J Mol Med (2011) 89:1207–1217
DOI 10.1007/s00109-011-0790-y

ORIGINAL ARTICLE

Increased hepatic apoptosis in high-fat diet-induced NASH in rats may be associated with downregulation of hepatic stimulator substance

Ying Jiang · Miaoyun Zhao · Wei An

Received: 3 January 2011 / Revised: 10 June 2011 / Accepted: 7 July 2011 / Published online: 4 August 2011

Abstract The mechanisms of progression from fatty liver to steatohepatitis and cirrhosis are not well elucidated. Hepatocellular apoptosis could be one of the key factors in the pathogenesis of non-alcoholic steatohepatitis (NASH). Hepatic stimulator substance (HSS) protects liver cells from various toxins. We previously reported that HSS is critically important for the survival of hepatocytes due to its mitochondrial association. This study aims to investigate the relationship between HSS and hepatocellular apoptosis in vivo models of high-fat diet-induced NASH and in vitro models of palmitic acid-induced hepatocyte injury. Sprague–Dawley rats were fed a high-fat diet for 8, 12 and 16 weeks. Hepatic histological lesions, liver function and apoptosis were examined. HSS expression, in association with caspase-3 and cytochrome *c* leakage, which are both indicators of cell apoptosis, was measured. Results showed that a high-fat diet altered liver function and histology in a manner resembling NASH. Hepatic protein and mRNA HSS expression was decreased as NASH progressed. Meanwhile, cell apoptosis increased as result of caspase-3 activation and cytochrome *c* release, indicating that HSS might be involved in NASH pathogenesis. Furthermore, in palmitic acid-induced hepatic cell damage, over-expression of HSS decreased cells apoptosis. In contrast, repression of HSS expression by siRNA increased cell apoptosis. In conclusion, these data imply that cell apoptosis contributes to the pathogenesis of NASH, during which HSS expression is downregulated. Increasing HSS expression in hepatocytes may forestall cell apoptosis as result of fatty acid insult.

Keywords Non-alcoholic steatohepatitis · Apoptosis · Hepatic stimulator substance

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common form of chronic liver disease in western countries, and it encompasses a wide spectrum of liver damage from steatosis to non-alcoholic steatohepatitis (NASH) and, finally, cirrhosis [1]. Liver injury in NASH is usually characterised by fat accumulation, infiltration of inflammatory cells and a varying extent of ballooning degeneration of hepatocytes in the absence of significant alcohol consumption [2]. NASH is currently considered a significant cause of cryptogenic cirrhosis. Emerging data have shown that hepatocellular apoptosis is significantly increased in patients with NASH and correlates with disease severity [3]. This implies that apoptosis could be involved in the aetiology of NASH, and anti-apoptotic therapy may be useful to treat this syndrome. Still, the pathogenesis of NASH as it relates to apoptosis remains poorly understood. Insight into the mechanisms responsible for the progression of NASH may be helpful in designing effective preventive and/or disease management strategies.

Recently, a liver growth-promoting factor, namely hepatic stimulator substance (HSS), has reported to

Ying Jiang and Miaoyun Zhao contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s00109-011-0790-y) contains supplementary material, which is available to authorized users.

Y. Jiang · M. Zhao · W. An (✉)
Department of Cell Biology and Municipal Laboratory for Liver Protection and Regulation of Liver Regeneration,
Capital Medical University,
10 You An Men Wai Xi Tou Tiao,
100069 Beijing, China
e-mail: anwei@ccmu.edu.cn

Y. Jiang
e-mail: jiangy@ccmu.edu.cn

protect the hepatocytes from apoptotic damage. HSS was initially identified in the liver of weanling rats [4] and was reported to stimulate remnant liver regeneration after partial hepatectomy [5, 6]. HSS has also been called as augmenter of liver regeneration (ALR) or hepatopoietin. Although HSS was synthesized in liver parenchymal cells [7], it has multiple-tissue expression [8], different subcellular localisations [9] and diverse functions [10]. HSS exerts two forms of either longer (with 205 amino acids) or shorter one (with only 125 amino acids). The shorter form of HSS is secreted as a cytokine that promotes hepatocyte growth [11], and the longer form of HSS that resides in the cell is a sulfhydryl oxidase that binds FAD in an unusual helix-rich domain containing a redox-active CXXC disulfide proximal to the flavin ring [12, 13]. In addition, the longer form of HSS could participate in oxidative trapping of reduced proteins entering the mitochondrial intermembrane space [14, 15]. Many studies have revealed that HSS protected the liver from acute injury caused by several compounds, including carbon tetrachloride [16], D-galactosamine [17], ethanol [18] and H_2O_2 [19]. It has also been proven that some alterations in HSS gene expression occur in relation to pathological states, such as acute liver disease, liver cirrhosis and hepatocellular carcinoma [20]. HSS possesses clinical implications because exogenous HSS administration to rats with thioacetamide-induced liver fibrosis/cirrhosis is able to significantly decrease fibrosis and suppress the onset of cirrhosis [21]. Thirunavukkarasu et al. reported that ALR is critically important for the survival of hepatocytes due to its association with mitochondria [22]. The results of our previous study [19, 23, 24] also demonstrated that the function of the *HSS* gene in liver cells may be related to its anti-apoptotic effects. The protective effect of HSS may be associated with blockade of the mitochondrial permeability transition.

Although hepatic apoptosis played an important role during the pathogenesis of NASH, the ability of HSS to prevent liver cell apoptosis, thereby alleviating NASH, remains unclear. In this study, we prepared an animal model of NASH using a high-fat diet and investigated the relationship between HSS and hepatocellular apoptosis. We found that marked cell apoptosis in livers was observed during NASH progress, which was accompanied with gradual reduction of hepatic HSS contents. In in vitro study, we transfected *HSS* gene into hepatoma cells and found that *HSS* over-expression could alleviate the cell apoptosis induced by fatty acids. Moreover, we decreased HSS expression by siRNA treatment and demonstrated that inhibition of HSS expression could enhance the cell apoptosis. These results indicated that the HSS down-regulation, although with unknown mechanism, might deteriorate hepatocellular apoptosis, leading to severity of NASH pathogenesis. Hence, upregulation of hepatic HSS expression seems to be a potential target strategy dealt with NASH experimental therapy.

Materials and methods

Animals and experimental protocols

All protocols for animal care and experiments were approved by the Ethical Committee of Capital Medical University, Beijing. Male Sprague–Dawley rats (110–130 g) were fed a standard diet (ingredients: 5% fat, 23% protein, 55% carbohydrate, 6% fibrate, 1.2% calcium, 0.8% phosphorus and 9% water) or a high-fat diet (88% standard diet plus 2% cholesterol and 10% lard) with ad libitum food intake as previously described [25]. After the 8, 12 and 16 weeks of dietary intervention, rats were killed by puncture of the abdominal aorta after overnight fasting, and the livers were removed rapidly, weighed and dissected. Serum alanine aminotransferase (ALT) levels were measured using an autoanalyser in the Clinical Chemistry Laboratory of the Youan Hospital, Capital Medical University, Beijing.

Histopathologic evaluation

Bouin-fixed, paraffin-embedded sections of the liver were stained with haematoxylin–eosin (H & E) and Masson's staining. Sections of frozen rat liver tissue were stained with oil red O for hepatic lipid droplets. Apoptosis was detected using the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) method. The TUNEL assay was carried out according to the manufacturer's protocol with in situ Cell Death/AP Detection kit (Roche Diagnostics, Mannheim, Germany). The results were scored semi-quantitatively by averaging the number of TUNEL cells/field for six fields/tissue sample.

Hepatic ATP levels

Mitochondria were isolated and protein concentrations of the lysates were determined using a bicinchoninic acid Protein Assay Kit (Pierce, Rockford, IL, USA). Mitochondrial ATP content was measured using a luciferin–luciferase assay from a commercial bioluminescence assay kit (Promega, Madison, USA).

Western blot analysis

Liver tissues were homogenised and cell cytosol or mitochondrial proteins were extracted using a Mito-Cyto Isolation Buffer Kit (Pierce). Proteins were sepa-

rated with 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes (Millipore, Billerica, MA, USA) for western blot analysis. Primary antibodies specific to caspase-3 (1:1,000, Cell Signalling Technology, Beverly, MA, USA), cytochrome *c* (Cyt *c*; 1:1,000, Santa Cruz, CA, USA), Bcl-2 (1:1,000, Cell Signalling Technology, Beverly MA, USA) and HSS (1:500, Santa Cruz) were incubated with the membrane at 4°C overnight. The positive reactions against these antibodies were visualised using enhanced chemiluminescence (ECL, Santa Cruz) reagent, followed by exposure to Kodak X-Omat X-ray film. After rinsing the membrane with acetonitrile for 10 min, the membranes were re-hybridised with antibodies against glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:10,000, Kangchen Company, China) or voltage-dependent anion channel (VDAC; 1:1,000, Cell Signalling Technology) as the loading controls. Relative density of protein bands was determined using Image J software (National Institutes of Health, USA).

mRNA analysis by real-time PCR

Total RNA from rat liver was extracted using TRIzol (Invitrogen, Carlsbad, CA USA). The first strand of cDNA was synthesized from 5 μg RNA (Superscript III cDNA Synthesis Kit, Invitrogen), and mRNA for HSS and 18S rRNA were estimated by quantitative real-time PCR using a SYBR Green PCR Kit (Applied Biosystems, Carlsbad, CA, USA). Quantitative RT-PCR was performed with a Prism 7300 Sequence Detecting System (Applied Biosystems). HSS primer sequences were as follows: forward primer 5′-AAAGGTTTGGAGCACGGGA-3′, reverse primer 5′-GAAGGAAAGCCCAGGTGTTG-3′ and 18S forward primer 5′-GTAACCCGTTGAACCCCATT-3′, reverse primer 5′-CCATCCAATCGGTAGTAGCG-3′.

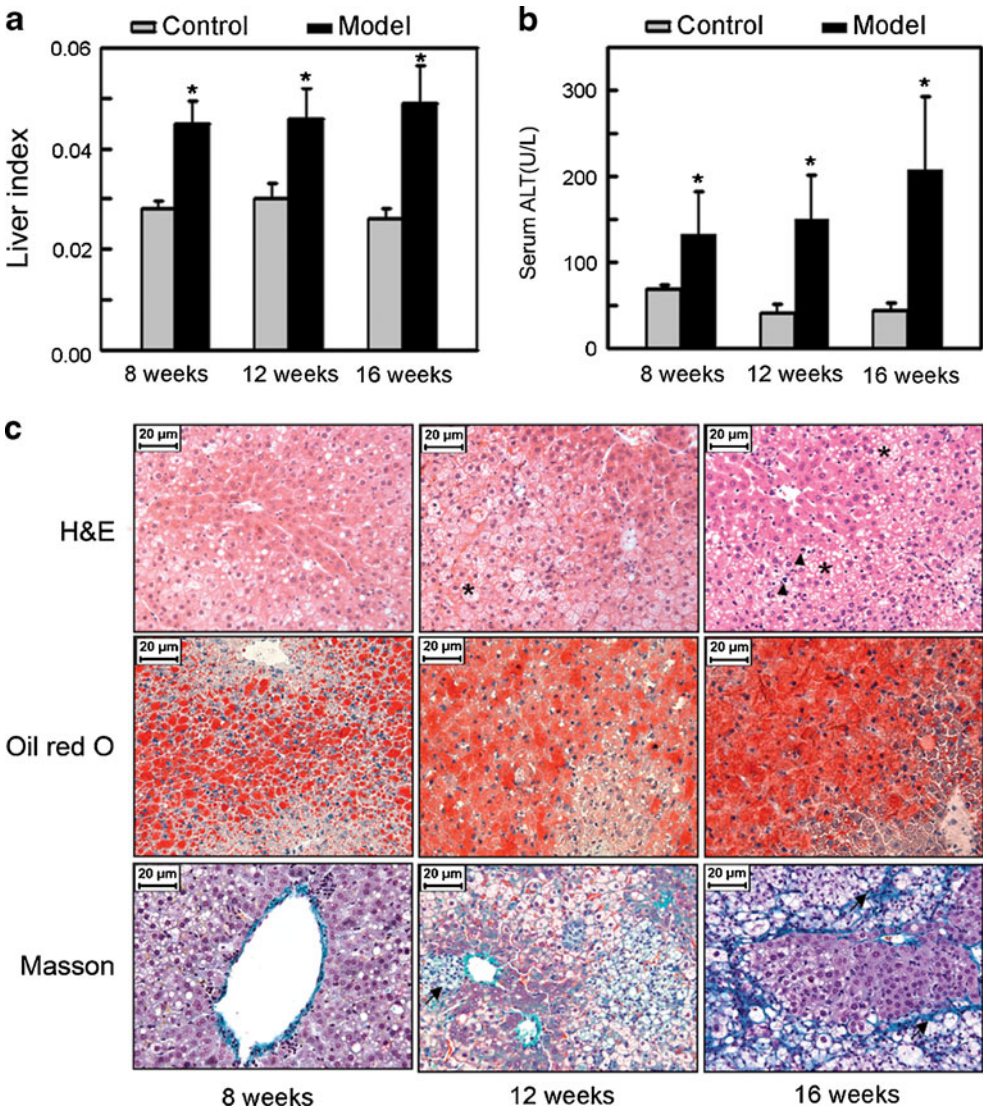

Fig. 1 **a** Liver index (the liver-to-body mass ratio) in rats fed a high-fat diet or normal diet at 8, 12 and 16 weeks. **b** Rat serum alanine aminotransferase (*ALT*) levels. Data are expressed as mean±SD of ten animals in each group. *$P<0.05$ vs. the control group. **c** Histochemical analysis of liver specimens stained with haematoxylin and eosin (H & E) from rats fed a high-fat diet for 8, 12 and 16 weeks. Rats fed a high-fat diet for 8 weeks show steatosis, predominantly as microvesicular fat in the acinar zones 1 and 2. (H & E and oil red O staining). Rats fed a high-fat diet for 12 and 16 weeks show pronounced hepatic steatosis (oil red O staining), ballooning degeneration, infiltration of inflammatory cells (*arrowhead*), piecemeal necrosis (*stars*, H & E staining), fibrous tissue deposition and formation of fat granulomas (*arrows*, Masson's staining). Objective lens, ×20

Cell culture and palmitic acid treatment

BEL-7402 cells, a human hepatoma cell line, were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) containing 10% foetal bovine serum Hyclone, Rockford, IL, USA), penicillin (100 U/ml) and streptomycin (100 U/ml). To induce cellular damage, 200 or 400 μM of palmitic acid (PA; Sigma, St. Louis, MO, USA) was added to serum-free DMEM medium after the cells grew to 70~80% confluences. Bovine serum albumin (BSA) was administrated as control as described previously [26].

Assessment of intracellular lipid accumulation

Intracellular lipid accumulation was assessed by Nile red staining [27]. PA-treated cells or control cells were fixed with 4% paraformaldehyde at room temperature for 15 min. Intracellular lipid was stained with Nile red (0.2 mg/ml) for 5 min at room temperature. Images were acquired with a laser scanning confocal microscope (LEICA TCS SP5).

HSS plasmid transfection

The HSS expression plasmid was constructed as previously described in detail [23]. The cells were transfected with 5 μg of either *HSS*-bearing vector or control vector with Lipofectamine 2000 (Roche Diagnostic) following the manufacturer's recommendations. Eight hours after transfection, cells were selected using a G418 sulphate (400 μg/ml) for 14 days. The cell colonies resistant to G418 were harvested. Stably expressing *HSS*-transfected cells were cultured for further study.

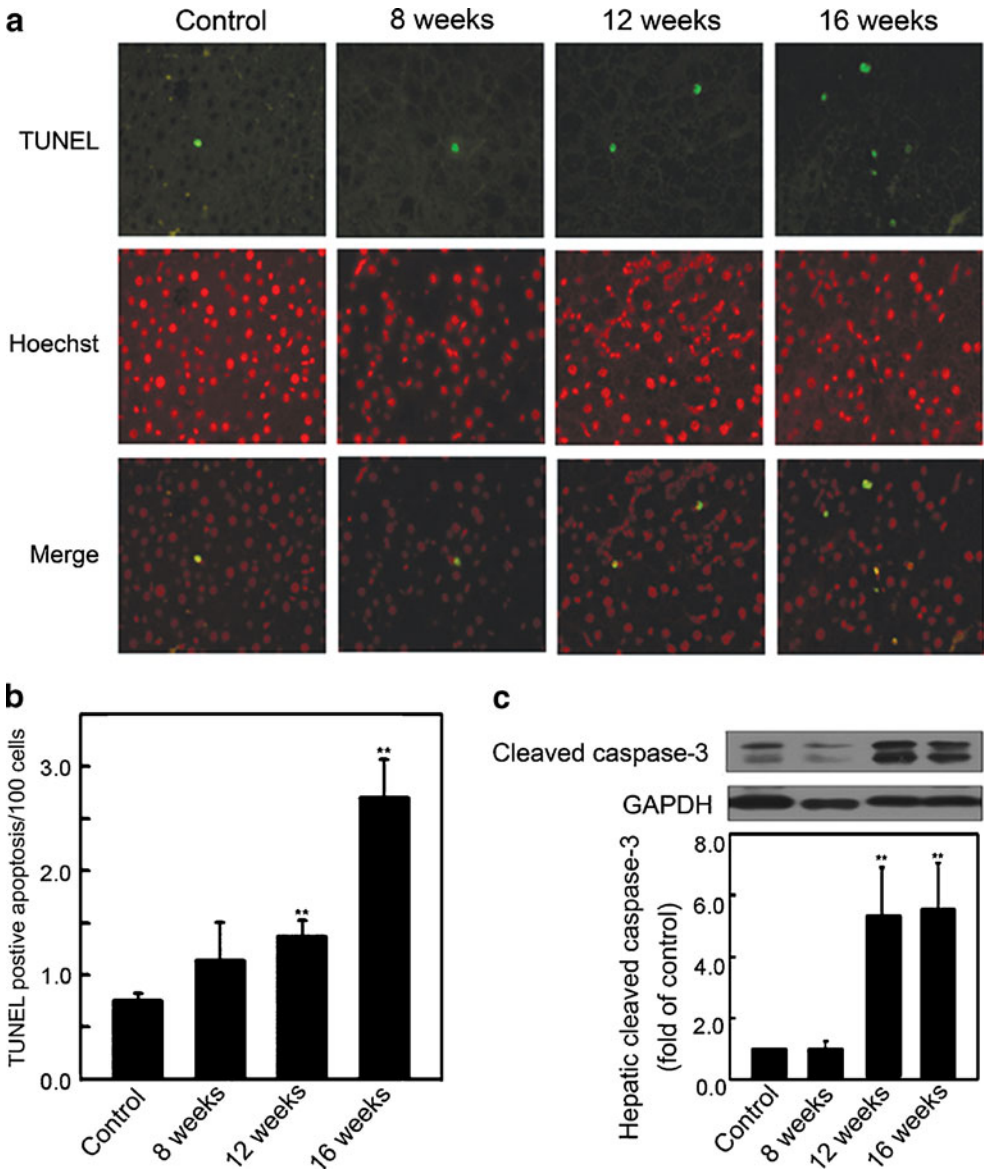

Fig. 2 Cell apoptosis in liver of rats fed a high-fat diet or control diet for 8, 12 and 16 weeks. **a**, **b** TUNEL-positive cells that were green under fluorescence microscopy were quantified from six randomly selected fields at ×400 magnification. Data were expressed as mean±SD of six animals in each group. **c** Representative of cleaved caspase-3 and GAPDH proteins expression by Western blot. Values are means± SD of ten animals in each group. *$P<0.05$; **$P<0.01$ vs. the control group

siRNA transfection

siRNA target sequences against HSS were designed according to a previous report [19]. BEL-7402 cells, grown in 10% FBS-DMEM, were seeded in 6- or 24-well plates and allowed to grow to 70~80% confluences. Subsequently, the cells were transiently transfected with *HSS*-specific siRNA or non-targeting control (scrambled) siRNA. The transfection of siRNA was preceded using the DharmaFECT 4 transfection reagent (Thermo Scientific) according to the manufacturer's instructions. After transfection, the cells were allowed to grow for 48 h and then were treated with 200 or 400 μM of PA for 16 h as indicated. Cells were then harvested for apoptosis analysis.

Apoptotic analysis

Cells were plated in 6-well plates. After attachment, cells were incubated with 200 or 400 μM of PA for 24 h. Cell apoptosis was analysed using the Annexin V-FITC/PI. Apoptosis Kit (Biosea, Beijing, China) according to its instructions by flow cytometry (Becton Dickinson FACScan, USA) as previously described in detail [19]. The data were analysed using Cellquest software (Becton Dickinson). To further assess apoptosis, caspase-3 activation was monitored using Quantikine Active Caspase-3 Immunoassay Kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Mitochondrial Cyt *c* and VDAC content were also analysed by western blot analysis. All data are presented as the mean of three determinations.

Statistical analysis

The results of multiple observations are presented as mean± SD. The data were analysed with statistics software SPSS 11.5 using a nonparametric analysis of variance test. Differences were considered significant if the P value is less than 0.05.

Results

Induction of NAFLD and histopathological lesions

Liver index of treated rats (the liver-to-body mass ratio) significantly increased at 8 weeks in comparison to control rats ($P<0.05$) and persisted at high levels until 12

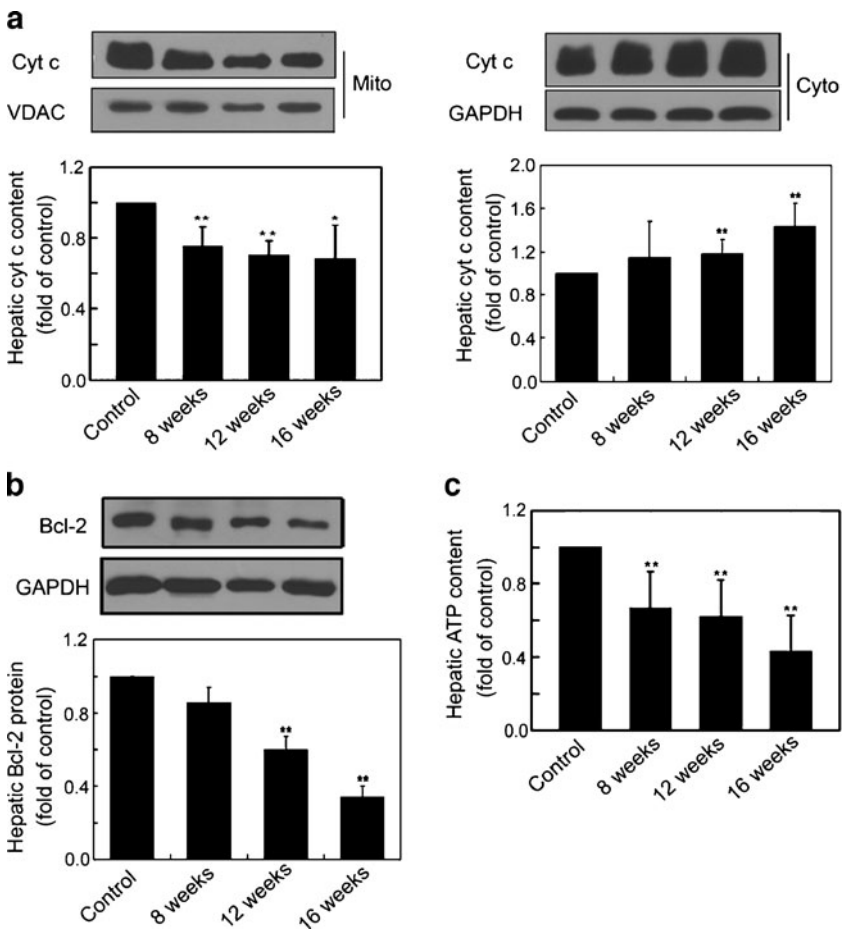

Fig. 3 **a** Cyt *c* protein levels in liver mitochondria and cytosol from rats fed a high-fat diet and rats fed control diet for 8, 12 and 16 weeks. The level of Cyt *c* protein was normalised to VDAC protein level, and this ratio was set to 100% in control mitochondria. The values are the mean±SD for independent mitochondrial preparations. Data were expressed as mean±SD of ten animals in each group. **b** Bcl-2 protein levels in liver from rats fed a high-fat diet and rats fed control diet for 8, 12 and 16 weeks. The level of Bcl-2 protein was normalised to GAPDH protein level, and this ratio was set to 100% in each control. **c** Hepatic ATP content in rats fed a high-fat diet or normal diet at 8, 12 and 16 weeks. Data are expressed as mean±SD of ten animals in each group. *$P<0.05$; **$P<0.01$ vs. the control group

and 16 weeks ($P<0.05$ vs. controls; Fig. 1a). ALT levels of treated rats significantly increased at 12 and 16 weeks as compared with control rats ($P<0.05$ vs. controls; Fig. 1b). Histochemical analysis of liver specimens (Fig. 1c) showed remarkable fat accumulation in the livers of rats fed high-fat diet for 8, 12 and 16 weeks. Foci of lobular inflammation, ballooning degeneration and piecemeal necrosis appeared scattered throughout the livers of rats fed high-fat diet for 12 and 16 weeks as previously described [28]. Masson's staining indicated that fibrous tissue deposition and formation of fat granulomas occurred in the treated rat livers at 16 weeks (Fig. 1c). Together, these observations suggest that hepatic manifestations, such as steatosis, inflammation and early fibrosis, in the rats fed high-fat diet for 8, 12 and 16 weeks closely resemble the hepatic changes observed in human NAFLD.

Cell apoptosis during NAFLD development

To assess hepatocellular apoptosis in rats with NAFLD, we performed the TUNEL assay on paraffin sections and assessed cleaved caspase-3 expression by western blot in the liver. High-fat diet administration significantly promoted hepatocellular apoptosis in the rat, as the numbers of TUNEL-positive cells increased at 12 and 16 weeks (Fig. 2a, b). The high-fat diet also enhanced the activation of caspase-3 in rat livers ($P<0.05$ vs. controls; Fig. 2c), further confirming that apoptosis was involved in NASH development.

Leakage of mitochondrial Cyt *c*, Bcl-2 activity and ATP depletion during NAFLD

Leakage of mitochondrial contents, such as Cyt *c,* was considered as a major event in cellular apoptosis. We next determined whether the leakage of Cyt *c* from the mitochondria takes place during NASH development. Mitochondria from livers with NAFLD and livers of control rats were separated. As shown in Fig. 3, leakage of Cyt *c* from the mitochondria was clearly observed as a consequence of high-fat diet administration. (Fig. 3a). The anti-apoptotic Bcl-2 protein levels were significantly decreased in the liver of rats fed high-fat diet for 12 and 16 weeks. To convince the cell apoptosis, Bcl-xl expression was also determined and the result was shown in S-Fig. 1 of the Electronic Supplementary Material (ESM). The ATP level was greatly reduced after high-fat diet for 12 and 16 weeks (Fig. 3b).

HSS downregulation during NAFLD

Hepatic HSS mRNA and protein expression were evaluated by real-time PCR and western blot. It was shown that both HSS mRNA and protein levels were reduced in the livers of rats with NASH after having received the high-fat diet for 12 and 16 weeks (Fig. 4a, b). Furthermore, we isolated mitochondrial proteins from NAFLD and control rat livers and found that HSS protein level significantly decreased 12 and 16 weeks after NASH development (data not shown).

HSS over-expression and anti-apoptotic effect in fatty acid-induced apoptosis

The anti-apoptotic effect of HSS was previously reported [19, 29]. The over-expression of HSS in cells confers resistance to H_2O_2 and radiation, as well as mechanisms involving mitochondrial injury. To further demonstrate that reduction of HSS levels is one of the major factors that leads to apoptosis in NAFLD, we first confirmed an model of cellular steatosis by incubating BEL-7402 cells with PA

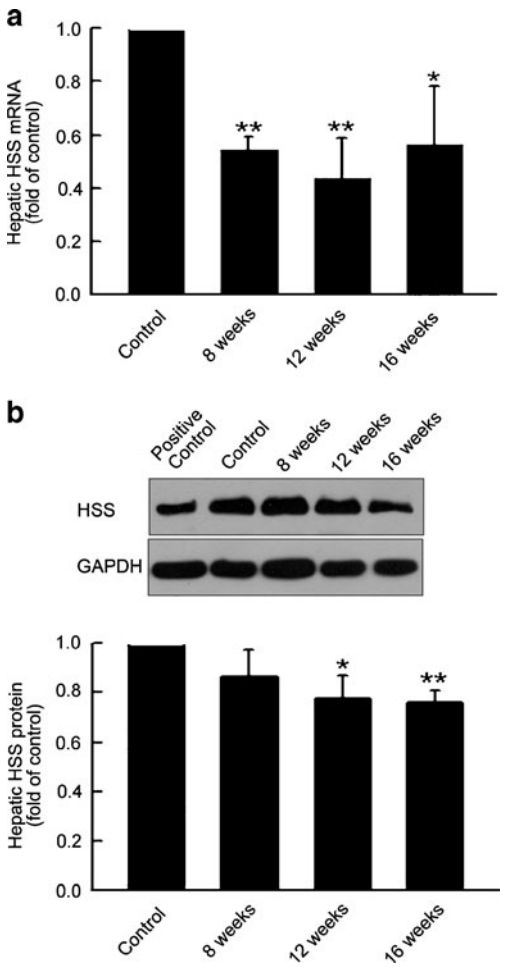

Fig. 4 Expression of HSS protein and mRNA by western blot and real-time PCR. **a** The level of HSS mRNA was normalised to 18S rRNA level and this ratio was set to 100% in the control. **b** The level of HSS protein was normalised to GAPDH protein level, and this ratio was set to 100% in the control. The positive control came from *HSS*-transfected HepG2 cell. Data are expressed as mean±SD of ten animals in each group. *$P<0.05$; **$P<0.01$ vs. the control group

in medium containing BSA as previously reported [26, 27]. As shown in Fig. 5a, intracellular lipid vacuoles visible under confocal microscope were confirmed by Nile red staining. Next, we tried to increase intracellular HSS expression by *HSS*-bearing plasmid transfection to ascertain whether anti-apoptotic effects against fatty acid was subsequently raised. HSS expression in the cells transfected with the *HSS*-bearing plasmid was much higher than cells treated with the control vector (data not shown). Fig. 5b showed that apoptosis induced by PA treatment (200 or 400 μM) was clearly visible in vector-transfected cells by Hochest staining. However, the apoptosis seemed to decrease in the *HSS*-overexpressing cells. Moreover, the apoptotic ratio as analysis with flow cytometry was reduced approximately 32% (200 μM PA induced) or 45% (400 μM PA induced) in *HSS*-overexpressing cells compared with the vector-transfected cells ($P<0.05$), implying a protective effect of HSS against apoptosis (Fig. 5c). Control analysis of BSA-induced apoptosis was shown in S-Fig. 2 of the ESM. To further understand the mechanism by which HSS expression was able to resist apoptotic injury induced by fatty acids, caspase-3 enzymatic activity and Cyt *c* leakage were detected by an enzyme-linked immunosorbent assay and western blot, respectively. As shown in Fig. 5d, PA treatment resulted in caspase-3 activation in vector-transfected cells (as control). However, the enzymatic activity of *HSS*-overexpressing cells was greatly reduced to approximately 50% of the control level ($P<0.05$). Likewise, upon PA treatment, Cyt *c* leakage was significantly preserved in *HSS*-transfected cells as compared with vector-transfected cells (Fig. 5e).

HSS downregulation and anti-apoptosis induced by fatty acid

Based upon the data above, upregulation of HSS prevents liver cell apoptosis caused by PA. We, thus, raised the question of whether reduction of intracellular HSS could deteriorate PA-induced apoptosis. If the answer was yes, this would provide more sound evidence that HSS prevents lipoapoptosis. Inhibition of HSS expression was achieved using siRNA. We first verified the efficacy and specificity of the respective siRNAs to target HSS in BEL-7402 cells. As demonstrated by western blot, the HSS level in the cells transfected with the *HSS*-specific siRNA was much lower than the cells transfected with a scrambled siRNA (Fig. 6a). Next, the cells targeted with *HSS* siRNA were treated with 200 or 400 μM of PA. Treatment of *HSS* siRNA-transfected cells with PA did not prevent block apoptosis. As shown in Fig. 6b, apoptosis rates in the *HSS* siRNA-transfected cells seem increased as compared with the scrambled siRNA-transfected cells (about 20% increase). As control, BSA-induced apoptosis was considered and the result was shown in S-Fig. 3 of the ESM. In addition, caspase-3 activity was greatly increased in the *HSS* siRNA-transfected cells (Fig. 6c), suggesting that knockdown of HSS expression destroyed cellular ability to resist PA-induced damage. As a result, the mitochondrial Cyt *c* content in *HSS* siRNA-transfected cells also declined when compared with the scramble transfection (Fig. 6d). These data demonstrate that cell survival after PA injury is, to some extent, HSS dependent.

Discussion

There is growing evidence that NASH may be considered a mitochondrial disease. NASH is characterised by the impairment of the electron transport chain and oxidative phosphorylation, as liver tissues are highly dependent on oxidative metabolism and, therefore, highly vulnerable to mitochondrial impairment. In addition, it is well established that mitochondria, apart from acting as cellular ATP generators, also function in the regulation of cell death. Mitochondrial dysfunction is directly and indirectly involved in cell death, including apoptosis, and a variety of other pathological states.

The present study demonstrated that rats fed a high-fat diet for a few months showed typical hepatic lesions and a high rate of hepatocellular apoptosis. Additionally, the activation of apoptosis-related enzymes, such as caspase-3, increased leakage of mitochondrial Cyt *c* and depletion of mitochondrial ATP also prove that apoptosis plays an important role in the pathogenesis and development of NASH. Our results, which showed that a high-fat diet could induce apoptosis, were also in agreement with findings from both human studies of NASH patients [3] and animal NASH models induced by feeding a methionine- and choline-deficient diet or a liquid high-fat diet [30, 31]. Although hepatocellular apoptosis may occur by mitochondrial and/or receptor-mediated pathways, the relationship of HSS and apoptosis had not been examined during NAFLD development and progression.

HSS was originally described as a hepatrophic factor that promotes liver regeneration. However, it has been reported that HSS is able to rescue acute liver failure by inhibition of hepatic natural killer cell activation [32]. Therapeutically, the administration of exogenous HSS protein stimulated hepatocyte proliferation [33] and reversed experimental hepatic fibrosis [21]. These results indicate that HSS functions not only as a growth-promoting factor to proliferate hepatocytes but also as a potential therapeutic agent to protect the liver from toxic injury [10]. Despite these, it is not yet clear whether HSS is associated with mechanisms of NAFLD development and progression. Therefore, we surmised that it would be of interest to study HSS expression in the liver of

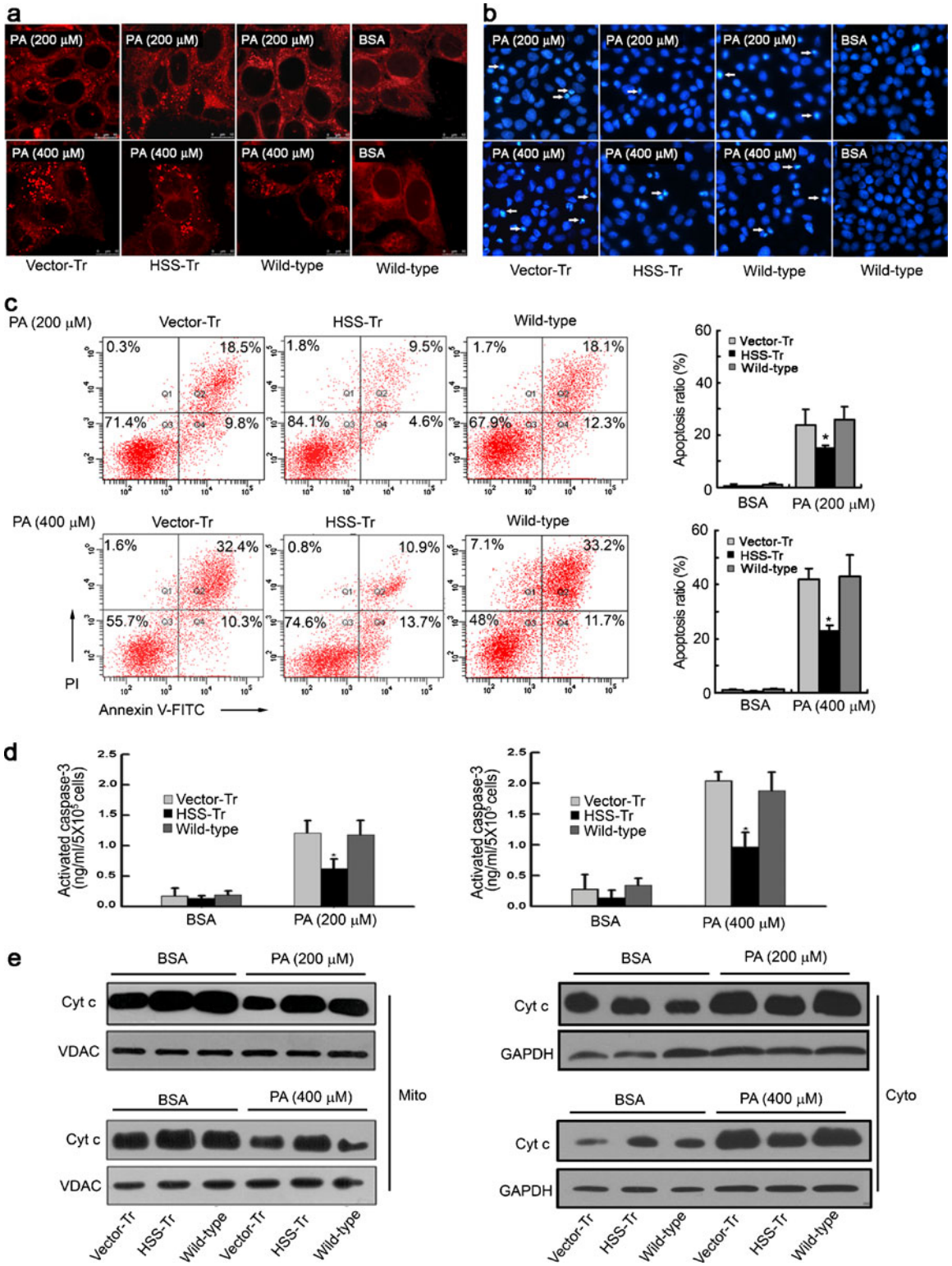

Fig. 5 Detection of palmitic acid (*PA*)-induced apoptosis. BEL-7402 cells were transfected with *HSS* plasmid (*HSS-Tr*) or control vector (*Vector-Tr*). **a** Intracellular lipid accumulation was assessed with Nile red staining under fluorescent photomicrographs; 200/400 μM PA-induced steatosis of BEL-7402 cells exhibit numerous small discrete bodies distributed throughout the cytoplasm (objective lens, ×63). **b** Cellular apoptosis (arrowhead) induced by 200/400 μM PA was assessed by Hoechst 33342 staining. **c** Cellular apoptosis induced by 200/400 μM PA was analysed by flow cytometry. Data are expressed as means±SD for each experiment. **d** Cell caspase-3 activity induced by 200/400 μM PA for 20 h was determined by ELISA. Data are expressed as means±SD for each experiment. **e** Western blot analysis of mitochondrial Cyt *c* and cytosol Cyt *c*. Mitochondria were isolated, and 15 μg of each sample were loaded. VDAC served as a loading control. All data presented here are representative of three separate experiments with consistent results. **P*<0.05 vs. Vector-Tr group

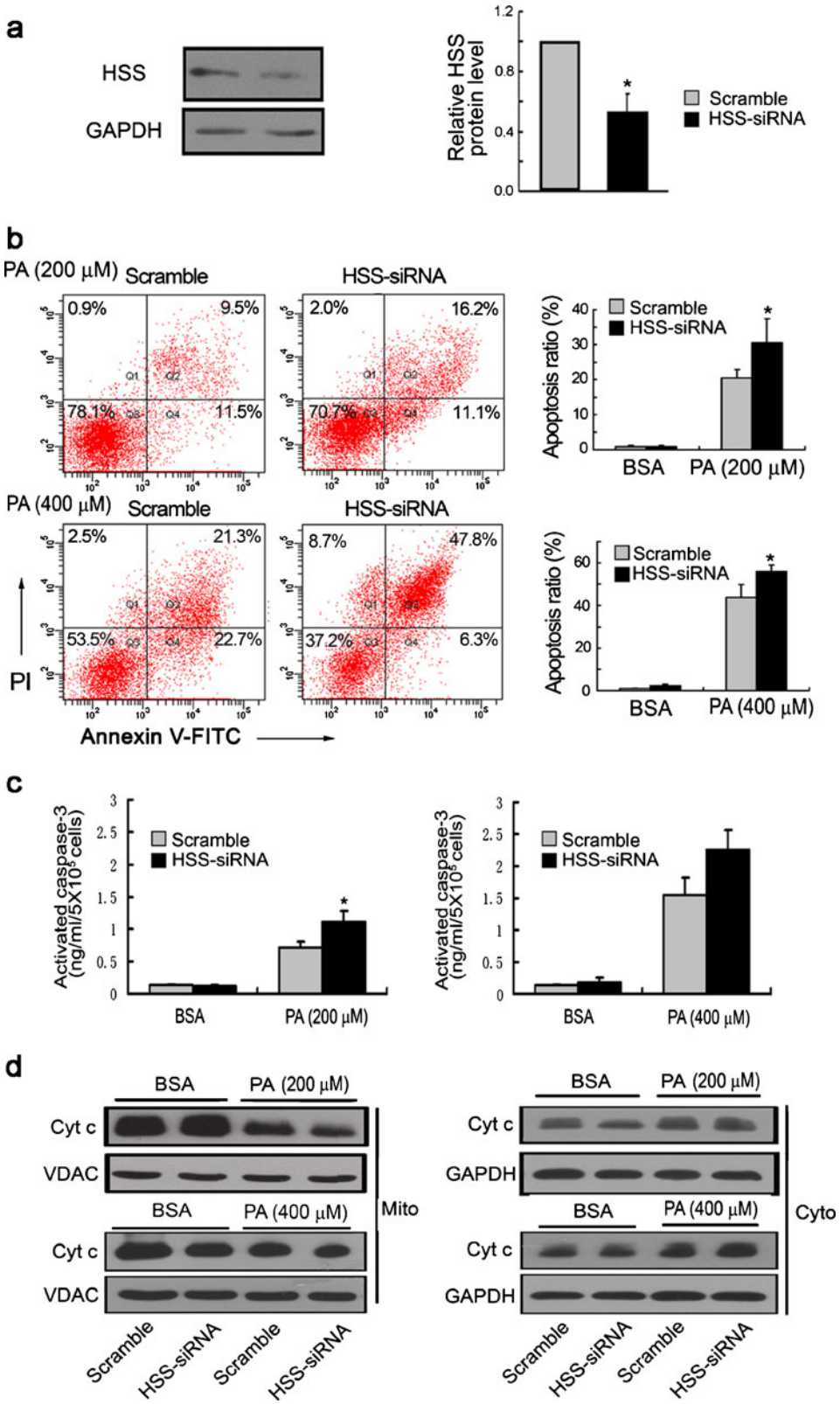

Fig. 6 Influence of knockdown *HSS* on lipoapoptosis. BEL-7402 cells were transfected with *HSS* siRNA, and apoptosis induced by 200/400 μM PA was detected using the following methods. **a** BEL-7402 cells were transfected with *HSS* siRNA (*HSS-siRNA*) or scrambled siRNA (scrambled). Cellular proteins were extracted 48 h after transfection and separated by 12% SDS-PAGE for immunoblot analysis. GAPDH served as a loading control. Data were quantified by densiometric scanning and are presented as mean±SD for three independent experiments. **b** Cell apoptosis induced by 200/400 μM PA was analysed by flow cytometry. **c** Caspase-3 activity was determined by ELISA. **d** Mitochondrial and cytosol Cyt *c* contents measured with western blot. **P*<0.05 vs. scrambled group

rats with NAFLD. The present study showed that a band of 23 kD form of HSS was visible in protein preparations of rat livers (Fig. 4b). The immunohistochemical staining demonstrated that HSS was mainly expressed in hepatocytes and cholangiocytes of liver (S-Fig. 4 of the ESM). Our present study revealed that both HSS mRNA and protein levels were downregulated in the rats with NAFLD induced by high-fat diet (Fig. 4). However, Thasler et al. reported that HSS was upregulated in cirrhosis, hepatocellular carcinoma or cholangiocellular carcinoma [20]. The inconsistency of HSS expression in two kinds of liver diseases is probably due to different pathogenesis attributed to the diseases. In hepatic carcinoma, HSS might act as a growth-promoting substance; therefore, it might be reasonable to see the increase of HSS in hepatocarcinoma. While in NASH, excessive fatty acid deposition within liver and consequent lipotoxicity to hepatocytes were considered to be prominent pathogenesis. Apoptosis appears to be one of the important events to the pathogenesis of lipotoxic injury in the liver [34]. In response to lipotoxic and apoptotic injury to liver, a number of factors such as anti-oxidative enzymes or anti-apoptotic genes are modulated to protect the liver cells. If the injury sustained, superoxide dismutase, glutathione peroxidase and catalase activities might be decreased as a result of depletion (S-Fig. 5 in the ESM). Whether HSS, as one kind of sulfhydryl oxidase, participates in anti-oxidation during NASH and thereby its content in liver is probably depleted, remain uncertain. Subsequently, we attempt to see if HSS downregulation might result from PA lipotoxicity. However, the data in S-Fig. 6 of the ESM suggest that HSS downregulation seems to be not directly affected by PA damage. Unquestionably, further extensive investigations are absolutely required to unmask the mechanism of HSS downregulation in NASH.

Recent studies [22, 35] also provide evidence of ALR's role as an intracellular survival factor for hepatocytes. Studies show that ALR is critically important for the survival of hepatocytes due to its association with mitochondria and regulation of ATP synthesis within hepatocytes. ALR depletion caused apoptosis and necrosis of hepatocytes. Over-expression of ALR significantly increased mitochondrial membrane potential, inhibited Cyt *c* release, and opposed the loss of intracellular ATP levels after radiation. Knockdown of ALR by siRNA resulted in decreased viability in the absence of exogenously added oxidative stress and radiation sensitisation in $HepG_2$ cells. Our previous study demonstrated that the protective effect of HSS in hepatoma apoptosis induced by H_2O_2 may be associated with a blockade of the mitochondrial permeability transition pore [23].

In this study, we demonstrated that, with increasing apoptosis rates, anti-apoptotic *Bcl*-2 or *Bcl-xl*, Cyt *c* content and ATP content in liver mitochondrial compartments were all decreased during NASH development and progression (S-Fig. 1 in the ESM; Fig. 3). All of these data implied that HSS protection against apoptosis was severely impaired as NAFLD progressed. Although this experiment was unable to provide further in vivo data by introducing the HSS transgenic model to see if apoptosis was subsequently blocked, we can infer a likely explanation from our in vitro results. It may be that diffuse hepatocellular fatty change leads to HSS downregulation. These probably results in opening of the mitochondrial permeability transition pore, release of apoptosis-stimulating compounds from the mitochondria (such as Cyt *c*), activation of pro-caspases (such as cleaved caspase-3) and apoptosis and reduced ATP synthesis. All of these data supported our results in vivo.

Next, we wanted to determine if HSS was associated with apoptosis induced by fatty acid in vitro. It was proven that PA was able to induce liver damage that resembles NAFLD in human and was characterised by increasing caspase-3 activity and prominent apoptosis [3]. Then, we altered the HSS expression levels in BEL-7402 hepatocytes either by transfection with an HSS-expressing plasmid or an HSS mRNA interference (RNAi) plasmid. As a result, HSS over-expression caused significantly decreased apoptosis rates and caspase-3 activity in the PA-treated cells while HSS RNAi resulted in an increase in apoptosis rates and caspase-3 activity. HSS over-expression also significantly preserved the Cyt *c* content of mitochondria in the PA-treated cells, while HSS RNAi resulted in a decrease in mitochondrial Cyt *c* content. In addition, we have demonstrated that HSS protects liver cells from PA-induced apoptosis in vitro by decreasing GRP78, a marker of endoplasmic reticulum stress (data not shown). Taking together, we predict that anti-apoptotic effect of HSS in vitro probably relies not only on its prevention on onset of mitochondrial permeability transition but also relates to its eventual inhibition of endoplasmic reticulum stress.

In summary, our present study shows that HSS was downregulated and hepatocellular apoptosis was increased in rats with high-fat diet-induced NASH. We show that hepatic apoptosis in NASH rats may be associated with a low HSS protein level. This mechanism, which is associated with a high rate of apoptosis and sustained anti-oxidative response, could result in reduced HSS in the livers of rats with NASH. The results provide evidence that HSS is an anti-apoptotic factor during liver injury secondary to NASH. The current data obtained from our experiments might also provide useful information regarding potential molecular targets for NAFLD prevention and treatment.

Acknowledgements This work was supported by the National Key Basic Science Project (2010CB5344903) and the National Natural Science Foundation of China (81070352).

References

1. Ong JP, Younossi ZM (2007) Epidemiology and natural history of NAFLD and NASH. Clin Liver Dis 11:1–16
2. James OF, Day CP (1998) Non-alcoholic steatohepatitis (NASH): a disease of emerging identity and importance. J Hepatol 29:495–501
3. Feldstein AE, Canbay A, Angulo P, Taniai M, Burgart LJ, Lindor KD, Gores GJ (2003) Hepatocyte apoptosis and Fas expression are prominent features of human non-alcoholic steatohepatitis. Gastroenterology 125:437–443
4. LaBrecque DR, Pesch LA (1975) Preparation and partial characterization of hepatic regenerative stimulator substance (SS) from rat liver. J Physiol 248:273–284
5. LaBrecque DR, Steele G, Fogerty S, Wilson M, Barton J (1987) Purification and physical-chemical characterization of hepatic stimulator substance. Hepatology 7:100–106
6. Hagiya M, Francavilla A, Polimeno L, Ihara I, Sakai H, Seki T, Shimonishi M, Porter KA, Starzl TE (1994) Cloning and sequence analysis of the rat augmenter of liver regeneration (ALR) gene: expression of biologically active recombinant ALR and demonstration of tissue distribution. Proc Natl Acad Sci 91:8142–8146
7. He F, Wu C, Tu Q, Xing G (1993) Human hepatic stimulator substance: a product of gene expression of human fetal liver tissue. Hepatology 17:225–229
8. Giorda R, Hagiya M, Seki T, Shimonishi M, Sakai H, Michaelson J, Francavilla A, Starzl TE, Trucco M (1996) Analysis of the structure and expression of the augmenter of liver regeneration (ALR) gene. Mol Med 2:97–108
9. Hofhaus G, Stein G, Polimeno L, Francavilla A, Lisowsky T (1999) Highly divergent amino termini of the homologous human ALR and yeast scERV1 gene products define species specific differences in cellular localization. Eur J Cell Biol 78:349–356
10. Pawlowski R, Jura J (2006) ALR and liver regeneration. Mol Cell Biochem 288:159–169
11. Wang G, Yang X, Zhang Y, Wang Q, Chen H, Wei H, Xing G, Xie L, Hu Z, Zhang C, Fang D, Wu C, He F (1999) Identification and characterization of receptor for mammalian hepatopoietin that is homologous to yeast ERV1. J Biol Chem 274:11469–11472
12. Lisowsky T, Lee JE, Polimeno L, Francavilla A, Hofhaus G (2001) Mammalian augmenter of liver regeneration protein is a sulfhydryl oxidase. Dig Liver Dis 33:173–180
13. Wu CK, Dailey TA, Dailey HA, Wang BC, Rose JP (2003) The crystal structure of augmenter of liver regeneration: a mammalian FAD-dependent sulfhydryl oxidase. Protein Sci 12:1109–1118
14. Wang W, Winther JR, Thorpe C (2007) Erv2p: characterization of the redox behavior of a yeast sulfhydryl oxidase. Biochemistry 46:3246–3254
15. Daithankar VN, Farrell SR, Thorpe C (2009) Augmenter of liver regeneration: substrate specificity of a flavin-dependent oxidoreductase from the mitochondrial intermembrane space. Biochemistry 48:4828–4837
16. Mei MH, An W, Zhang BH, Shao Q, Gong DZ (1993) Hepatic stimulator substance protects against acute liver failure induced by carbon tetrachloride poisoning in mice. Hepatology 17:638–644
17. Ho DW, Fan ST, To J, Woo YH, Zhang Z, Lau C, Wong J (2002) Selective plasma filtration for treatment of fulminant hepatic failure induced by D-galactosamine in a pig model. Gut 50:869–876
18. Liatsos GD, Mykoniatis MG, Margeli A, Liakos AA, Theocharis SE (2003) Effect of acute ethanol exposure on hepatic stimulator substance (HSS) levels during liver regeneration: protective function of HSS. Dig Dis Sci 48:1929–1938
19. Wu Y, Zhang J, Dong LY, Li W, An W (2010) Hepatic stimulator substance mitigates hepatic cell injury through suppression of the mitochondrial permeability transition. FEBS J 277:1297–1309
20. Thasler WE, Schlott T, Thelen P, Hellerbrand C, Bataille F, Lichtenauer M, Schlitt HJ, Jauch KW, Weiss TS (2005) Expression of augmenter of liver regeneration (ALR) in human liver cirrhosis and carcinoma. Histopathology 47:57–66
21. Gribilas G, Zarros A, Zira A, Giaginis C, Tsourouflis G, Liapi C, Spiliopoulou C, Theocharis SE (2009) Involvement of hepatic stimulator substance in experimentally induced fibrosis and cirrhosis in the rat. Dig Dis Sci 54:2367–2376
22. Thirunavukkarasu C, Wang LF, Harvey SA, Watkins SC, Chaillet JR, Prelich J, Starzl TE, Gandhi CR (2008) Augmenter of liver regeneration: an important intracellular survival factor for hepatocytes. J Hepatol 48:578–588
23. Wu Y, Chen L, Yu H, Liu HJ, An W (2007) Transfection of hepatic stimulator substance gene desensitizes hepatoma cells to H_2O_2-induced cell apoptosis via preservation of mitochondria. Arch Biochem Biophys 464:48–56
24. Li W, Zhang J, An W (2010) The conserved CXXC motif of hepatic stimulator substance is essential for its role in mitochondrial protection in H_2O_2-induced cell apoptosis. FEBS Lett 584:3929–3935
25. Jiang Y, Zhang H, Dong LY, Wang D, An W (2008) Increased hepatic UCP2 expression in rats with nonalcoholic steatohepatitis is associated with upregulation of Sp1 binding to its motif within the proximal promoter region. J Cell Biochem 105:277–289
26. Yang YH, Jiang Y, Wang YT, An W (2010) Suppression of ABCA1 by unsaturated fatty acids leads to lipid accumulation in HepG2 cells. Biochimie 92:958–963
27. Malhi H, Bronk SF, Werneburg NW, Gores GJ (2006) Free fatty acids induce JNK-dependent hepatocyte lipoapoptosis. J Biol Chem 281:12093–12101
28. Wu J, Zhao MY, Zheng H, Zhang H, Jiang Y (2010) Pentoxifylline alleviates high-fat diet-induced nonalcoholic steatohepatitis and early atherosclerosis in rats by inhibiting AGE and RAGE expression. Acta Pharmacol Sin 31:1367–1375
29. Cao Y, Fu YL, Yu M, Yue PB, Ge CH, Xu WX, Zhan YQ, Li CY, Li W, Yang XH (2009) Human augmenter of liver regeneration is important for hepatoma cell viability and resistance to radiation-induced oxidative stress. Free Radic Biol Med 47:1057–1066
30. Farrell GC, Larter CZ, Hou JY, Zhang RH, Yeh MM, Williams J, dela Pena A, Francisco R, Osvath SR, Brooling J (2009) Apoptosis in experimental NASH is associated with p53 activation and TRAIL receptor expression. J Gastroenterol Hepat 24:443–452
31. Wang Y, Ausman LM, Russell RM, Greenberg AS, Wang XD (2008) Increased apoptosis in high-fat diet-induced nonalcoholic steatohepatitis in rats is associated with c-Jun NH2-terminal kinase activation and elevated proapoptotic Bax. J Nutr 138:1866–1871
32. Tanigawa K, Sakaida I, Masuhara M, Hagiya M, Okita K (2000) Augmenter of liver regeneration (ALR) may promote liver regeneration by reducing natural killer (NK) cell activity in human liver disease. J Gastroenterol 35:112–119
33. Zhang M, Song G, Minuk GY (1996) Effects of hepatic stimulator substance, herbal medicine, selenium/vitamin E, and ciprofloxacin on cirrhosis in the rat. Gastroenterology 110:1150–1155
34. Neuschwander-Tetri BA (2010) Hepatic lipotoxicity and the pathogenesis of nonalcoholic steatohepatitis: the central role of nontriglyceride fatty acid metabolites. Hepatology 52:774–788
35. Polimeno L, Pesetti B, Annoscia E, Giorgio F, Francavilla R, Lisowsky T, Gentile A, Rossi R, Bucci A, Francavilla A (2011) Alrp, a survival factor that controls the apoptotic process of regenerating liver after partial hepatectomy in rats. Free Radic Res 45:534–549