Amelioration of Non-alcoholic Fatty Liver Disease by Hepatic Stimulator Substance via Preservation of Carnitine Palmitoyl Transferase-1 Activity

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Running Title: hepatic stimulator substance and mitochondrial CPT-1

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Abstract

Non-alcoholic steatohepatitis (NASH) is the progressive form of non-alcoholic fatty liver disease and so-far supposed to be related with mitochondrial impairment. Hepatic stimulator substances (HSS) has been defined as a liver protective factor promoting hepatocyte DNA synthesis and hepatic proliferation after liver intoxication. We previously reported that HSS ameliorated hepatocyte death probably due to its preservation of mitochondria. This study is aiming to explore if HSS could protect carnitine palmitoyl transferase-1 (CPT-1), an essential enzyme responsible for \( \beta \)-oxidation of free fatty acids (FFAs) in mitochondria, from lipotoxicity, thus alleviating hepatic lipid deposition. To test this, the HSS gene was delivered into C57BL/6J mice and efficiently expressed in the liver. NASH mice were prepared with high fat diet or methionine-choline deficient diet. The results showed that hepatic inflammation and liver functions were alleviated in the HSS-transfected mice, meanwhile, the activity of CPT-1 was obviously protected. Moreover, oleic acid (OA) treatment resulted in remarkable lipid accumulation in HepG2 cells; this deposition was improved by HSS-transfection. Simultaneously, the CPT-1 activity, which was impaired by OA treatment, was profoundly rescued in the HSS-expressing cells. CPT-1 activity was more severely impaired if the OA treatment was combined with S15176, a CPT-1 inhibitor. However, this impairment was effectively reduced by the HSS-transfection, and the effect was enhanced by C75, a CPT-1 activator. Interestingly, if the cells were transfected with HSS siRNA, the preservation of CPT-1 provided by HSS was again diminished. In conclusion, HSS reduces lipotoxicity to mitochondria most likely via preservation of CPT-1.

Keywords: hepatic stimulator substance (HSS); nonalcoholic steatohepatitis (NASH); methionine-choline-deficient diet (MCD); oleic acid (OA); carnitine palmitoyl transferase-1 (CPT-1)
Introduction

Non-alcoholic fatty liver disease (NAFLD) is regarded as a hepatic manifestation of the metabolic syndrome which is associated with obesity, insulin resistance, dyslipidemia and hypertension (25). NAFLD ranges from simple steatosis to nonalcoholic steatohepatitis (NASH), advanced fibrosis and cirrhosis. As a progressive stage of NAFLD, NASH is characterized by steatosis and inflammation, i.e., accumulation of triacylglycerols within hepatocytes along with infiltration of inflammatory cells (mainly neutrophils) and ballooning degeneration (35). NASH is a serious condition, as approximately 15% of these patients progress to cirrhosis, with complications including portal hypertension, liver failure and hepatocellular carcinoma (1, 11, 26). The development of NASH is frequently described by the “two-hits” mechanism where liver steatosis constitutes the “first hit” and is accompanied by obesity as well as a metabolic disorder that causes excessive hepatic lipid accumulation (8). The hepatic lipid accumulation occurs as a result of the availability of excess free fatty acids (FFA) from high-energy/high fat diets, release from adipose tissue, or an increase in hepatic fatty acid synthesis and a decrease in degradation (5). Impairment of triglyceride secretion by very low density lipoprotein (VLDL) can also worsen the lipid metabolic disorder in some conditions (12). The “second hit” is proposed to be a multifactorial process likely involving a combination of apoptosis and necrosis, massive production of reactive oxidative species (ROS) and the subsequent injuries, such as lipid peroxidation, dysregulated adipokine expression and mitochondrial dysfunction (32).

Even though the mechanisms of the progression from simple steatosis to NASH are not completely understood, mitochondrial dysfunction has been proposed as one of key factors (6, 37). Therefore, therapeutic approaches that address protection of mitochondria from lipotoxicity would be expected to be a practical strategy against NASH. It is reported that hepatic mitochondrial function is altered in rats with NASH that has been induced by a methionine-choline deficient (MCD) diet (37). Rodents fed an MCD diet develop steatohepatitis with hepatic lesions and changes in the liver redox balance, mimicking the impairments observed in patients with NASH (4, 20). Fat accumulation within hepatocytes enhances mitochondrial ROS production (13), which, in turn, may cause oxidative stress. The most important cellular damage caused by ROS is peroxidation of membrane lipids resulting
in a generalized alteration of the membrane function (39). Lipid peroxidation products can react with functional groups of amino acids in proteins and enzymes to form adducts that may alter protein function (43). CPT-1, the mitochondrial gateway for fatty acid entry into the matrix, is the main controller of the hepatic mitochondrial β-oxidation flux (10). In the liver, CPT-1 exerts approximately 80% of the control under physiological conditions (3). The impairment of β-oxidation in mitochondria due to down-regulation of hepatic CPT-1 may be a crucial event in the pathogenesis of hepatic steatosis in mice (7). It has been reported that feeding rats an MCD diet for 4 weeks led to a reduction in CPT-1 activity both in isolated mitochondria and in hepatocytes, thus producing pronounced effects on hepatic lipid metabolism that appear much more severe than those caused by a simple inhibition of triglyceride (TG) secretion (38).

Hepatic stimulator substance (HSS) is extracted initially from the liver cytosol of weanlings or partially hepatectomized adult rats and was first described by LaBrecque and Pesch (19). A major function of this protein is to promote hepatocyte proliferation and liver regeneration after partial hepatectomy (14, 18). HSS has been shown to protect the liver from acute injury caused by several compounds, including CCl₄ (28), D-galactosamine (16), ethanol (23), H₂O₂ (44) and cadmium (42). Additionally, HSS has been shown to induce mitochondrial gene expression and to enhance the oxidative phosphorylation capacity of the liver mitochondria (33). Further, HSS has been proven to protect mitochondria from mitochondrial permeability transition (MPT) and to act as a potential MPT inhibitor, preventing the cell damage from apoptosis caused by an MPT-inducing agent, carbonyl cyanide m-chlorophenyl hydrazone (CCCP) (45). Impairment of mitochondrial β-oxidation plays an essential role in the pathogenesis of NASH induced by an MCD diet (2), and HSS may play a role in the inhibition of NASH progression. Therefore, we are aiming to investigate whether HSS protection of the hepatic mitochondria is linked to mitochondrial metabolism, particularly activity of CPT-1, the key rate-limiting enzyme of mitochondrial β-oxidation, to see if CPT-1 could be one of the effector molecules responsible for HSS protection.

Here, in an in vivo study, mice were fed an MCD diet to induce NASH. The therapeutic effect of using the HSS gene, delivered by injection into the femoral vein, was evaluated. In an in vitro study, HepG2 cells were stably transfected with the HSS plasmid and were
subjected to lipid injury by oleic acid (OA) to determine the protection provided by HSS. The
ability of HSS to protect cellular and mitochondrial CPT-1 activity in the HSS-expressing
cells was specifically analyzed. The results demonstrated that HSS could protect the liver
from MCD/OA-induced hepatic lipotoxicity, ameliorating the severe decrease in CPT-1
activity resulting from NASH. The protection provided by HSS appeared not only in the
HSS-transfected cells but also in isolated mitochondria treated with recombinant HSS. In
addition, the hepatic CPT-1 activity was decreased as NASH progressed and was further
diminished by its specific inhibitor, S15176. However, HSS could rescue the S15176-induced
CPT-1 activity decrease in a dose-dependent manner. Moreover, HSS was able to augment
the increase caused by C75, a specific activator of CPT-1, in a dose-dependent manner. In
conclusion, HSS protects the liver cells from MCD/OA-induced steatosis most likely via the
regulation of CPT-1 activity.
Materials and Methods

Animals

Male C57BL/6J mice weighing 18 to 20 g were purchased from the Academy of Military Medical Sciences (Beijing, China) and maintained at a controlled room temperature (22-25°C) on a 12 h light-dark cycle. All animals received humane care at the Capital Medical University in compliance with the Guide for the Care and Use of Laboratory Animals. NASH was induced in mice by feeding them a MCD diet for 4 weeks as described previously (36). Mice were fed with high-fat-diet (HF) for 16 weeks (31). The MCD diet and HF diet were purchased from the Beijing HFK Bioscience Co., Ltd (Beijing, China) and Research Diets Inc. (New Brunswick, NJ, USA) respectively. The food intake was evaluated for all the mice twice a day in every morning and every evening. The MCD/HF diet animals were assigned randomly into the following dietary groups: the mice in the control group (n=30) were fed a normal chow diet; the mice in the MCD/HF diet group (n=90) received either the HSS gene (n=30, nominated as the MCD+HSS-Tx /HF+HSS-Tx group, for protocols see below) or the vector (n=30, nominated as the MCD+vector-Tx /HF+vector-Tx group) or a 0.9% NaCl solution (n=30, nominated as the MCD/HF group); The body weights and the liver weights/body weights of all mice were recorded weekly throughout the experiment. In addition, the histological changes in the liver were observed.

Gene delivery and expression assay

A total of 1×10⁹ pAdxsi-GFP plasmids were dissolved in 500 μl of normal saline and this aliquot was injected into C57BL/6 mice through the femoral vein as previously described (21). After two weeks, the target tissues were obtained, and 5-μm-thick sections were prepared, then observed under a confocal laser scanning microscope (Leica DM 5000B, Wetzlar, Germany) to evaluate efficiency of gene transfection. After confirming the delivered gene being expressed, pAdxsi vector containing HSS gene plus Flag-tag was applied for the followed experiments.

Then a total of 1×10⁹ pAdxsi-Flag-HSS plasmids were dissolved in 500 μl of normal saline and this aliquot was injected into MCD-diet-fed mice. Following the injection, the mice were sacrificed and the levels of HSS expression were measured in different organs, including the liver, heart, spleen, lungs and kidneys within 48 h after gene delivery. The HF-diet-fed mice
were injected with $1 \times 10^9$ pAdxsi-Flag-HSS plasmids dissolved in 100 μl of normal saline by cauda vein every 2 weeks. The mice were sacrificed after 16 weeks.

For the liver, the tissues from the median, the left and right lobes were collected and frozen in the Tissue Tek OCT compound (Electron Microscopy Sciences, Hatfield, PA, USA) for cryosectioning. Sections 10 μm thick were prepared, examined and photographed using a fluorescence microscope (Leica DM 5000B, Wetzlar, Germany). For an accurate assay of gene transfer, tissues were prepared from three animals, and the examinations were assessed by two individuals.

For Western blotting, the tissues from the liver were harvested and homogenized with lysis buffer (0.5% sodium deoxycholate, 0.5% Triton X-100, 50 mM Tris, 150 mM NaCl, 62.5 mM sucrose, 5 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride). Then, the homogenate was centrifuged at 12,000 g for 15 min at 4°C, and the supernatant was collected for Western blotting analysis. The protein concentration was determined using the bicinchoninic acid (BCA) method (Pierce Biotechnology, Rockford, IL, USA) with bovine serum albumin (Thermo Fisher Scientific, Waltham, MA, USA) as a standard. Thirty micrograms of hepatic protein was separated by 12% SDS (sodium dodecyl sulfate)-polyacrylamide gel electrophoresis and electrotransferred onto a nitrocellulose membrane. The blots were blocked with 5% nonfat milk for 2 h, and then the membranes were briefly washed with Tris-buffered saline containing 0.05% Tween 20 and incubated with anti-FLAG antibody (1:7000) (Sigma-Aldrich, St. Louis, MO, USA) at 4°C overnight. The membranes were stained with goat-anti-mouse IgG secondary antibodies (1:10,000) (Cell Signaling Technology, Danvers, MA, USA) and then developed with the enhanced chemiluminescence reagents (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

**Liver histology**

When the mice were sacrificed, one specimen each from the median, the left and right lobes of the liver was harvested, and the small pieces were fixed immediately in 10% formalin. After paraffin embedding, 5-μm-thick sections were prepared, stained with either hematoxylin-eosin or Oil-Red O (Sigma-Aldrich). Immunohistochemistry was performed to detect the response of F4/80 in sections using a rat anti-mouse F4/80 antibodies (1/500) (Proteintech, Chicago, IL, USA). The tissue morphology was examined under light
microscopy (Leica, Germany). The inflammatory foci were quantified by image analysis software Image-Pro Plus 6.0.

**Lipid measurement**

The hepatic tissues (150 mg) were homogenized, and the triglyceride (TG), cholesterol (CHO) and free fatty acid (FFA) levels were examined according to the manufacturer's instructions for the Triglyceride/Cholesterol/Free Fatty Acid Quantification Kit (Applygen Technologies, Beijing, China). Serum samples were obtained according to previous method (21), and the TG, CHO and FFA levels were determined as well.

**Determination of ATP levels and CPT-1 activity**

The isolation of the mitochondrial fractions was performed using the Cytosol/Mitochondria Isolation Kit (Applygen) according to the manufacturer’s instructions. Fresh tissues were quickly removed, chopped into small pieces (1 mm³) and placed in ice-cold mitochondrial isolation buffer. After homogenization, the homogenate was centrifuged at 800 g for 5 min at 4°C. Next, the supernatant was collected and further centrifuged at 800 g for 10 min at 4°C. The pellet was gently resuspended in isolation buffer and centrifuged at 12,000 g for 10 min at 4°C. Subsequently, the pellet was resuspended in isolation buffer and centrifuged at 12,000 g for 10 min at 4°C. The mitochondrial pellet was resuspended in the same isolation buffer, and the protein concentration was determined using the BCA method. The isolation of mitochondria from the cultured cells was performed according to the manufacturer’s instructions for the Mitochondria/Cytosol Isolation Kit. The cells were harvested and homogenized in 1.5 ml of ice-cold Mito-Cyto Buffer using a Dounce homogenizer. Following homogenization, the remaining steps of the protocol are same as those for isolating mitochondria from liver.

The level of ATP was determined using the Cell Titer-Glo Luminescent Cell Viability Assay Kit (Promega, Madison, WI, USA) according to the manufacturer’s instructions. Briefly, 100 μg of mitochondrial protein was added to 96-well plates. The reaction was initiated by adding mitochondrial buffer and 100 μl of reaction liquid in a final volume of 200 μl and incubating at room temperature for 30 min. Next, the mixture was incubated at 21°C for 2 min and then incubated at room temperature for another 10 min. The 96-well plate was then centrifuged at 12,000 g for 10 min at 4°C, and the supernatant was collected to another
96-well plate. Subsequently, the ATP levels were assayed using an ELx800 microplate reader (BioTek Instruments, Winooski, VT, USA).

The CPT-1 activity was measured using a CPT-1 assay kit (Genmed Scientific, Arlington, MA, USA) according to the manufacturer’s instructions. Briefly, GENMED buffer, reaction buffer and substrate buffer were added together in turn, incubated for 3 min at 25°C, then the protein (either 50 μg of protein from the liver tissue extract, or 40 μg of protein from the cultured cells or 20 μg of protein from the mitochondria) was added to mix respectively. Finally, the mixture was observed using an ELx800 microplate reader (BioTek Instruments) to evaluate the CPT-1 activity level.

Detection of oxidative stress, β-hydroxybutyrate (β-HB) and malonyl-CoA concentrations

Hepatic MDA/cellular ROS was tested by MDA assay kit /ROS assay kit (both from Beyotime, Shanghai, China) according to the manufacturer’s instructions. Serum β-HB concentrations was detected by β-HB assay kit (Biovision, San Diego, CA, USA ) according to the manufacturer’s instructions. The hepatic tissues (100 mg) were homogenized, and the supernatant was obtained, and then hepatic β-HB levels were determined as above and the levels of malonyl-CoA were determined by ELISA.

HSS gene transfection and expression in cells

To confirm the HSS protective effect obtained in an in vitro experiment, HepG2 cells were stably transfected with the HSS construct as previously described (44). Briefly, the cells were cultured at 37°C in a 5% CO₂ humidified atmosphere incubator with DMEM medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. A total of 2×10⁶ cells were allowed to grow to 50-70% confluence. To overexpress HSS, the cells were transfected with 5 μg of HSS-pcDNA 3.1 plasmid using Lipofectamine 2000 (Invitrogen, Carlsbad, CA,USA) (15). The pcDNA 3.1 vector was simultaneously transfected as a control. Eight hours post-transfection, the cells were selected using G418 (400 μg/ml) (Sigma-Aldrich, St. Louis, MO, USA) for 14 days. The cells resistant to G418 were used for further study.

To inhibit the HSS expression, HepG2 cells were stably transfected with HSS-specific siRNAs (Dharmacon, Lafayette, CO, USA) according to standard protocols (45). Briefly, the
cells were seeded in a 6-well plate (3×10⁵ cells per well) and grown in DMEM hepatocyte basal culture medium without antibiotics for 24 h to 70-80% confluence. For the transfection, the cells were then incubated with the siRNA and DharmaFECT 4 transfection reagent at 37°C for 24 h. After 24 h, the transfection was terminated by the addition of DMEM hepatocyte basal culture medium. The scrambled siRNAs were transfected in parallel as an internal control.

To measure cellular HSS expression, real-time PCR was used to measure the mRNA level of HSS. Total RNA was extracted from the HSS-expressing cells, the vector-transfected cells and the wild-type cells by using the QIAamp RNA Purification Kit (Qiagen, Hilden, Germany) and the HSS mRNA expression was evaluated as previously described (44). The specific primers for real-time amplification of the HSS mRNA are listed in Supplemental Table 1. The 18S rRNA was amplified as an internal standard.

For the HSS protein level assay, a Western blotting was performed as described above except that the blot membrane was hybridized with anti-HSS antibody (1:1000, Santa Cruz Biotechnology) at 4°C overnight. The hybridization with the secondary antibody and visualization of the positive signals were performed similarly to that described above.

**Hepatocyte steatosis**

A total of 3.0×10⁴ HepG2 cells suspended in 1 ml of culture medium were added to each well in 24-well plates. The cells were treated with oleic acid (OA, 300 μM) for 24 h (24) and then stained with Oil-Red O. The cells not subjected to OA treatment were used as a control. Then, TG, CHO and FFA levels were measured as described above. The experiments were carried out in triplicates.

To confirm inflammation during hepatic steatosis, mRNAs of inflammatory factors such as tumor necrosis factor-beta (TNF-α), interleukin-1 beta (IL-1β) and interleukin-6 (IL-6) were recorded. The RT-PCR method is similar as described above. In addition, the mRNA level of lipogenesis gene acetyl-coenzyme A carboxylase 1 (ACC1), fatty acid synthase (FAS) was also monitored. The specific primers for real-time amplification of the specific gene are listed in Supplemental Table 1.

**Detection of Bcl-2 and Bax expression**
After treatment with 250 μM palmitic acid (PA) for 24 h, the cytosol proteins were extracted and Western blotting was performed as described above. Anti-Bcl-2 antibody (Cell signal technology, Boston, MA, USA) and anti-Bax antibody (Cell signal technology) were used as 1:1000. Actin (Santa Cruz, USA) was used as internal standard.

**Measurement of cytosolic and mitochondrial CPT-1 activity**

After treatment with 250 μM OA for 24 h for the HSS-transfected cells and the HSS-siRNA cells, the proteins were extracted from the cytosol and the mitochondria compartment. To optimize the measurement of the CPT-1 activity, C75 (an activator of CPT-1) and S15176 (an inhibitor of CPT-1, both from Sigma-Aldrich) were applied. Various concentration of C75 (5, 20, 50, or 100 μM) or S15176 (20, 40, or 80 μM) were added into the cell culture or into 20 μg of the mitochondrial aliquot. The reaction was carried out at 37°C for 10 min (for cell culture) and 5 min (for isolated mitochondria), respectively, followed by centrifugation for 10 min at 12,000 g at 4°C. The buffer was decanted, and the resulting pellet was carefully washed with 0.2 ml of ice-cold binding buffer and centrifuged again at 12,000 g at 4°C for 10 min. Then, the CPT-1 activity was measured as described above.

To effectively inhibit CPT-1 expression, specific siRNA was transfected to HepG2 cells. The control transfection was conducted with a scrambled siRNA transfection in parallel. The expression of the CPT-1 after siRNA knockdown was detected by Western blotting and real-time PCR.

**Statistical analyses**

All data were expressed as the mean ± standard deviation (SD), and the statistical significance of the differences between groups was assessed using Student’s t-test. A level of $P < 0.05$ was regarded as statistically significant.
Results

The HSS expression after pAdxi-Flag-HSS transfection in mice

The GFP expression indicated that approximately 10% of hepatocytes were transfected with the indicated gene at 15 days after injection of $1 \times 10^9$ pAdxi-GFP plasmids (Fig. 1A). The GFP expression was apparent in the heart, kidneys and lungs, but the expression was less visible in the spleen (Fig. 1A). Moreover, the exogenous HSS expression in the liver of C57BL/6J mice fed for 1-4 weeks was assayed by Western blotting analysis, after injection of the HSS-bearing plasmids (Fig. 1B). The endogenous HSS expression was detected by Western blotting analysis after MCD diet at the 2nd and 4th week (Fig. 1C). The results demonstrated the exogenous FLAG-HSS was effectively expressed from 1-4 weeks, and the endogenous HSS was deceased in MCD-fed mice.

HSS expression reduced hepatic steatosis in the MCD-fed mice

Some C57BL/6J mice were subjected to the control diet, whereas the MCD diet was fed to non-transfected animals, vector-transfected animals and the HSS gene-transfected animals, and the development of NASH in these mice was recorded. As shown in Fig. 2A, the body weights of mice fed the control diet increased steadily to approximately 28 g within 4 weeks, while, by contrast, the body weights of the MCD-fed mice markedly decreased in all the groups. However, the decrease in body weight of mice subjected to the HSS gene transfection was less severe than in the vector-transfected or non-transfected mice, indicating that the HSS transfection significantly improved the MCD diet-induced body weight loss. Histological analyses (Fig. 2B, upper panel and middle panel) demonstrated that the hepatic steatosis was significantly more serious in the livers of MCD-fed mice compared with the livers of mice that underwent the HSS gene transfection 4 weeks after starting the MCD diet. As shown in Fig. 2B, upper panel and middle panel), significantly more lipid accumulated in the liver tissue from the vector-transfected or non-transfected mice compared with the control or HSS-transfected mice. In order to estimate the hepatic inflammation in MCD-fed mice and the effect of HSS on inflammation upon HSS gene transfection, macrophage staining (F4/80 staining) and the measurement of mRNA levels of inflammation factor IL-1β, IL-6 and TNF-α were conducted. There was the positive staining in the liver of MCD diet mice, by
contrast it was almost not detected in HSS-expressing mice (Fig. 2B lower panel), the quantification of inflammatory foci demonstrated that it was increasing to 2.2%, and obviously decreased into 0.48% (Fig. 2E). Meanwhile, the mRNA levels of inflammation factors, IL-1β, IL-6 and TNF-α, were dramatically elevated to 178.16%, 156.16% and 210.05% in MCD-mice compared with control, and notably downregulated in HSS-transfected mice by 47.81%, 50.07% and 51.16% compared with vector-transfected mice (Fig. 2D). The results showed that the inflammation was significantly more severe in MCD-mice, and it was lightened in HSS-expressing mice. Serum transaminase levels (ALT, AST) were detected to evaluate the liver function, The result demonstrated that they were seriously higher in MCD-mice at 1-4 weeks and 3-4 weeks, respectively, as compared with control, and it was significantly relieved in 2-4 weeks in HSS-expressing mice as compared with vector-treated mice (Fig. 2C). To assess the oxidative stress, the hepatic MDA levels at the 4th week were tested, and the results showed that MDA levels were significantly elevated in MCD-diet mice by 345.18% as compared with control, and HSS could observably reduce the MDA levels that were induced by MCD diet by 51.49% if comparing with vector transfected animals (Fig. 2F). β-HB concentrations in liver and serum have been obviously reduced (Fig. 2G) by HSS transfection.

Consistent with these histological findings, the hepatic lipid depositions such as triglycerides and free fatty acids (Fig. 3D-E), were more obvious in the vector-transfected or non-transfected mice than the HSS-expressing animals. The hepatic cholesterol content, was significantly decreased in the first week after MCD diet, but not in the week 2-4 in HSS-expressing animals (Fig. 3C). In contrast to the hepatic steatosis findings, the serum levels of triglycerides and free fatty acids are higher in the HSS-expressing mice than in the non-transfected mice (Fig. 3A-B).

HSS preserved ATP production and protected CPT-1 activity in the MCD-fed mice

The hepatic ATP levels were greatly decreased in the MCD-fed mice including the vector-transfected group and the HSS-expressing group (Fig. 4A). However, compared with the vector-transfected mice, the ATP content in the livers of the HSS-transfected mice was significantly elevated by 37.51% (P<0.01) at 3 weeks and by 86.16% (P<0.01) at 4 weeks of
the diet treatment. These results suggested that in the HSS-expressing mice, energy production was less impaired by lipotoxicity to mitochondrial β-oxidation. CPT-1 is a mitochondrial enzyme responsible for the formation of acyl carnitines by catalyzing the transfer of the acyl group of a long-chain fatty acyl-CoA from coenzyme A to l-carnitine. Because CPT-1 plays an important role in fatty acid metabolism (27, 34), we next measured the alteration of hepatic CPT-1 activity in the different groups of mice fed the MCD-diet for 4 weeks. As indicated in Fig. 4B, the hepatic CPT-1 activities were severely reduced as a result of the MCD diet from the 1st week to the 4th week. However, the enzymatic activities were significantly restored by the HSS-transfection to 229.17%, 180.25%, and 191.17% at the 1st, 2nd and 4th week, respectively, compared with vector transfection, although the decrease in activity remained significant relative to the control mice which were fed a chow diet (Fig. 4B).

To evaluate effect on the lipogenesis by HSS, in addition to FFA content, we measured the mRNA levels of two lipogenic enzymes, ACC1 and FAS. The results suggested that HSS had no significant effect on the mRNA levels of ACC1 and FAS, but it significantly lessened the hepatic FFA content (data not shown). These results suggested that the decrease in hepatic FFA content by HSS was not due to the inhibition of lipogenesis. Then the concentrations of hepatic malonyl-CoA, the metabolite known to inhibit CPT1 activity, in mice fed with MCD diet were detected. The results demonstrated that there was no significant difference in hepatic malonyl-CoA levels between groups (data not shown), suggesting that the CPT-1 activity was improved by HSS, but not regulated by malonyl-CoA.

**HSS expression reduced hepatic steatosis in the HF-fed mice**

One hundred and twenty mice of strain C57BL/6J were divided into 4 groups, the control group was subjected to the regular diet, whereas the HF diet was fed to the other three groups including non-transfected animals, vector-transfected animals and the HSS gene-transfected animals. The development of NASH in all mice was prepared. As shown in Fig.5A, the body weights of mice fed the control diet increased steadily to approximately 31 g within 16 weeks, while, the body weights of the HF-fed mice markedly increased to approximately 50 g. However, the increase in body weight of mice transfected with the HSS gene was less severe than in the vector-transfected or non-transfected mice, indicating that the HSS transfection
significantly lessened the HF diet-induced adiposity. Liver weights were recorded, and they were markedly heavier in HF diet fed mice for 16 weeks than those of control mice, and the average liver weight was 2.91 g in HF group, while it was 1.71 g in control group. And the average liver weight for the HSS-transfected group and vector-transfected group were 1.93 g and 2.85 g, respectively (Fig.5B). The liver/body weight ratio was increased at week 16 after high fat diet and decreased by HSS expression (Fig.5C). Serum transaminase (ALT/AST) levels were risen by HF diet and were relieved by HSS (Fig.5D, E). The hepatic lipid depositions such as triglycerides and FFA were also accumulated after HF diet for 16 weeks and were notably reduced in HSS-expressing animals (Fig. 5H, I). In contrast to the hepatic steatosis, the serum levels of triglycerides and cholesterol are higher in the HSS-expressing mice than in the non-transfected mice (Fig. 5F, G).

Histological analyses (Fig. 6A upper panel and middle panel) demonstrated that the hepatic steatosis was significantly more serious in the livers of HF fed mice subject to vector transfection compared with that underwent the HSS transfection 16 weeks after starting the HF diet. As shown in Fig. 6A upper panel and middle panel, significantly more lipid accumulated in the liver tissue from the vector-transfected or non-transfected mice compared with the control or HSS-transfected mice. The hepatic inflammation and oxidase stress index were also evaluated in HF-fed mice. Macrophage staining (F4/80) and the mRNA levels of inflammation factors, TNF-α, IL-6 and IL-1β, indicated the inflammation was more severe in HF-diet mice, and it was lightened in HSS-expressing mice (Fig.6A lower panel, B). The hepatic MDA levels in week 16 were tested, the results showed that MDA levels were augmented in HF-diet mice by 238.16% as compared with control and HSS could significantly reduce the MDA level by 28.17% as comparing with vector transfection (Fig. 6C).

The effect of HSS on OA-induced steatosis and PA-induced lipotoxicity

As demonstrated in the in vivo study above, feeding mice the MCD diet for 4 weeks led to severe damage to the host livers and massive accumulation of lipids in the hepatocytes, which decreased the ATP production and the mitochondrial CPT-1 activity. However, this lipotoxicity to hepatocytes could be rescued by HSS gene transfection. It had previously been
reported that HSS could protect hepatocytes from injuries caused by various toxins (17, 23, 45). Therefore, it is reasonable to question if the protection provided by HSS is due to preservation of CPT-1 activity; if so, more additional supporting data are required from an in vitro experiment. First, HSS was successfully transfected and expressed in the cultured HepG2 cells, as demonstrated by the real-time PCR and Western blotting analysis (Fig. 7A).

After incubation with 300 μM OA for 24 h, the HSS-expressing cells appeared to be resistant to the OA-induced lipid accumulation observed within the control cells (Fig. 7C-E, Fig. 9D). Lipotoxicity has been then evaluated by the incubation of cells with 250 μM PA for 24 h. The production of ROS was severely up-regulated to 145.32% by PA as compared with control, and notably alleviated by 50.16% in HSS-expressing cells as a contrast to vector treatment cells (Fig. 7F). Bcl-2 and Bax which involved in apoptosis were detected by Western blotting, the expression of Bax was sharply enhanced after PA treatment as compared with control and was reduced in HSS-expressing cells. The expression pattern of Bcl-2 is opposite (Fig.7G).

The results showed that HSS alleviated apoptosis caused by lipotoxicity, compared with control cells transfected with vectors.

**HSS increased CPT-1 activity in cells and isolated mitochondria**

As the mitochondrial gateway for fatty acid entry into the matrix, CPT-1 is an essential enzyme for FFA metabolism. To clarify the mechanism of lessening lipid accumulation which induced steatosis by HSS with CPT-1, the CPT-1 activity was analyzed. The results showed that the overexpression of HSS promoted CPT-1 activity by approximately 33.64% \( (P<0.05) \), whereas the knockdown of HSS inhibited CPT-1 activity by approximately 13.83% \( (P<0.05) \), as compared with the vector-transfected cells, respectively (Fig. 8A). Interestingly, recombinant HSS (rHSS), if added into the culture medium, could rescue the CPT-1 activity destroyed by 250 μM of OA in a dose-dependent manner. The maximum effect appeared at 50 μM of rHSS, and at this point, the rescue could be augmented further by adding 80 μM of C75, a specific activator of CPT-1 enzyme, into the culture medium (Fig. 8B). After down-regulation of CPT-1 by siRNA (Fig. 7B), the OA-induced lipid accumulation were not improved (Fig. 7C-E).
To explore the possibility that the HSS-induced alleviation of the OA-induced hepatocyte steatosis is due to enhancement of the mitochondrial CPT-1 activity, the HSS-expressing cells and isolated mitochondria were employed. As shown in Fig. 8C, the cellular CPT-1 activity was markedly decreased after the addition of 250 μM OA. To further examine the CPT-1 activity following OA-induced lipotoxic injury, various doses of S15176 and C75 (a specific inhibitor and activator of CPT-1, respectively) were used. As shown in Fig. 8C, the OA-induced toxicity to CPT-1 activity was worsened when S15176 was added to the cells at a concentration up to 100 μM. However, the stepwise inhibition of CPT-1 activity by OA could be effectively reversed in a dose-dependent manner by treating the cells with C75. Similarly, the restoration of CPT-1 activity could also be observed in the HSS-transfected cells. More importantly, a synergistic effect was observed when C75 was added into the medium of HSS-transfected cells.

In order to verify the protective effect of HSS on CPT-1 activity, we added various doses of S15176 and C75 to the isolated mitochondria subjected to OA injury. After the addition of 20 μM of S15176 to the isolated mitochondria, the CPT-1 activity was quickly and significantly decreased (Fig. 8D). However, this reduction of the enzyme activity was significantly mitigated in the mitochondria extracted from HSS-expression cells. The CPT-1 activity was restored by 45.16% (P<0.01) compared with the mitochondria treated with 20 μM of S15176 (Fig. 7B). S15176 (20 μM) caused a decrease in CPT-1 activity in the isolated mitochondria, and this decrease was reduced by treating mitochondria with HSS (Fig. 8D). C75 (at 20 μM) caused an increase in CPT-1 activity in the mitochondria, and this increase was further enhanced in the mitochondria of the HSS-transfected cells. These data clearly suggest that HSS may play an important role in the regulation of the CPT-1 activity by amplifying C75 activation and by diminishing the inhibitory effect of S15176.

**HSS increased the mRNA and protein expression levels of CPT-1 in HepG2 cells**

Finally, we are interested to see if the preservation of the CPT-1 activity in the HSS-expressing cells will help the cells exclude lipid droplets from accumulating in the cytosol. The elevated or declined expression of HSS was achieved by the transfection of HSS gene or HSS-specific siRNA, respectively. As shown in Fig. 9A-C, OA treatment itself did not
cause drastic change the cellular CPT-1 expression, but its protein and mRNA expression was clearly increased by approximately 43.76% ($P<0.01$) and 49.39% ($P<0.01$) in the HSS-expressing cells, respectively, and decreased by approximately 50.24% ($P<0.01$) and 49.99% ($P<0.01$) in the HSS-siRNA cells, respectively, as compared with the control. Although the mechanism of HSS regulation on CPT-1 expression or synthesis remains unclear, it is no doubt that the elevated CPT-1 activity in the HSS-transfected cells would help the cells increase the metabolism of fatty acids within the mitochondria and, as a result, fewer lipids would be present to be stained by oil red O (Fig. 9D). By contrast, the decline in CPT-1 activity in the HSS-siRNA cells reduced mitochondrial $\beta$-oxidation, resulting in the increased accumulation of lipids in the cytosol (Fig. 9D). These results demonstrated that HSS protects hepatocytes from steatotic injury most likely by regulating the activity of CPT-1, resulting in an alleviation of the accumulation of lipid droplets.

To realize CPT-1 alteration could actually affect NAFLD in an animal model, mice with CPT-1 insufficiency were created by hydrodynamic tail vein injection of shRNA-CPT-1-GFP-pGPU6 plasmid(21). The hepatic CPT-1 levels were analyzed with Western blot. Fig. 10A showed that CPT-1 expression was effectively decreased in CPT-1-knockdown mice, and meanwhile, lipid accumulation (triglyceride and cholesterol) and hepatic lipid deposition were deteriorated which came along with liver function injury and inflammation (Fig. 10B-F), suggesting that CPT-1 plays a vital function in preventing hepatocytes from steatosis.
It has been reported that 5% of NAFLD patients develop liver cirrhosis (1). To date, few therapeutic strategies have been proven effective for patients with NASH, the progressive form of NAFLD (46). Hepatic steatosis accompanied with inflammation is a well-known histological and biochemical feature of NASH. Progressive steatosis causes liver insufficiency and portal hypertension which is a risk factor for developing fibrosis and cirrhosis as well. Therefore, any possible interventions that could protect the liver from steatosis would be important therapeutic targets for patients with NASH. HSS has been confirmed to effectively promote liver protection and regeneration, however, little is known about its effect on hepatic steatosis. Therefore, the purpose of the current study is to investigate if HSS is able to rescue hepatocytes from lipotoxicity, and if so, to determine if the mechanism of action could be via HSS promotion of lipid metabolism. One of key points we address is whether HSS could help the hepatocytes eliminate excessive fat deposition in the liver. CPT-1, residing in the mitochondrial outer membrane, is a key rate-limiting enzyme of mitochondrion β-oxidation. It catalyzes the conversion of long-chain acyl-CoA to acylcarnitine, which enters the mitochondrial matrix and undergoes β-oxidation. It has been reported that CPT-1 is closely related with the pathogenesis of NAFLD. Hepatic CPT-1 expression was significantly inhibited in liver injury and fat accumulation rats (40). Our results also showed that the CPT-1 knockdown mice fed with MCD diet had more severe hepatic lipid accumulation and inflammation (Fig.10), which is identical to the results found in high-fat diet-fed CPT-1 heterozygote knockout mice(29). On the contrary, the upregulation of CPT-1 expression could attenuate NAFLD through mobilizing fatty acid oxidation in the liver (30). Maja Stefanovic-Racic et al. also showed that overexpression of CPT-1 prevented the metabolic disorders caused by MCD diet, including reducing hepatic triglyceride levels(41). These data suggest a close relationship between alternation of CPT-1 and development of NAFLD.

In an in vivo experiment, it has been demonstrated that the hepatic steatosis induced by the MCD-diet was alleviated in the mice that received the HSS gene transfection. Our assessment of pAdxsi-Flag-HSS (pAdxsi-GFP) delivery indicated that enough HSS protein was produced to exert protective effects on mice with NASH. In agreement with our previous studies, the
results showed that the MCD diet led to weight loss, severe steatosis as assessed by HE/Oil-Red O staining, liver injury as assessed by transaminase (AST, ALT) levels, hepatic inflammation index, hepatic lipid levels, oxidative stress and energy production (Figs. 1-4). It is demonstrated from these indexes that liver function and morphology were improved by HSS transfection. HF diet induced hepatic steatosis and the alternation of these parameters exhibited similar with those observed in MCD-mice (Figs. 5,6). It is interesting to note that the hepatic activity of CPT-1 significantly decreased in MCD-fed mice or in OA-treated cells. However, the HSS gene transfection effectively mitigated these decreases and resulted in an amelioration of the hepatic steatosis and an elevation of the hepatic activity of CPT-1, resulting in preservation of the ATP levels. By expanding these findings, our results suggest for the first time that HSS could preserve mitochondrial CPT-1 activity and facilitate the clearance of excessive hepatic lipids during NASH formation. These novel data provide a fresh outlook for evaluating the function of HSS, which had previously only been known as a co-mitogen for augmenting the liver growth response when combined with other growth factors or cytokines (9, 22).

To further confirm the hypothesis that HSS preserves the CPT-1 activity, additional experiments exploring the effects of the HSS-gene transfer and HSS-siRNA inhibition were performed in the hepatocytes and in the isolated mitochondria. The HSS protection of HepG2 cells from OA-induced lipotoxic injury was successfully demonstrated. Meanwhile, the OA-induced impairment of mitochondrial CPT-1 activity and ATP production was clearly rescued (Figs. 5-8). Of particular interest, the HSS protection of CPT-1 activity was compared with the effects of S15176 and C75, a known inhibitor and activator of CPT-1, respectively. The purpose of employing these two agents is to clarify whether HSS protection of CPT-1 activity is specific. S15176 and C75 explore the specific influence of CPT-1 activity and indicate that HSS can compensate for the decrease in CPT-1 activity caused by S15176 and enhance CPT-1 activity caused by C75 (Fig. 7). Therefore, we predict that the protection of HSS on CTP-1 could be with reasonably high specificity; more detailed experiments are certainly required. To verify the data obtained from the gene transfections, we also used recombinant HSS (rHSS) protein to check if it could preserve mitochondrial CPT-1 activity. As shown in Fig. 6B, the rHSS did protect mitochondrial CPT-1 activity in a dose-dependent
manner. Further supporting data are seen in Fig. 6A and Fig. 8, as HSS-siRNA treatment could reverse the HSS protective effect and result in lipid droplet accumulation in HepG2 cells, strengthening the findings that HSS promotes lipid removal via the regulation of CPT-1 activity.

CPT-1 is one of the key enzymes regulating fatty acid metabolism in the mitochondrial membrane. With increment of the CPT-1 activity, large amount of FFA could be transported into mitochondria where it is subject to beta-oxidation. In this case, FFA content was reasonably reduced. If CPT-1 expression was inhibited by siRNA, suppressive effect of HSS on hepatic lipid accumulation could not be preserved (Fig. 7E), suggesting that the prevention of HSS from hepatic lipid deposition might be partially via its regulation on CPT-1 activity which could finally enhance FFA transportation into mitochondria where it is subject to degradation.

In current study, compared with vector injected mice, the HSS-expressing mice partially appeared the weight loss during high fat diet (Fig. 5A), which may be caused either by decrease in food intake or increase in energy expenditure. However, there is no significant difference in food intake in mice, hence, it is predicted that the weight loss in the HSS-expressing mice was partially due to an increase in CPT-1 activity modulated by HSS and subsequently boosting of fatty acid beta-oxidation in mitochondria.

In conclusion, HSS protects the liver against NAFLD injury through the regulation of the mitochondrial lipid metabolism via enhancement of CPT-1 activity.
Acknowledgments

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**Fig. 1. The expression of HSS in mice induced with pAdxsi-Flag-HSS plasmids.** (A), transfection of the reporter gene pAdxsi-GFP via the femoral vein. GFP was expressed in the liver 15 days after the injection of $1 \times 10^9$ pAdxsi-Flag-HSS plasmids, whereas GFP was also visible in the heart, lungs, spleen and kidney. Liver biopsies from saline-treated mice were used as controls in the heart, lung, spleen, kidney and liver ($\times$40). (B), HSS expression in the liver at different time points (1-4 weeks) after injection of $1 \times 10^9$ pAdxsi-Flag-HSS plasmids was assayed by Western blotting analysis (n=6). The HSS gene was tagged with Flag, and anti-Flag tag monoclonal antibody was used to exclusively detect exogenous HSS. Antibodies against GAPDH were used for normalization of the loading controls. (C) The endogenous HSS expression in the liver was detected by Western blotting analysis.

**Fig. 2. The effect of HSS expression on steatosis in MCD-fed mice.** (A), the change of body weight in each group over four weeks. HSS expression in the liver rescued mice from the weight loss caused by the MCD diet. a, $P<0.05$ versus control, aa, $P<0.01$ versus control; b, $P<0.05$ versus vector. (B), the hepatic Oil-Red O staining of frozen liver sections (upper panel), HE staining (middle panel) and immunochemistry for F4/80, murine macrophage marker (bottom panel) (n=6) ($\times$200). (C), serum ALT level (left panel), serum ALT level (right panel) (D), mRNA levels of IL-1β (left panel), IL-6 (middle panel) and TNF-α (right panel) in liver of mice. (E), quantification of inflammatory foci in F4/80. (F), hepatic MDA levels. (G), serum and hepatic β-HB level. (n=6), Control, mice fed the regular diet; MCD, mice fed the MCD-diet; vector-Tx, mice transfected with the empty vector; HSS-Tx, mice transfected with $1 \times 10^9$ pAdxsi-Flag-HSS. Serum samples were prepared every week. Values are means ± SD, a, $P<0.05$ versus control, aa, $P<0.01$ versus control; b, $P<0.05$ versus vector, bb, $P<0.01$ versus vector.

**Fig. 3. The effect of HSS expression on lipids levels in MCD-fed mice.** Serum cholesterol (A), serum triglyceride (B), hepatic cholesterol (C), hepatic triglyceride (D) and hepatic free fatty acid (E) levels are shown. Control, mice fed the control diet; MCD, mice fed the MCD-diet; vector-Tx, mice transfected with the control vector; HSS-Tx, mice transfected with $1 \times 10^9$ pAdxsi-Flag-HSS. Serum samples were prepared every week. Values are means ±
Fig. 4. HSS expression preserved mitochondrial respiratory function. Mitochondria freshly isolated were used to measure the ATP levels (A), and the CPT-1 activity (B). a, P<0.05 versus control, aa, P<0.01 versus control; b, P<0.05 versus vector, bb, P<0.01 versus vector.

Fig. 5. The effect of HSS expression on lipids levels in the HF-fed mice at week 16. (A), the change of body weight in 16 weeks. HSS expression in the liver lessened the weight caused by the HF diet; (B), liver weights at week 16; (C), liver weight/body weight at week 16. Serum ALT (D); serum AST (E); serum cholesterol (F); serum triglyceride (G); hepatic free fatty acid (H); and hepatic triglyceride (I). Control, mice fed the control diet; HF, mice fed the high-fat-diet; vector-Tx, mice transfected with the control vector; HSS-Tx, mice transfected with 1×10⁹ pAdxsi-Flag-HSS. (n=6), a, P<0.05 versus control, aa, P<0.01 versus control; b, P<0.05 versus vector, bb, P<0.01 versus vector.

Fig. 6. HSS expression reduced hepatic steatosis in the HF-fed mice at week 16. (A), the hepatic Oil-Red O staining of frozen liver sections (upper panel), HE staining (middle panel), immunohistochemistry for F4/80 (lower panel) (×200); (B), mRNA levels of IL-1β (left panel), IL-6 (middle panel) and TNF-α (right panel) in the liver of mice; (C), hepatic MDA levels. (n=6). aa, P<0.01 versus control; b, P<0.05 versus vector, bb, P<0.01 versus vector.

Fig. 7. The effect of HSS on OA-induced steatosis and PA-induced lipotoxicity. (A), the levels of HSS RNA in the three types of cells was quantified using real-time PCR. The expression of HSS in cells stably transfected with the HSS expression construct is significantly greater than in wild-type cells and vector-Tx cells (upper panel). The differential expression of HSS in the three cell lines was also assayed. Mitochondrial protein extracts (25 g) from each of the cell cultures were analyzed using Western blotting and an antibody against HSS (lower panel); (B), mRNA level (upper panel) and protein expression (lower
panel) of CPT-1 were measured in cells transfected with CPT-1-siRNA. After incubation with 300 μM OA for 24 h, the OA-induced lipid accumulation including cellular cholesterol (C), triglyceride (D), and free fatty acid (E) levels was observed. Lipotoxicity has been then evaluated by the incubation of cells with 250 μM PA for 24 h (F,G); cellular ROS (F); expression of Bcl-2 and Bax (G). a, P<0.05 versus control, aa, P<0.01 versus control; b, P<0.05 versus vector, bb, P<0.01 versus vector.

Fig. 8. HSS increased CPT-1 activity in cells and isolated mitochondria. Equal cell numbers from different cultures were treated with OA (250 μM) for 24 h, then the mitochondria were isolated. (A), HSS expression increased the CPT-1 activity, rescuing the OA-induced toxicity, whereas in the HSS-siRNA group, the activity of CPT-1 decreased remarkably. a, P<0.05 versus vector, aa, P<0.01 versus vector. (B), recombinant HSS (rHSS) protein was added to the mitochondria as indicated, and the effects on the CPT-1 activity were analyzed. a, P<0.05 versus treatment with OA. b, P<0.05 versus control. The results from three independent experiments were statistically evaluated. (C), equal cell numbers from different cultures were treated with OA (250 μM) for 24 h. C75 or S15176 were added to the cells at the concentration indicated, and their effects on CPT-1 activity were analyzed. The activity was increased or decreased in a dose-dependent manner; a, P<0.05 versus treatment with OA, aa, P<0.01 versus treatment with OA, bb, P<0.01 versus treatment with OA+S15176. cc, P<0.01 versus treatment with OA. dd, P<0.01 versus treatment with OA+OA+C75. e, P<0.05 versus treatment with OA+C75+HSS. (D), Equal cell numbers from different cultures were treated with OA (250 μM) for 24 h. The mitochondria were then isolated, C75 or S15176 were added to the mitochondria, and their effects on the CPT-1 activity were analyzed. The CPT-1 activity increased or decreased in a dose-dependent manner. a, P<0.05 versus treatment with OA; bb, P<0.01 versus treatment with OA+S15176; cc, P<0.01 versus treatment with OA; d, P<0.05 versus treatment with OA+C75. ee, P<0.01 versus treatment with OA+C75+HSS. The results from three independent experiments were statistically evaluated.
Fig. 9. HSS increased the mRNA and protein expression levels of CPT-1 in HepG2 cells.
(A, B), the protein expression of CPT-1 in cells. The expression of CPT-1 in cells stably transfected with the HSS DNA construct is significantly greater than in wild-type cells and vector-Tx cells, whereas the expression of CPT-1 in cells stably transfected with the HSS-siRNA construct is significantly lower than in vector-Tx cells. (C), the levels of CPT-1 RNA in the five types of cells was quantified using real-time-PCR. aa, $P<0.01$ versus vector-Tx. (D), Oil-Red O staining of HepG2 cells stably transfected with Flag-HSS or HSS-siRNA or vector (×200).

Fig. 10. The effect of shRNA-CPT-1 transfection on hepatic steatosis/inflammation in MCD-fed mice at week 2. Mice were injected with 0.2 mg shRNA-CPT-1-GFP-pGPU6 plasmids after MCD-fed mice for 2 weeks, and then sacrificed after 3 days. (A), CPT-1 expression in the liver was assayed by Western blotting analysis (n=6). (B), serum ALT level. (C), serum AST level. (D), Serum cholesterol level. (E), Serum triglyceride level. (F), the hepatic Oil-Red O staining of frozen liver sections (upper panel), HE staining (middle panel) and immunochemistry for F4/80 (bottom panel) (n=6) (×200). Control, mice fed the regular diet; MCD, mice fed the MCD-diet; shRNA-CPT-1, mice transfected with the shRNA-CPT-1-GFP-pGPU6 plasmid; shRNA-scramble, mice transfected with shRNA-scramble-GFP-pGPU6 plasmid; Values are means ± SD, a, $P<0.05$ versus control, aa, $P<0.01$ versus control; b, $P<0.05$ versus shRNA-scramble, bb, $P<0.01$ versus shRNA-scramble.
References


19. LaBrecque DR and Pesch LA. Preparation and partial characterization of hepatic regenerative


35. Rezaadzeh A, Yazdanparast R, and Molaei M. Amelioration of diet-induced nonalcoholic
steatohepatitis in rats by Mn-salen complexes via reduction of oxidative stress. J Biomed Sci 19:
26,2012.
expression is involved in the development of nonalcoholic steatohepatitis in a dietary murine model.
and Altomare E. Uncoupling protein-2 (UCP2) induces mitochondrial proton leak and increases
susceptibility of non-alcoholic steatohepatitis (NASH) liver to ischaemia-reperfusion injury. Gut 57:
Altomare E, and Gnoni GV. Oxidation of hepatic carnitine palmitoyl transferase-I (CPT-I) impairs fatty
40. Song M, Schuschke DA, Zhou Z, Chen T, Pierce WM, Jr., Wang R, Johnson WT, and McClain CJ.
High fructose feeding induces copper deficiency in Sprague-Dawley rats: a novel mechanism for
41. Stefanovic-Racic M, Perdomo G, Mantell BS, Sipula UJ, Brown NF, and O'Doherty RM. A
moderate increase in carnitine palmitoyltransferase 1a activity is sufficient to substantially reduce
42. Tziriogiannis KN, Panoutisopoulos GI, Demonakou MD, Hereti RI, Alexandropoulou KN, and
Mykoniatis MG. Effect of hepatic stimulator substance (HSS) on cadmium-induced acute
43. Uchida K and Stadtman ER. Modification of histidine residues in proteins by reaction with
desensitizes hepatoma cells to H2O2-induced cell apoptosis via preservation of mitochondria. Arch
45. Wu Y, Zhang J, Dong L, Li W, Jia J, and An W. Hepatic stimulator substance mitigates hepatic cell
non-alcoholic steatohepatitis - a case for personalised treatment based on pathogenic targets. Aliment
Pharmacol Ther 39: 3-14,2013.
A

Oil-Red O staining

H&E staining

F4/80 staining

B

Relative IL-1β mRNA level (fold of control)

Relative IL-6 mRNA level (fold of control)

Relative TNF-α mRNA level (fold of control)

C

Hepatic MDA level (fold of control)
<table>
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