. 1.00 ± 0.00, $P < 0.01$), but *CHREBP* mRNA showed no change in expression [Figure 5d]. XBP-1s upregulation also significantly increased SCD-1 (0.93 ± 0.06 vs. 0.26 ± 0.05, $P < 0.01$), ACC (0.98 ± 0.09 vs. 0.43 ± 0.03, $P < 0.01$), and FAS (0.90 ± 0.33 vs. 0.71 ± 0.02, $P = 0.04$) protein content compared with the untransfected group [Figure 5e].

DISCUSSION

Chronic fructose consumption is considered as a contributing factor in the development of NAFLD.[31] There is now a great interest in the mechanism by which high fructose induces fatty liver. Acquired and accumulated excessive TGs in hepatocytes may be considered as the first step of the "two hits" in the progression of NAFLD.[32] Although considerable progress has been made in understanding the molecular mechanisms underlying hepatic steatosis,[33] satisfactory treatment modalities remain limited. The liver is the predominant organ in the body delegated to *de novo*

lipogenesis. This process, in which fatty acids are newly synthesized from acetyl coenzyme A (converted from glucose through the tricarboxylic acid cycle), usually contributes about one-third of all TG in hepatocytes, but this contribution increases by approximately 6-fold in the abnormal state.[34] Two key transcription factors, *SREBP-1c* and *ChREBP*, and three key target enzymes, ACC, FAS, and SCD-1, participate in the lipogenesis process. In the present study, by determining TG levels and by histologic analysis, we demonstrated that fructose increased lipid accumulation in HepG2 cells in a dose-dependent manner. This study also demonstrated that factors associated with *de novo* TG synthesis (i.e., ACC, FAS, SCD-1, SREBP-1c, and ChREBP) were increased in HepG2 cells incubated with high fructose (20 mmol/L), compared with those incubated with PA (a saturated free fatty acid). Consequently, the findings suggest that chronic intake of fructose induces lipid accumulation through lipogenesis.

The UPR is an adaptive signaling pathway triggered in response to perturbations in ER homeostasis, conditions referred to as ERS.[13,19] Recent animal and human studies have revealed on the one hand that obesity and fatty liver are associated with ERS-associated factors[21,35,36] and that these diseases can be attenuated by chemical chaperone 4-phenylbutyric acid, *Schisandra chinensis* extract, or gastric bypass surgery.[21,37,38] On the other hand, lipotoxic stress leads to ERS activation in the liver,[4] so the causal relationship
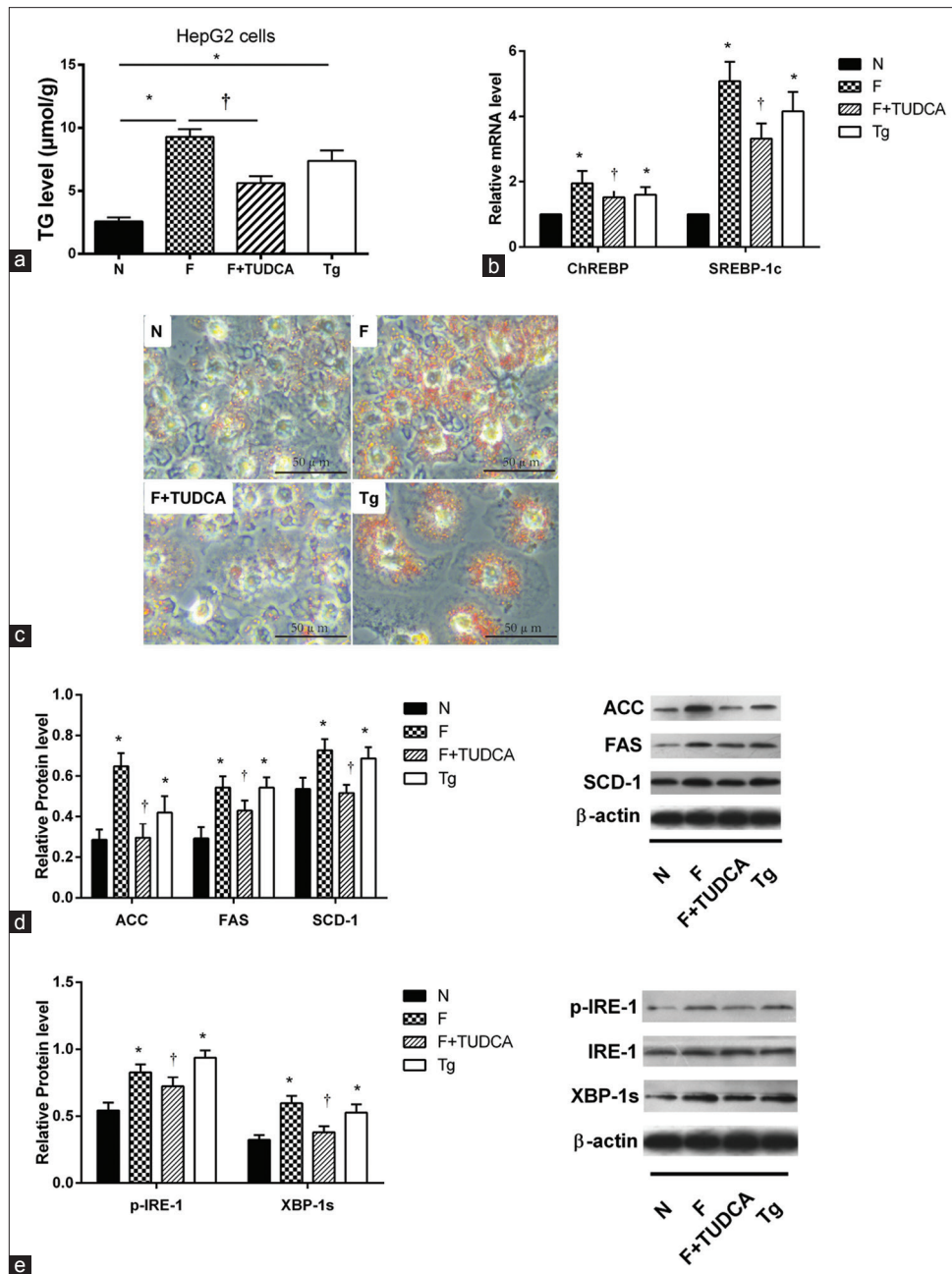
Figure 3: The effect of selective ERS inhibitor-TUDCA and inducer-Tg on IRE/XBP-1 pathway and lipogenesis. (a) TG level was significantly lower in TUDCA group than F group but increased in Tg group compared to N group ($n = 3$), *$P < 0.05$ versus N group, †$P < 0.05$ versus F group. (b) *SREBP-1c* and *ChREBP* were significantly lower in F+ TUDCA group compared to F group and were significantly increased in Tg group compared to N group ($n = 6$), *$P < 0.05$ versus N group, †$P < 0.05$ versus F group. (c) Oil Red O straining was alleviated in F+ TUDCA group compared to F group and was sharpened in Tg group compared to N group. Scale bar = 50 μm. (d) Protein levels of three key enzymes (SCD-1, FAS, and ACC) were upregulated in F group compared to N group but were suppressed by TUDCA. They were significantly increased in Tg group compared to N group ($n = 3$), *$P < 0.05$ versus N group, †$P < 0.05$ versus F group. (e) Protein expression of p-IRE-1/IRE-1 and XBP-1s were significantly increased in HepG2 cells in F group compared to N group. They were lower in F+ TUDCA group compared to F group, but increased in Tg group ($n = 3$), *$P < 0.05$ versus N group, †$P < 0.05$ versus F group. Cells cultured with normal medium, 20 mmol/L fructose, 20 mmol/L fructose plus 0.2 mmol/L TUDCA, or 600 nmol/L thapsigargin were assigned into N, F, F+TUDCA, or Tg group respectively. Data are shown as mean ± standard deviation. TUDCA: Tauroursodeoxycholic acid; Tg: Thapsigargin; TG: Triglycerides; *SREBP-1c*: Sterol regulatory element-binding protein 1c; *ChREBP*: Carbohydrate response element-binding protein; SCD-1: Stearoyl-CoA desaturase-1; FAS: Fatty acid synthase; ACC: Acetyl-CoA carboxylase.

between ERS and lipogenesis remains to be clarified. Whereas these previous studies were based on human and animal experiments, the present study examined the role of ERS in

lipogenesis at a cellular level. To investigate the role of ERS in fructose-induced lipogenesis, we first tested whether the putative chemical chaperone TUDCA (an ERS inhibitor)
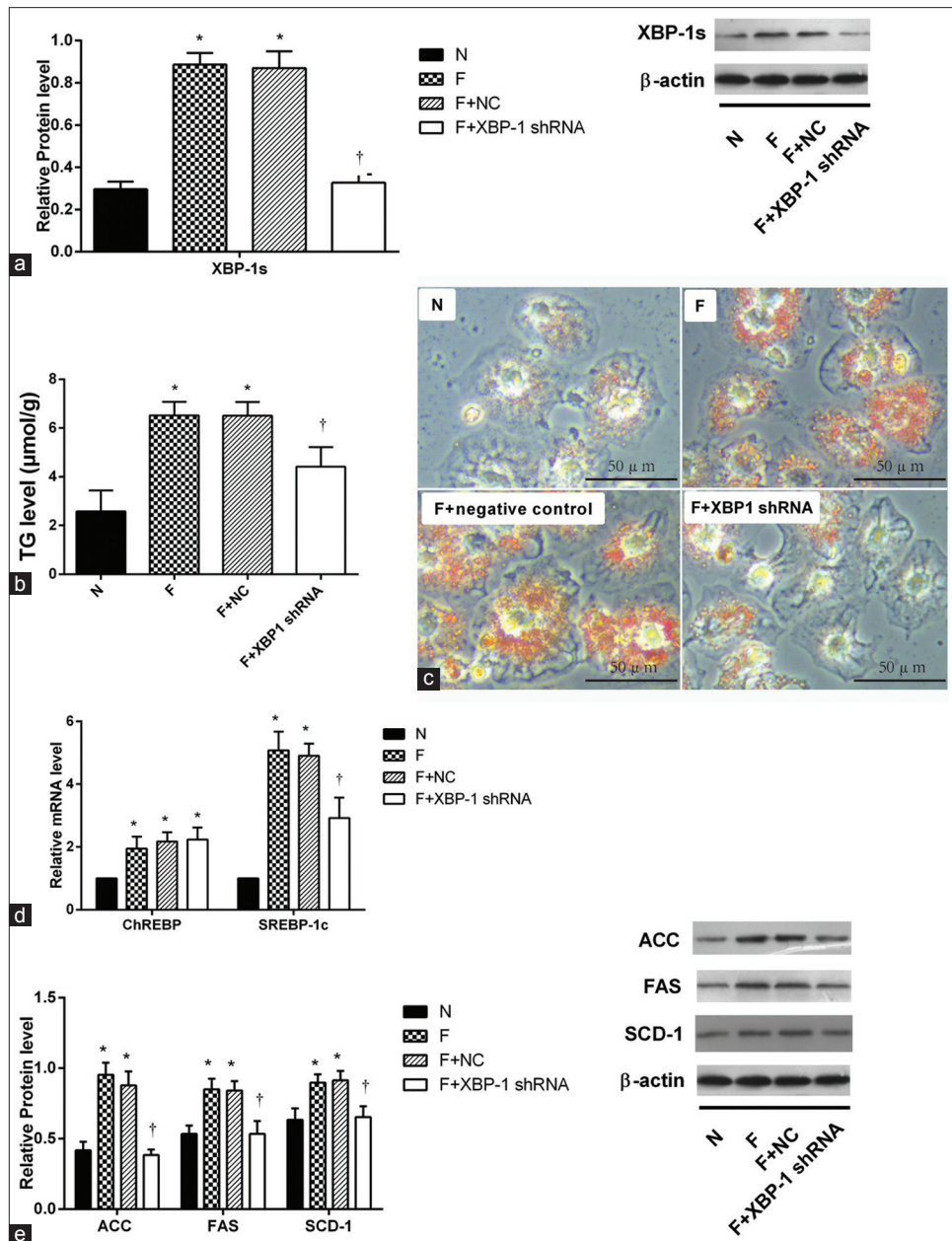
Figure 4: Lipid accumulation and expression of critical enzymes in the lipogenic pathway in HepG2 cells were decreased after transfection with XBP-1s shRNA. (a) Protein expression level of XBP-1s 8 h after transfection decreased significantly ($n = 3$), *$P < 0.05$ versus N group, †$P < 0.05$ versus F group. (b) TG induced by high fructose was decreased after transfection of XBP-1s shRNA ($n = 3$), *$P < 0.01$ versus N group, †$P < 0.01$ versus F group. (c) Oil Red O straining was alleviated in F+ XBP-1s shRNA group. Scale bar = 50 μm. (d) Level of *SREBP-1c* and *ChREBP* mRNA were tested by PCR in different groups ($n = 6$), *$P < 0.05$ versus untransfection group, †$P < 0.05$ versus F group. (e) Expression of critical enzymes in the lipogenic pathway proteins (SCD-1, FAS, and ACC) in HepG2 cells in different groups were tested by Western blotting, β-Actin expression is a loading control. Fold induction represents relative expression level compared to that of control group ($n = 3$), *$P < 0.05$ versus untransfection group, †$P < 0.05$ versus F group. Cells cultured with normal medium, 20 mmol/L fructose, 20 mmol/L fructose followed by transfection with empty vector, or 20 mmol/L fructose followed by transfection with shRNA targeting XBP-1 were assigned into N, F, F+NC, or F+XBP-1 shRNA group respectively. Data are shown as mean ± standard deviation. TG: Triglycerides; *SREBP-1c*: Sterol regulatory element-binding protein 1c; *ChREBP*: Carbohydrate response element-binding protein; SCD-1: Stearoyl-CoA desaturase-1; FAS: Fatty acid synthase; ACC: Acetyl-CoA carboxylase; PCR: Polymerase chain reaction.

protected against experimental ERS in HepG2 cells cultured in the presence of fructose. Pretreatment of HepG2 cells with TUDCA suppressed fructose-induced ERS (IRE-1 and XBP-1) and *de novo* lipogenesis (mRNA expression of *SREBP-1c* and *ChREBP* and protein content of ACC, SCD-1, and FAS). Conversely, thapsigargin (an ERS inducer) increased the

expression of PERK, eIF2α, IRE-1, XBP-1, and factors involved in lipogenesis. Although other studies have demonstrated that ERS may interfere with insulin signaling, inhibit insulin action in liver, and thereby affect lipid accumulation,[20] this study provides evidence for a direct effect of ERS on lipogenesis. On this basis, we speculate
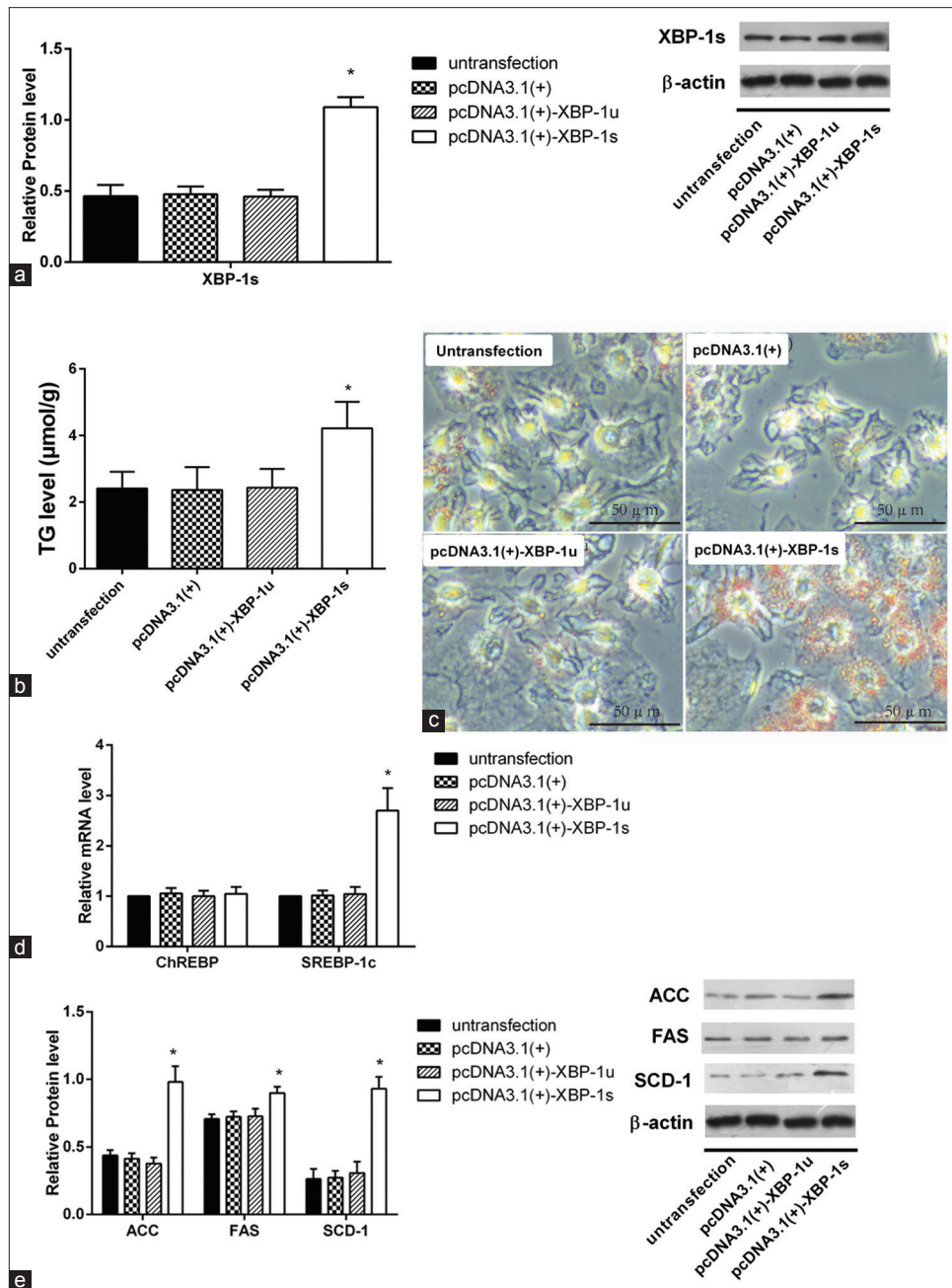
Figure 5: Expressions of critical enzymes in the lipogenic pathway were increased in XBP-1s overexpressed HepG2 cells. (a) Protein expression level of XBP-1s increased after transfection, *$P < 0.05$ versus untransfection group, †$P < 0.05$ versus pcDNA3.1(+)-XBP-1u group. (b) TG increased after transfection of XBP-1s plasmid, ($n = 3$), *$P < 0.05$ versus untransfection group. Scale bar = 50 μm. (c) Oil Red O straining was aggravated after XBP-1s upregulation. Scale bar = 50 μm. (d) Level of *SREBP-1c*, *ChREBP* mRNA was tested by PCR in different groups, and they were induced in pcDNA 3.1-XBP-1s group ($n = 6$), *$P < 0.05$ versus untransfection group. (e) Expression of critical enzymes in the lipogenic pathway proteins (SCD-1, FAS, and ACC) in HepG2 cells in different groups was tested by Western blotting inclined compared to that of control group ($n = 3$), *$P < 0.05$ versus untransfection group. Cells cultured with normal medium, transfecting with empty vector pcDNA3.1(+), vector pcDNA3.1(+)-XBP-1u or vector pcDNA3.1(+)-XBP-1s were assigned into untransfection, pcDNA3.1(+), pcDNA3.1(+)-XBP-1u, or pcDNA3.1(+)-XBP-1s group respectively. Data are shown as mean ± standard deviation. TG: Triglycerides; *SREBP-1c*: Sterol regulatory element-binding protein 1c; *ChREBP*: Carbohydrate response element-binding protein; SCD-1: Stearoyl-CoA desaturase-1; FAS: Fatty acid synthase; ACC: Acetyl-CoA carboxylase; PCR: Polymerase chain reaction.

that there may be a positive feedback loop between ERS and lipogenesis, and therefore that reducing ERS is a key to reducing lipogenesis and reversing NAFLD.

IRE1/XBP-1 is one of the three classic ERS pathways associated with growth, differentiation, and cellular apoptosis.[13] Recent studies in animals and cells have shed light on the role of XBP-1 in numerous diseases, such as obesity, insulin resistance, and metabolic syndrome.[39] XBP-1 protein expression in mice was elevated after mice were fed carbohydrates, and deletion of XBP-1 resulted in

marked hypocholesterolemia and hypotriglyceridemia.[30] Fatty liver induced by a high-calorie diet could be protected against by decreasing XBP-1.[12] The 3T3-L1 cells with *XBP1* or *IRE1α* knockdown and *XBP1*-deficient mouse embryonic fibroblasts showed significantly weakened adipogenesis.[40] Further studies showed that selectively knocking down the *XBP1* gene in the liver decreased critical genes involved in fatty acid synthesis.[30] Our study showed that in the presence of high fructose (20 mmol/L), there was significant upregulation of XBP-1s (active form) and downregulation of XBP-1u (inactive form). To elucidate the underlying mechanisms, we investigated hepatic regulation of TG synthesis by activating XBP-1. XBP-1s overexpression increased cellular TG accumulation, which was accompanied by increased mRNA expression of *SREBP-1c* and protein content of three key target enzymes associated with lipogenesis, ACC, FAS, and SCD-1, but not by a change in *ChREBP* mRNA expression. Again, silencing of XBP-1s expression effectively prevented against the lipid deposition in HepG2 cells induced by high fructose, by decreasing the transcriptional expression of *de novo* lipogenesis genes to alleviate further lipotoxic stress. Therefore, we explored the causal relationship between XBP-1s and lipid accumulation and found that high fructose-induced cellular lipid deposition in NAFLD was partially regulated by XBP-1s.

SREBP-1c and ChREBP are mediators of the transcriptional effects of lipogenic enzyme genes. Lipogenic genes such as *FAS*, *ACC*, and *SCD-1* are activated by both SREBP-1c and ChREBP. SREBP-1c mainly targets glucokinase, the first enzyme of the glycolytic pathway, as well as FAS and ACC.[41] SREBPs are synthesized as inactive precursors bound to the membranes of the ER and thus must undergo proteolytic cleavage to liberate their N-terminal domain, which constitutes the mature transcription factor. ERS could induce cleavage of the precursor form of SREBP-1c (increasing the expression of the mature form of SREBP-1c in the nucleus) and expression of SREBP-1c target genes independent of insulin, but the role of XBP-1 is unclear.[42] Lee *et al.*'s research shows that the genetic deletion of XBP-1 in liver leads to a decrease in *de novo* lipid synthesis.[30] Our research indicates that XBP-1 is associated with SREBP-1c without ChREBP, but further experiment need to be done to research the exact mechanism. However, our study convinced that the high fructose accelerates ERS to enhance *de novo* lipogenesis.

Our study had several limitations. First, we investigated the effect of XBP-1 on lipogenesis *in vitro*; rodent experiments are needed to verify our results *in vivo*. Second, ERS includes three pathways, and research has shown that transcription factor-4 activation contributes to lipid accumulation; thus, XBP-1 may be only partially responsible for ERS aggravation.

The present study provides novel insight into the mechanisms involved in fructose-mediated hepatic hypertriglyceridemia and identifies ERS as a potential therapeutic target for NAFLD. Furthermore, we show that XBP-1 plays a critical role in *de novo* lipogenesis during NAFLD.

To sum up, exposure of HepG2 cells to fructose resulted in increased hepatic lipid deposition via acceleration of *de novo* lipogenesis compared with PA exposure. In addition, the findings suggest that XBP-1 is crucial for lipid synthesis. Thus, using compounds that selectively target XBP-1 may be beneficial in the treatment of NAFLD.

Conflicts of interest

There are no conflicts of interest.

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X-盒结合蛋白-1在高果糖诱导的HepG2细胞脂质从头合成中的作用研究

摘要

背景：高果糖易导致肝脏的脂质沉积，其中20-30%的甘油三酯来源于脂质从头合成。有的研究证实内质网应激(Endoplasmic reticulum stress，ERS)参与脂质沉积的过程，而有的研究发现高脂环境可诱发ERS。因此本研究的目的旨在深入探讨ERS和脂质合成之间的关系，及ERS的重要因子X-盒结合蛋白-1（X-box binding protein-1，XBP-1）在高果糖诱发的脂质沉积中的作用。

方法：首先收集不同浓度的果糖干预处理后的HepG2细胞，应用PCR的方法测定其脂质从头合成的上游调控因子碳水化合物反应元件结合蛋白(carbohydrate response element-binding protein，ChREBP) 和（sterol regulatory element-binding protein 1c，SREBP-1c），应用Western方法测定了脂质从头合成的三个关键酶乙酰-CoA 羧化酶（acetyl-CoA carboxylase，ACC）、脂肪酸合酶（fatty acid synthase，FAS）、硬脂酰-CoA 去饱和酶（stearoyl-CoA desaturase-1，SCD-1）。然后HepG2 细胞中加入ERS诱导剂（thapsigargin），或是ERS抑制剂牛磺熊去氧胆酸 （tauroursodeoxycholic acid，TUDCA）加入到高果糖培养的HepG2 细胞中，再通过上述方法测定脂质从头合成的相关因子的表达水平。最后，采用细胞转染技术分别上调和下调XBP-1后，测定脂质从头合成的相关因子的表达。

结果： 高果糖以时间和浓度依赖性增加细胞内脂质沉积，增加细胞内*SREBP-1c*和*ChREBP*的mRNA的表达水平，及FAS、ACC和SCD-1 的蛋白表达水平，并促进了XBP-1转化为活性形式。而且这一过程可以被ERS的抑制剂TUDCA所抑制，相反，ERS的诱导剂却促进脂质从头合成。下调XBP-1组甘油三酯的水平较高果糖组明显下降（4.41±0.54 μmol/g vs. 6.52±0.38 μmol/g，$P<0.001$），且脂质从头合成的上游调控因子SREBP-1c的mRNA （2.92±0.46 vs. 5.08±0.41，$P<0.01$）和FAS（0.53±0.06 vs. 0.85±0.05，$P=0.01$）、SCD-1（0.65±0.06 vs. 0.90±0.04，$P=0.04$） ACC（0.38±0.03 vs. 0.95±0.06，$P<0.01$）的蛋白水平明显下降。与之相反的是上调XBP-1s组的甘油三酯的水平较未转染组却明显升高（4.22±0.54 μmol/g vs. 2.41±0.35 μmol/g，$P<0.001$），且上游调控因子SREBP-1c的mRNA （2.70±0.33 vs. 1.00±0.00，$P<0.01$）和SCD-1（0.93±0.06 vs. 0.26±0.05，$P<0.01$）、ACC（0.98±0.09 vs. 0.43±0.03，$P<0.01$）、FAS（0.90±0.33 vs. 0.71±0.02，$P=0.04$)的蛋白表达水平明显增加。

结论：ERS与脂质从头合成有关，且XBP-1在高果糖诱导的HepG2细胞的脂质沉积的发挥着一定的作用，可能是与上调了脂质从头合成有关。