The chemical chaperon 4-phenylbutyric acid ameliorates hepatic steatosis through inhibition of de novo lipogenesis in high-fructose-fed rats

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Abstract. Non-alcoholic fatty liver disease caused by dietary factors such as a high fructose intake is a growing global concern. The aim of this study was to investigate the intervention effects of an endoplasmic reticulum stress (ERS) inhibitor 4-phenylbutyric acid (PBA) on liver steatosis induced by high-fructose feeding in rats and the possible underlying mechanisms. Wistar rats were divided into the control, high-fructose group (HFru) and PBA intervention (HFru-PBA) groups. PBA intervention was initiated following 4 weeks of high-fructose feeding. After 8 weeks of feeding, the ERS markers p-PERK, p-eIF2α, p-IRE-1, spliced XBP-1, ATF-6 were measured by western blotting. Liver triglyceride contents and morphological changes were examined. The protein expression of lipogenic key enzymes (ACC, FAS and SCD-1) and upstream transcriptional factors (SREBP-1c and ChREBP) were measured. The ERS-related cell events, oxidative stress and apoptosis, were evaluated by standard methods. Results demonstrated that PBA intervention significantly resolved hepatic ERS and improved liver steatosis induced by high-fructose feeding in rats. The protein expression of ACC, FAS, SCD-1 and SREBP-1c was upregulated in high-fructose-fed rats, whereas it decreased following PBA intervention. Oxidative stress and apoptosis were observed in livers of high-fructose-fed rats, but were alleviated by PBA intervention. ERS is involved in the development of fatty liver induced by a high fructose intake. ERS inhibition by PBA can therefore ameliorate liver steatosis through inhibition of hepatic lipogenesis.

Introduction

Non-alcoholic fatty liver disease (NAFLD) has gained increasing attention worldwide due to its prevalence and its association with insulin resistance and metabolic syndrome (1,2). Hepatic steatosis is the basic pathophysiological change occurring throughout the development of NAFLD. Dietary effects on whole-body metabolism and its regulation via the effects on lipid metabolic pathways are considered to be crucial in the pathogenesis of hepatic steatosis (3,4). The effect of high fructose intake on the pathogenesis of hepatic steatosis due to an increase in daily fructose intake and its harmful impact on hepatic lipid metabolism has gained much attention (5-7). High-fructose feeding leads to significant lipid accumulation in livers of rodents (7-9). One important reason for this is that dietary fructose stimulates endogenous de novo lipogenesis within the liver (10). However, the clear underlying mechanisms by which fructose induces liver steatosis remain to be clarified.

Previous studies demonstrated that endoplasmic reticulum stress (ERS) and unfolded protein response (UPR), which occurs following ERS, have a regulatory effect on lipid synthesis in liver (11-13). On the other hand, ERS is associated with the development of NAFLD. Specifically, hepatic ERS is accompanied by a fatty liver in genetically obese or chronic high-fat-fed rodents (14,15). An intervention study on ERS inhibitors, 4-phenylbutyric acid (4-PBA) and tauroursodeoxycholic (TUDCA) found that resolved ERS is able to ameliorate hepatic steatosis in ob/ob mice (15,16). In a short-term study, it was suggested that ERS is involved in the development of lipid accumulation in liver in mice fed a high fructose diet (17). Few studies have investigated the role of ERS in hepatic steatosis induced by long-term high-fructose feeding.

The aim of the present study was to clarify the role of ERS in the development of fatty liver induced by long-term high fructose intake. Fructose is a dietary factor that highly stimulates lipogenesis, while ERS has a regulatory effect on lipogenesis. We hypothesized that alleviation of ERS is able to ameliorate hepatic steatosis in high-fructose-fed rats through regulation of de novo lipogenesis. To prove this hypothesis, 4-phenylbutyric acid was used to inhibit the ERS induced by

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high-fructose feeding in liver in Wistar rats. Lipid content, ERS and lipogenic markers in liver were detected in order to investigate the association between ERS and fructose-induced fatty liver. Oxidative stress and hepatocyte apoptosis were also observed to study the role of ERS in the development of NAFLD.

Materials and methods

Animals. Male Wistar rats supplied from the Experimental Animal Center of Hebei Province (Shijiazhuang, China) were conditioned in communal cages for 1 week at 22.0±0.5°C with a 12/12-h light/dark cycle (lights on 06.00 a.m.). Experimental procedures were approved by the Animal Ethics Board of the Hebei Research Institute for Endocrine and Metabolic Diseases and were in accordance with China's National Code of the Animal Care for Scientific Experimentation. After an acclimatization period, the rats (250-300 g) were divided into three groups. The control group (Con) was fed a standard laboratory diet (18). The high-fructose (HFru) and 4-phenylbutyric acid (PBA) intervention (HFru-PBA) groups were fed a high-fructose diet (35% calories from fructose, 35% calories from starch, ~9% calories from fat and 21% calories from protein; based on a recipe described in a study by Ren et al (17)). PBA [dose: 0.35 g/kg.day, based on a previous study (15)] was administered to the HFru-PBA group by oral gavage subsequent to 4 weeks of high-fructose feeding. After 8-weeks, the rats were sacrificed and liver tissues were collected.

Plasma glucose concentrations were determined using a glucometer (Accu-Check Active, Roche Diagnostics GmbH, Mannheim, Germany). Plasma insulin was measured using a radioimmunoassay kit (Linco Research, St. Charles, MO, USA). The enzymatic activities of ALT and AST were determined using a Biochemical Analyzer (Beckman Coulter, Brea, CA, USA). Plasma FFA concentration was determined using a Biochemical Analyzer (Beckman X20, USA). The enzymatic activities of ALT and AST were determined using commercial assay kits according to the manufacturer's instructions. The MDA level was expressed as nmol/mg protein. The activities of antioxidant enzymes were expressed as U/mg protein.

Histology staining. Small liver sections, fixed in 10% buffered formalin, were processed for embedding in paraffin. Sections of 5-6 mm were cut for histopathological evaluation. Liver sections were stained with hematoxylin and eosin (H&E staining) using a standard protocol and then analyzed by light microscopy.

Hyperinsulinemic-euglycaemic clamp. The study was conducted between 09.00 and 12.00 a.m. at week 8 in animals that had been fasted for 12 h. After being anaesthetized with pentobarbitone (40 mg/kg, i.p.), the rat was placed on a warm table to maintain rectal temperature at 37°C. Catheters were inserted into the left femoral vein (for infusion of glucose and insulin) and the femoral artery (for blood sampling). After a basal period of 30 min, a hyperinsulinemic-euglycaemic clamp was performed, as previously described (19). In brief, human insulin (Actrapid; Novo-Nordisk, Beijing, China) was infused at a constant rate of 4.1 mU/kg per min to achieve physiological hyperinsulinaemia (100-150 mU/l) for a period of 2 h. The blood glucose concentration was clamped at the basal level by infusing glucose at variable rates. Under these conditions, the glucose infusion rate (GIR) required to maintain euglycaemia (usually calculated between 60 and 120 min) reflects whole-body insulin sensitivity.

Western blot analysis. Liver samples were homogenized in ice-cold lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X-100, 10 mM NaF, 100 mM NaF, 2 mM Na orthovanadate, 1 mM EDTA, 1 mM EGTA, 10% glycerol), supplemented with protease inhibitor cocktail tablets (Roche) and DL-dithiothreitol, and solubilized for 30 min at 4°C. Protein samples were then denatured in SDS sample buffer (125 mmol/l Tris-HCl, pH 6.8, 50% glycerol, 2% SDS, 5% β-mercaptoethanol and 0.01% bromophenol blue). Equal amounts of tissue lysates were resolved by SDS-PAGE and immunoblotted with antibodies against the following proteins: ERS markers, phosphorylated pancreatic ER kinase (p-PERK) (Thr980, Cell Signaling Technology, Danvers, MA, USA), total- and phospho (Ser51)-eukaryotic translation initiation factor 2α (eIF2α, Santa Cruz Biotechnology, Santa Cruz, CA, USA), inositol-requiring kinase 1 (IRE1, Abcam, Cambridge, MA, USA), phospho-IRE1 (Ser724, Abcam), X-box banding protein 1 (XBPI, Santa Cruz Biotechnology), activation transcriptional factor 6 (ATF6, Santa Cruz Biotechnology), C/EBP homologous protein (CHOP, Cell Signaling Technology); upstream transcriptional factors of lipogenesis: sterol regulatory element-binding protein-1c (SREBP1c, Santa Cruz Biotechnology), carbohydrate responsive element binding protein (ChREBP, Santa Cruz Biotechnology); downstream lipogenic enzymes, acetyl-CoA carboxylase (ACC, Upstate, Lake Placid, NY, USA), fatty acid synthase (FAS, Abcam) and stearoyl-CoA desaturase 1 (SCDI, Cell Signaling Technology). Antibodies against β-actin (Santa Cruz Biotechnology) were blotted in each gel as the loading control.

Determination of oxidative parameters. Tissue homogenates were centrifuged for 15 min at 15,000 x g, and then the clear supernatants were removed for analysis. Lipid peroxidation in the liver was measured by the formation of malondialdehyde (MDA). The levels of MDA and the activities of antioxidant enzymes including SOD, GSH-Px and CAT were assayed using commercial assay kits according to the manufacturer's instructions. The MDA level was expressed as nmol/mg protein. The activities of antioxidant enzymes were expressed as U/mg protein.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling method (TUNEL) assay. Paraffin sections (6-μm) were collected on poly-L-lysine-coated glass slides, and the nuclear DNA fragmentation of apoptotic cells was labeled in situ with the ApopTag Peroxidase in situ Apoptosis Detection kit (Intergen Co., Purchase, NY, USA). DNA fragmentation was determined using a TUNEL assay as described by Boncompagni et al (20). TUNEL-positive nuclei were counted.

Statistical analyses. The results are presented as means ± SE. Differences were considered significant when p<0.05 tested
by one-way analysis of variance (ANOVA). When significant variations were found, the Tukey-Kramer multiple comparisons test was applied.

Results

Baseline characteristics and plasma parameters in three groups of rats fed for 8 weeks. After 8 weeks of feeding, no difference was observed in body mass for the three groups of rats. Visceral white adipose tissue (WAT) was increased in the HFru group compared with the Con group (p<0.05), while visceral WAT was significantly decreased in the HFru-PBA group compared with the HFru group. Total cholesterol (21), triglyceride (TG) and free fatty acids in plasma were all increased in the HFru group compared with the Con group, but improved in the HFru-PBA group. No difference was observed in plasma ALT and AST for the three groups. Basal plasma glucose and insulin concentrations were both increased in the HFru group compared with the Con group, but decreased in the HFru-PBA group compared with the HFru group (Table I).

Systemic insulin resistance was induced by high-fructose feeding but ameliorated by PBA intervention after 8 weeks of feeding. After 8 weeks of feeding, the GIR during the hyperinsulinemic-euglycemic clamp study was decreased by 46% in the HFru group compared with the Con group (p<0.01) but did not change with PBA intervention (Fig. 1).

ERS markers were activated by high-fructose feeding but normalized by PBA intervention after 8 weeks of feeding. After 8 weeks of feeding, the protein expression of the activation forms of ERS markers, including phosphorylated PERK (p-PERK), phosphorylated eIF2α (p-eIF2α), phosphorylated IRE-1 (p-IRE-1), spliced XBP1 (shown as the ratio of spliced XBP1 to unspliced XBP1; spliced XBP1 is the activated form of XBP1) and ATF6, were all upregulated in rat livers in the HFru group (all p-values <0.01); while the protein expression of the above markers in liver were significantly inhibited in the HFru-PBA group (all p-values <0.01) (Fig. 3).

Protein expression of lipogenic enzymes were stimulated by high-fructose feeding but were decreased by PBA intervention after 8 weeks. Compared with the Con group, protein levels of the key lipogenic enzymes including ACC, FAS and SCD1 were significantly increased by 2.8, 5.7 and 3.8-fold, respectively, in the liver tissues in rats after 8 weeks of high-fructose feeding (all p-values <0.01). After PBA intervention, the proteins levels of ACC, FAS and SCD1 were almost normalized in rat livers in the HFru-PBA group (all p-values <0.01) (Fig. 4).

Upregulated protein expression of SREBP1c induced by high-fructose feeding was decreased by PBA intervention after 8 weeks. Protein contents of SREBP1c in rat livers in the HFru group were upregulated by 1.9 and 4.3-fold, respectively, but downregulated by 66 and 57%, respectively, with PBA intervention (both p-values <0.01). Compared with the Con group, the protein expression of ChREBP was increased by 66% in the HFru group (p<0.01) but did not change with PBA intervention (Fig. 5).

Oxidative stress in liver tissue in high-fructose-fed rats was relieved by PBA intervention. The MDA level was significantly increased in the HFru group compared with the Con group after 8 weeks of feeding. By contrast, PBA intervention decreased the MDA level significantly in the HFru-PBA group (both p-values <0.01) (Fig. 6A). Compared with the Con group, the activities of SOD, GSH-px and CAT were significantly increased by 2.8, 5.7 and 3.8-fold, respectively, in the liver tissue in rats after 8 weeks of high-fructose feeding (all p-values <0.01). After PBA intervention, the activities of SOD, GSH-px and CAT were almost normalized in rat livers in the HFru-PBA group (all p-values <0.01) (Fig. 6B).
in high-fructose-fed rat livers were significantly decreased by 26.4, 16.4 and 23.1%, respectively. In the HFrui-PBA group, the activities of the abovementioned parameters in livers were recovered by 18.2, 11.1 and 21.5%, respectively, following PBA intervention (all p-values <0.05) (Fig. 6B-D).

Changes in TUNEL assay and the protein expression of CHOP indicate that apoptosis in hepatocytes was increased in livers in high-fructose-fed rats while it was relieved in livers in high-fructose-fed rats following PBA intervention. Compared with the Con group, the percentage of TUNEL-positive cells in livers was significantly increased (1.4 vs. 23.8%, p<0.01) in the HFru group. In the HFrui-PBA group, PBA treatment decreased the percentage of TUNEL-positive cells in livers to 11.0% (p<0.01) (Fig. 7A). Compared with the Con group, the protein contents of CHOP in livers were increased by 59% (p<0.01) in the HFru group. PBA treatment decreased the protein contents of CHOP by 21% in rat livers in the HFrui-PBA group (p<0.05) (Fig. 7B).

Discussion

Animal studies have shown that a high intake of fructose leads to hepatic steatosis and whole-body insulin resistance (8,9). In the present rat study, 8 weeks of high-fructose feeding induced liver lipid accumulation, increased glucose and insulin levels in plasma and decreased GIR during hyperinsulinaemic-euglycaemic clamp study, which are consistent with previous studies (9). The mechanisms by which fructose induces hepatic steatosis remain to be clarified. From previous studies (17), ERS is possibly involved in the development of fatty liver induced by high-fructose feeding.

ERS involves the disruption of endoplasmic reticulum homeostasis. Unfolded protein response (UPR) is the self-protective mechanism in endoplasmic reticulum required to cope with ERS. Since no direct marker of ERS is currently available, the transcription factors of the three pathways of UPR are used as indirect ERS markers, including the PERK-eIF2α, IRE-1-XBP1 and ATF6 pathways. ERS has been shown to be involved in the development of NAFLD and insulin resistance in genetically obese mice models or high-fat-fed rodent models (14,15). Inhibition of ERS is able to ameliorate hepatic steatosis in ob/ob mice (15,16). Additionally, ERS is induced in mice fed on a short-term high-fructose diet in hepatic steatosis (17). However, few studies have been conducted on the role of ERS in hepatic steatosis induced by long-term intake of fructose. Results of the present study show that the hepatic lipid accumulation induced by long-term high-fructose feeding was accompanied by ERS in liver in Wistar rats, as reflected by the activation of 3' UPR pathways. However, resolved ERS by PBA intervention ameliorated hepatic steatosis, indicating that ERS is involved in the pathogenesis of fatty liver induced by high-fructose feeding. On the other hand, PBA intervention improved the whole-body glucose metabolism and insulin
Fructose is known to be a highly lipogenic dietary factor, and increased hepatic lipogenesis is an important mechanism by which fructose induces hepatic steatosis (22). Studies (11-13) have shown that ERS and UPR have a regulatory effect on the lipid synthesis in the liver. In obese rodents, the ERS-induced dissociation of ERS chaperon GRP78 leads to an upstream lipogenic transcriptional factor SREBP1c maturation and result in hepatic steatosis, indicating ERS has a regulatory effect on SREBP1c (12). On the other hand, the ERS marker along the IRE-1-XBP1 pathway of UPR, XBP1 has been established as a novel transcription factor governing hepatic lipogenesis (13). Since it has been proven that ERS and UPR have a regulatory effect on the lipid synthesis in liver (11-13), we hypothesized that the alleviation of ERS decrease lipid contents in liver by inhibiting de novo lipogenesis. As shown in the results, the key lipogenic enzymes including ACC, FAS and SCD1 were all upregulated by high-fructose feeding, which reflects increased de novo lipogenesis, while in the Fru-PBA group, the protein expression of ACC, FAS and SCD1 was significantly decreased. This finding confirms our hypothesis and suggests that resolved ERS by PBA is capable of inhibiting de novo lipogenesis and thus ameliorating hepatic steatosis. We then assessed the abovementioned upstream transcriptional factors, including SREBP1c, ChREBP [another well-known upstream transcriptional factor of de novo lipogenesis in liver (23)] and spliced XBP1. The results demonstrated that long-term fructose intake increased the protein expression of SREBP1c, ChREBP and spliced XBP1, whereas PBA...

Figure 3. ERS markers were activated by high-fructose feeding but were normalized by PBA intervention after rats were fed for 8 weeks. Protein levels of (A) phosphorylated PERK (p-PERK), (B) phosphorylated eIF2α (p-eIF2α), (C) phosphorylated IRE-1 (p-IRE-1), (D) spliced XBP-1 and (E) ATF-6 in livers in three groups of rats. Data are presented as means ± SE, and two representative blots of each group were presented for the protein. *P<0.05 vs. Con rats; **P<0.01 vs. Con rats; ††P<0.01 vs. HFru rats (n=6).
intervention decreased the protein levels of SREBP1c and spliced XBP1, but not ChREBP. Combined with the observation that ACC, FAS and SCD1 in liver were downregulated following PBA intervention, the results indicate that ERS has a regulatory effect on the lipogenesis. Decreased expression of SREBP1c and XBPI may contribute to the decreased protein expression of key lipogenic enzymes, resulting in improved hepatic steatosis. Of note, the protein expression of ChREBP was not changed by PBA intervention. However, the association between ChREBP and ERS remains to be clarified. Our results show that ERS inhibition does not affect the expression of ChREBP, suggesting that ERS does not have a regulatory effect on ChREBP.

Another finding in our study is that ERS and oxidative stress occur simultaneously in livers in high-fructose-fed rats. Inhibition of ERS by PBA alleviated oxidative stress in liver induced by high-fructose feeding, as reflected by the increased activity of SOD, GSH-px and CAT following PBA intervention compared with that in the high-fructose group. These results suggest that ERS is connected to oxidative stress in fatty liver in high-fructose-fed rats. Previous studies have suggested that there is a complicated interaction between endoplasmic reticulum and oxidative stress. Specifically, free radicals, including reactive oxygen species (24), are one of the key messengers between the two cell events (25). Evidence suggests that endoplasmic reticulum is a potent source of ROS production. ROS produced in endoplasmic reticulum under ERS has been estimated to account for ~25% of all ROS generated in cells. Sustained ERS can result in the accumulation of reactive oxygen species and promotes oxidative stress (26,27). In addition, C/EBP homologous protein (CHOP) activation seems to enhance oxidative stress: as previously shown, CHOP dele-
tion reduces oxidative damage in mouse models of diabetes (28). By contrast, UPR in ERS has a protective effect against oxidative stress response via the PERK and IRE-1-XBP1 pathways (29). The PERK pathway provides protection from ROS via eIF2α. Mice lacking the ability to phosphorylate eIF2α are characterized by a severe diabetic phenotype that can be attenuated by a high antioxidant diet, suggesting a role for eIF2α phosphorylation in the prevention of oxidative stress (30). XBP1 may also protect cells from oxidative damage. Mouse embryonic fibroblasts deficient in XBP1 were more prone to cell death and less able to activate antioxidant defenses following exposure to hydrogen peroxide (31). The results in the present study support that ERS and oxidative stress are cross-linked in the development of fatty liver and that inhibition of ERS can affect oxidative stress. However, the interaction and the cause-effect relationship between the two events remain unclear. Further investigations are needed to clarify the relationship between the two stress responses in the development of hepatic steatosis.

Besides relieving oxidative stress, apoptosis in liver cells has been significantly decreased by PBA inhibition in high-fructose-fed rats. Apoptosis is a cell event occurring in the development of NAFLD and is associated with ERS and oxidative stress (32-34). CHOP is best known as an important mediator of ERS-induced cell death. Chronic ERS promotes apoptosis, at least in part through the activation of CHOP (32). In addition to ERS, oxidative stress is a key mechanism responsible for liver cell death and liver damage (34). The accumulation of ROS (24) also contributes to the apoptosis of hepatocytes (35). Therefore, the decreased apoptotic rate and decreased expression of CHOP in liver in the HFru-PBA group may be the results of the improvement of ERS and oxidative stress following PBA intervention.

Figure 6. Oxidative stress in liver tissue in high-fructose-fed rats was relieved by PBA intervention. (A) MDA levels. The enzyme activities of (B) SOD, (C) GSH-px and (D) CAT in livers in three groups of rats. Data are presented as means ± SE. *P<0.01 vs. Con rats; †P<0.05 vs. HFru rats (n=6).

Figure 7. TUNEL assay and the protein expression of CHOP in three groups of rats after 8 weeks of feeding. (A) TUNEL assay of livers in the three groups of rats. (B) Protein levels of CHOP in the three groups of rats. Data are presented as means ± SE, and two representative blots of each group were provided for CHOP protein. *P<0.05 vs. Con rats; **P<0.01 vs. Con rats; †P<0.05 vs. HFru rats; ‡P<0.01 vs. HFru rats (n=6).
Findings of this study have proven that long-term high-fructose intake is capable of inducing ERS in liver with the occurrence of hepatic steatosis. PBA intervention is able to improve the lipid accumulation induced by high-fructose feeding through inhibition of the upstream lipogenic transcriptional factors SREBP-1c and XBP1 and downregulation of the lipogenic key enzymes. Besides ameliorating steatosis, oxidative stress and apoptosis in liver were also alleviated when ERS was resolved by PBA inhibition. The present study sheds new light on the role of ERS in the development of fatty liver. ERS is therefore a potential target for the prevention and treatment of NAFLD.

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