Enhanced endoplasmic reticulum SERCA activity by overexpression of hepatic stimulator substance gene prevents hepatic cells from ER stress-induced apoptosis

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Zhang J, Li Y, Jiang S, Yu H, An W. Enhanced endoplasmic reticulum SERCA activity by overexpression of hepatic stimulator substance gene prevents hepatic cells from ER stress-induced apoptosis. Am J Physiol Cell Physiol 306: C279–C290, 2014. First published November 27, 2013; doi:10.1152/ajpcell.00117.2013.—Although the potential pathogenesis of nonalcoholic fatty liver disease (NAFLD) is unclear, increasing evidence indicates that endoplasmic reticulum (ER) stress may link free fatty acids to NAFLD. Since we previously reported that hepatic stimulator substance (HSS) could protect the liver from steatosis, this study is aimed to investigate whether HSS protection could be related with its inhibition on ER stress. The HSS gene was stably transfected into BEL-7402 hepatoma cells and effectively expressed in ER. The palmitic acid (PA)-induced heptocyte lipotoxicity was reproduced in the HSS-transfected cells, and HSS alleviation of the ER stress and apoptosis were subsequently examined. The results showed that PA treatment led to a heavy accumulation of fatty acids within the cells and a remarkable increase in reactive oxygen species (ROS). However, in the HSS-expressing cells, production of ROS was inhibited and ER stress-related marker glucose-regulated protein 78 (GRP-78), sterol regulatory element-binding protein (SREBP), anti-phospho-PRK-like ER kinase (p-PERK), anti-phospho-eukaryotic initiation factor 2 alpha (p-eIF2α), and anti-C/EBP homologous protein (CHOP) were downregulated compared with the wild-type or mutant HSS-transfected cells. Furthermore, PA treatment severely impaired the activity of sarco-endoplasmic reticulum Ca2+-ATPase (SERCA), leading to imbalanced calcium homeostasis during ER stress, which could be rescued in the HSS-transfected cells. The protection provided by HSS to the SERCA is identical to that observed with N-acetyl-L-cysteine (NAC) and sodium dimercaptopropane sulfonate (Na-DMPS), which are two typical free radical scavengers. As a consequence, the rate of ER stress-mediated apoptosis in the HSS-expressing cells was significantly reduced. In conclusion, the protective effect of HSS against ER stress may be associated with the removal of ROS to restore the activity of the SERCA.

nonalcoholic fatty liver disease; hepatic stimulator substance; augmented liver regeneration; ER stress; SERCA

NONALCOHOLIC FATTY LIVER DISEASE (NAFLD) is a chronic metabolic disorder that is characterized by hepatic fat accumulation in the absence of excessive alcohol consumption (11). The prevalence of NAFLD has nearly doubled since 1980, and this condition is becoming one of the most common hepatic disorders in China. Clinically, NAFLD encompasses a disease spectrum of hepatic derangements, ranging from simple hepatic steatosis to nonalcoholic steatohepatitis (NASH); if insufficient intervention is adopted, the latter will develop into fibrosis and cirrhosis (44). Liver injury in NAFLD is usually characterized by fat accumulation, the infiltration of inflammatory cells, and a varying extent of ballooning degeneration of the hepatocytes (48). Steatosis is the earliest and most prevalent stage of NAFLD and is often referred to as the “first hit;” during this stage, free fatty acids (FFAs) accumulate in the cells, and lipid peroxidation occurs, producing large amounts of reactive oxygen species (ROS) and free radicals. It has been proposed that the lipotoxicity of FFAs increases the vulnerability of the liver to a “second hit,” involving environmental and/or genetic factors, which ultimately can lead to end-stage liver disease (7). Although the underlying pathogenesis of NAFLD is unclear, increasing evidence suggests that endoplasmic reticulum (ER) stress may link saturated fatty acids to NAFLD (31, 33, 35). ER stress causes the activation of the unfolded protein response (UPR) and the sterol regulatory element-binding protein (SREBP) pathway (5, 55). Once activated, the UPR increases the expression of intraluminal ER chaperones, especially glucose-regulated protein-78 (GRP-78), to cope with the accumulation of unfolded or misfolded proteins; in contrast, SREBP-1 acts as a transcription factor that regulates the genes that control the synthesis of fatty acids and the cellular uptake of lipoproteins. If greatly prolonged, the ER stress overwhelms the hepatocyte tolerance, apparently resulting in cellular apoptosis and accompanied by pathological consequences, including hepatic fat accumulation and inflammation (2, 27). This scenario implies that any substance that opposes ER stress may be useful for treating NAFLD. Insight into the mechanisms responsible for the progression of NAFLD may be helpful in designing effective preventive and disease management strategies.

Recently, hepatic stimulator substance (HSS) has been reported to protect hepatocytes from various toxins. HSS was initially identified in the liver cytosol of weanling rats by LaBrecque and Pesch (24). As a unique growth factor that can specifically activate hepatic-origin cells to grow, regardless of the animal species, HSS is able to stimulate the proliferation of hepatocytes and hepatoma cells in vitro and in vivo to promote liver regeneration after partial hepatectomy and to protect the liver from the acute liver failure induced by chemical poisons and drugs, such as carbon tetrachloride (CCL4), d-galactosamine, cadmium, acetaminophen, thioacetamide, and ethanol (28, 34, 43, 45). A partial purification of the HSS protein indicated that the molecular size of HSS is 15–18 kDa and that the protein is charac-

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terized by resistance to heat and pH but is protease sensitive (24). The biological function of the HSS protein is believed to be the stimulation of cellular DNA synthesis and mitosis (23). Intriguingly, several in vitro studies have demonstrated that although HSS stimulates cell growth in dividing hepatocytes, it is unable to stimulate proliferation in the resting adult liver. Instead, this protein augmented the mito-
genic effects of other growth factors when used in combination with such as epidermal growth factor (EGF) or transforming growth factor-α (TGF-α) (12, 13). Based on its growth-augmenting activity, HSS has also been named as augmenter of liver regeneration (ALR). It belongs to the new ALR/ERV1 (essential for respiration and vegetative growth) protein family (37), members of the ERV/ALR family function in a range of diverse cellular localization, including mitochondria, endoplasmic reticulum, and nucleus (14, 15, 36). The data suggest that ALR has diverse func-
tions, for instance, it regulates flavine adenine dinucleotide (FAD)-linked, oxidative phosphorylation-generating disul-
phide bridges in proteins (29), mitochondrial gene expres-
sion, and electron transfer via FAD to cytochrome c (9). The crystal analysis of ALR structure confirmed that it is a mammalian FAD-dependent sulphydryl oxidase (SOX) with cytochrome c reductase activity (9, 49). Furthermore, this SOX molecule has a specific motif at its COOH-terminal (C62XXC65) that is essential for cell survival and biogen-
esis of cytosolic Fe/S proteins (25). Our previous observa-
tions (26, 50) demonstrated that HSS protects the liver cells from H2O2-induced apoptosis via preservation of mitochond-
ria, and the conserved CXXC motif is essential for SOX activity and mitochondrial protection. In this study, the Western blotting and confocal microscopy analysis indicated that HSS could localize to ER and mitochondria as well; this result sheds a new light on the potential function of HSS, and it is likely to have multiple roles in the regulation of cell growth and cell protection.

Clinical studies have indicated that patients suffering from NAFLD display markers of ER stress in the liver (39, 42). The potential reason for this observation is that the extensive accumulation of fatty acids within the hepatocytes could pro-
voke the peroxidation of unsaturated fatty acids, producing large amounts of harmful substances, such as free radical molecules and ROS, which disrupt the structures and functions of the endomembrane system and also trigger the occurrence of ER stress. ER calcium disequilibrium is considered to be one of the initial and pivotal events of ER stress-mediated cell death (18). ROS produced in combination with peroxidation is able to attack the sarco-endoplasmic reticulum Ca2+-ATPase (SERCA) directly. The impairment of SERCAs can further lead to Ca2+ release from the ER lumen, the accumulation of unfolded proteins in the ER lumen, and, ultimately, the disruption of ER homeostasis, i.e., ER stress (4, 41).

Recently, our laboratory reported that HSS is able to protect hepatocytes from various liver toxins, including FFAs. Fur-
thermore, the downregulation of HSS expression by small interfering RNA aggravated the hepatic injury induced by fatty acid (21), implying that HSS might play a role in the protection of liver cells from lipid peroxidation. However, whether the HSS-mediated alleviation of lipotopic injury is associated with the suppression of ER stress remains largely unknown. In the present study, we aimed to investigate whether the transfection of hepatocytes with HSS is able to stabilize the ER structure and function and inhibit ER stress. We intend to emphasize a more in-depth consideration of the SERCA, a potential target protein of ER stress. In particular, the functional variations of the SERCAs in the HSS or nontransfected cells in response to ER stress were investigated during FFA-induced ER stress. We demonstrated that PA induced ER stress and clearly inhibited the activity of SERCAs, leading to a heavy calcium overload in the cytosol of the nontransfected cells. In contrast, the calcium overload was significantly overcome in the HSS-transfected cells, and this improvement was accompanied by an increase in SERCA activity. HSS also assisted in the scavenging of ROS and the suppression of the ER stress-related signal pathway. It is expected that all of these functions would contribute to the efficient protection of hepatocytes from cell death as a result of ER stress.

MATERIALS AND METHODS

Reagents. DMEM and G418 (a gentamycin analog) were pur-
chased from Gibco (Paisley, UK), and fetal bovine serum was pur-
chased from Hyclone (Victoria, Australia). The DNA plasmid transfection kit was purchased from Boehringer Mannheim (Basel, Switzerland). The animal cell high-purity ER divide kit, SERCA activity testing kit, and ER-Tracker were purchased from Genned Sciences (Boston, MA). The bicinechonic acid (BCA) kit was pur-
chased from Pierce (Rockford, IL). The ENLITEN total ATP rapid detection kit was purchased from Promega (Madison, WI). The anti-HSS, anti-protein disulfide isomerase (PDI), anti-glyc-
eraldehyde-3-phosphate dehydrogenase (GAPDH), anti-SREBP-1, anti-GRP-78, anti-phospho-PRK-like ER kinase (p-PRK), and anti-PRK antibodies and antibody enhanced chemiluminescence (ECL) kit were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-histone H3, anti-cytochrome c oxidase IV (COX IV), anti-eukaryotic initiation factor 2α (eIF2α), anti-p-
eIF2α, and anti-C/EBP homologous protein (CHOP) antibodies and the peroxidase-conjugated, affinity-purified goat anti-mouse immunoglobulin (IgG) antibody, and donkey anti-goat IgG fluo-
rescence antibody were purchased from Cell Signaling Technology (Danvers, MA). The bishenizimide Hoechst 33342 stain, 3-(4,5-
dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), bovine serum albumin (BSA), palmitate, 4,6-diamidino-2-phe-
nylindole (DAPI), dimethyl sulfoxide (DMSO), N-acetyl-l-cys-
teine (NAC) and sodium dimercaptopropane sulfonate (Na-
DMPs), Fluor-3AM probe, 2’7’-dichlorodihydrofluorescein diacetate (DCFH-DA), and Nile Red stain were purchased from Sigma (St. Louis, MO). Fluorescein isothiocyanate-conjugated annexin V was purchased from Biosea (Beijing, China). The free fatty acid (FFA), caspase-4 colorimetric assay kit, and triglycerin (TG) quantification kits were purchased from BioVision ( Palo Alto, CA). MitoTracker Red CMXRos was purchased from Invitrogen (Eugene, OR). The human FAD-linked sulphydryl oxidase ALR (GFER) ELISA kit was purchased from CUSABIO (Wuhan, Hu-
bei, China).

Cell culture and plasmid DNA transfection. BEL-7402 hepatoma cells were cultured at 37°C in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 U/ml streptomycin in a 5% CO2 humidified-atmosphere incubator. A total of 2 × 107 BEL-
7402 cells were seeded and allowed to grow to 50~70% confluence. HSS cDNA and the mutant-HSS cDNA (cysteine-to-serine mutations of HSS at positions 62/65 of the CXXC motif) were reserved in our laboratory. The cells were transfected with 5 μg of either HSS pcDNA 3.0, mutant-HSS pcDNA 3.0, or empty pcDNA 3.0 vector with Lipofectamine 2000, following the manufacturer’s recommendations. At 8 h posttransfection, the cells were selected using G418 (600
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µg/ml) for 14 days. Those cells that were resistant to G418 were used for further study.

Palmitic acid treatment and assessment of intracellular lipid accumulation. Palmitic acid (PA; 12.8 mg) was primarily dissolved in 500 µl NaOH (0.1 M), and the liquid container was then placed in boiling water for thorough dissolution. Afterward, 9.5 ml BSA/phosphate-buffered saline (PBS; 100 mg/ml) were added and the mixture was heated at 55°C for 15 min. As a result, the concentration of PA was 5 mM, and this PA stock solution was subsequently stored in −20°C after filtration sterilization.

To reproduce hepatocellular steatosis, after the cells grew to 70~80% confluence, serum-free DMEM medium with a different dosage of PA stock solution was added. The exposure time to PA was determined based on the cell morphology and viability (as described below). BSA was administered as a control, as described previously (53). The intracellular lipid vacuoles were assessed by laser scanning confocal microscope and transmission electron microscopy (TEM). 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. Cells were seeded in 10-mm culture dishes; after incubation with 100 µM PA for 24 h, the cells were harvested and fixed with 2.5% glutaraldehyde in 0.1 M PBS (pH 7.4). The cells were then postfixed with 1% osmium tetroxide in PBS and dehydrated with a graded ethanol series, followed by propylene oxide treatment. After being embedded in epoxy resin, ultrathin samples were sectioned and examined at 80 kV using a JEM-1200EX electron microscope (Jeol, Tokyo, Japan). The cellular FFA and TG contents were evaluated using the FFA and TG quantification kits with standard procedures.

Isolation of ER and mitochondria. The isolation of the ER was performed according to the instructions for the animal cell high-purity ER divide kit. After centrifugation at 200 g for 10 min, the cells were harvested and then homogenized in 5 ml of ice-cold lysis buffer using a Dounce homogenizer. After centrifugation at 1,000 g for 10 min at 4°C, the supernatant was collected, transferred to an ice-cold 15-ml Falcon tube, and centrifuged at 12,000 g for 15 min at 4°C. Subsequently, the supernatant was carefully transferred to a fresh tube and centrifuged at 100,000 g for 60 min at 4°C. The pellet, which contained the ER, was resuspended in 500 µl of reserve buffer; the isolated ER was stored on ice before the experiments. Mitochondrial fractions were isolated as described previously (51).

Measurement of intracellular ROS generation. The wild-type, the vector-transfected, and the HSS-transfected cells were plated in black 96-well plates at 2.5×10^5 cells per well. The cells were treated with 100 µM PA for 30 min. After the cells were washed gently with DPBS for 2 times, DCFH-DA at final concentration of 10 µM was added to cells and incubated at 37°C for 30 min in the dark. DCFH-DA is diffused into cells and is deacetylated by cellular esterases to nonfluorescentDCF, which is rapidly oxidized to highly fluorescent 2′,7′-dichlorofluorescein (DCF) by ROS. The fluorescence intensity is directly proportional to the ROS levels within the cell cytosol. Fluorescence measurement was performed on Fluoroskan Ascent FL (Thermo, Boston, MA) at 480-nm excitation and 538-nm emission. The relative fluorescence intensity was presented by the value that was calculated as follows: the measuring values of different groups divided the preliminary value of the control, which was considered as the normalization.

Determination of caspase-4 activity. Caspase-4 activity was analyzed using a caspase-4 colorimetric assay kit. After exposure to 100 µM PA for 24 h, 5×10^4 cells were suspended in 50 µl of chilled lysis buffer, incubated for 10 min on ice, and centrifuged at 10,000 g at 4°C and then the supernatants were retained and assayed protein concentration. Two-hundred micromgrams of cell lysate were diluted into 50 µl of lysis buffer for each assay. Each diluted sample was mixed with 50 µl of 2× reaction buffer (containing 10 mM DTT) and 5 µl of LEVD-pNA substrate. Following incubation at 37°C for 60 min, samples were read at 405 nm in a microplate reader. The reaction mixture without protein was referred to the background and was subtracted from samples, meanwhile the caspase-4 activation level in control cells was normalized to 1.

Western blotting. After treatment, 2×10^5 cells were harvested using a scraper and then lysed with 100 µl of lysis buffer [10 mM HEPEs (pH 7.4), 0.15 M NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, and 0.5% NP-40, with protease inhibitor cocktail freshly added]. The extracts were incubated for 30 min on ice and centrifuged at 12,000 rpm at 4°C, and the supernatants were retained. For the electrophoresis, 150 µg of ER proteins or 50 µg of total cellular proteins were separated by 6, 12, or 5~15% gradient gel SDS/PAGE and electrotransferred onto PVDF membranes. The blots were blocked with 5% BSA at 4°C for 1 h. The membranes were brieﬂy washed with Tris-buffered saline containing 0.05% Tween 20 (TBS-T), incubated with the appropriate primary antibodies (anti-HSS at 1:500, anti-PTD at 1:1,000, anti-GAPDH at 1:10,000, anti-GRP-78 at 1:3,500, anti-SREBP-1 at 1:600, anti-p-PERK at 1:1,000 and anti-PERK at 1:2,000, anti-FLAG at 1:6,000, anti-histone H3, anti-COX IV, anti-eIF2α, anti-p-eIF2α at 1:1,000, and anti-CHOP at 1:500) overnight, and subsequently stained with the corresponding IgG secondary antibodies (1:10,000) for 1 h. The membranes were then developed with ECL reagents.

Confocal microscopy analysis. To observe the subcellular localization of HSS after transfection, the HSS-transfected or mutant-HSS-transfected hepatoma cells were seeded in culture dishes specialized for confocal microscopy observations. After attachment, the cells were fixed with 4% paraformaldehyde at room temperature for 15 min. After being washed twice with PBS, the cells were incubated with 0.5% Triton X-100 for 20 min. The cells were then incubated with primary antibodies (anti-Flag antibody at 1:3,000) overnight and subsequently stained with a goat anti-mouse fluorescent secondary antibody (1:400). Next, the cells were stained with ER-Tracker at 37°C for 20 min and re-stained with DAPI to label the nuclei at 37°C for 5 min. For the track of mitochondria, the viable cells were stained with MitoTracker Red CMXRos at 37°C for 15 min and then proceeded to the fixation and other staining steps. A confocal microscope was also used to evaluate the concentration of free cytosolic Ca2+ and ROS. Cells were seeded in the culture dishes, and after attachment, two dishes were incubated with 10 mM NaCl/10 mM Na-DMPS (dissolved in PBS) for 1 h. Next, Fluoro-3AM or DCFH-DA, at a final concentration of 5 µg/ml (dissolved in DMSO), was added to the medium. The mixture was incubated in the dark at room temperature for 1 h, and the cells were washed with d-Hanks’ solution and rinsed with Hanks’ solution. After being washed, the cells were incubated in Hanks’ solution at room temperature for a further 30 min in the dark. The dyed cells were analyzed using a Leica NT fluorescence confocal microscope (Leica, Wetzlar, Germany), and the data were analyzed using the LAS AF Lite software.

Flow cytometric analysis. Cells were seeded in 35-mm culture dishes, and after attachment, the cells were incubated with 100 µM PA for 24 h. After being washed twice with PBS, the cells were resuspended in binding buffer [10 mM HEPEs/NaOH (pH 7.4), 140 mM NaCl, and 2.5 mM CaCl2] by 10.220.32.246 on July 6, 2017 http://ajpcell.physiology.org/ Downloaded from

Morphological assessment of apoptosis. Cells were plated in 24-well plates at 1×10^5 cells/well; 16 h after plating, the cells were treated with 100 µM PA for 24 h. Next, 1.5 µl of Hoechst 33342 stain (10 mg/ml), a DNA-specific fluorescent dye, was added to each well, and the plates were incubated at 37°C for 10 min. The stained cells were then observed using a Leica DMLH fluorescence microscope (DM 5000 B; Leica, Wetzlar, Germany). TEM was used for the
further characterization of the ultrastructure of the ER, and the software “Leica QWin” was used to quantify the dilation of the ER cisternae. In each figure, a piece of 1,000-nm line was depicted just along the ER cisternae in concave side, after crossing the gap to the other side the line was extended along the convex until it reached the beginning spot and closed the circle, and the area of this circle was accounted as quality of ER cisternae. The statistical evaluation of the cross sectional area was performed based on 10 different visual fields.

**Determination of the intracellular ATP level.** The intracellular ATP contents were measured using the Cell-Titer-Glo luminescent cell viability assay kit. The HSS-expressing cells were plated in 96-well plates at 2.5×10^4 cells per well. The cells were treated with 100 μM PA for 24 h and subsequently lysed with 100 μl of lysis buffer. After vortexing and centrifugation, the ATP contents were immediately measured using a Glomax 96 Microplate Luminometer (Promega, Madison, WI).

**Real-time quantitative PCR.** Total RNAs were isolated from the wild-type BEL-7402 cells using the RNeasy Mini Kit according to the manufacturer’s protocol. The cDNA was generated from total RNA samples by using the cDNA reverse transcription kit. Real-time PCR was performed using the Power SYBR Green Master kit. The HSS gene was amplified using the ABI Prism 7300 Sequence Detection System (Applied Biosystems) using specific oligonucleotide primers. 18S rRNA was used as an endogenous control. Primers were as follows: human HSS: sense, 5'-TGA AGC CCA AAT GAA ACG C-3'; antisense, 5'-CAC AGA GGG ATG GAA CAA GC-3'; 18S rRNA: sense, 5'-GTA ACC CGT TGA ACC CCA TT-3'; antisense, 5'-CCA TCC AAT CGG TAG TAGCG-3'.

**Detection of HSS concentrations in the culture medium and cell lysates.** Detection of HSS concentrations was performed according to the instructions for the human FAD-linked sulfhydryl oxidase ALR (GFER) ELISA kit. Each sample of cell lysates was extracted from
10^7 cells, and the samples of culture medium were collected after 10^7 cells were cultured for 24 h.

**Statistical analysis.** All of the results that were based on multiple assays or observations are presented as the means ± SD. The data were analyzed using the statistics software, SPSS 11.5. The differences were considered to be significant if the *P* value was <0.05.

## RESULTS

**Expression of gene delivered HSS in the ER and mitochondria.** The HSS expression levels in the ER of three types of cells (wild-type, vector-transfected and HSS-transfected cells) were assessed by Western blot analysis. As shown in Fig. 1A, a 15 kD band was clearly observed in the ER compartment extracted from the cells transfected with HSS-plasmid DNA. In contrast, HSS expression was barely detected in the ER of the wild-type and vector-transfected cells. The same result was observed in mitochondrial fractions (Fig. 1B). After transfection into BEL-7402 cells, the green signal produced by HSS in the cytoplasmic compartments was partially localized to the same regions as the red signal generated by the ER-Tracker (Fig. 1C), indicating that HSS was able to localize to the ER in the HSS-transfected cells. Meanwhile, confocal analysis of mitochondria showed that HSS presented colocalization in mitochondria (Fig. 1D). The mutation in CXXC motif did not alter the HSS localization.

**Transfected HSS did not secrete extracellularly.** After confirmation of HSS intracellular localization, we were interested in knowing if HSS could secret as cytokine. The HSS concentration in culture medium was measured. Surprisingly, cytosolic HSS concentration in the HSS-transfected cells increased onefold compared with the wild-type cells; however, HSS was still undetectable in culture medium (Fig. 1E). Meanwhile, PA treatment did not result in any change of HSS level in the culture medium (data not shown). These results suggest that the function of HSS provided by transfected HSS is probably due to its effect inside the cells.

**PA-induced hepatocyte steatosis and cell damage.** To produce a proper cellular model of hepatic steatosis, cells were incubated in the medium containing 100 μM PA. Intracellular fat accumulation, which forms lipid vacuoles, was observed under confocal microscope (by Nile Red staining) and TEM. The PA treatment significantly increased the fat accumulation within the BEL-7402 cells (Fig. 2A). Next, we tried to evaluate the cellular FFA and TG concentrations. The results showed that both FFA and TG levels were significantly increased after the PA treatment, suggesting that fat accumulation in the BEL-7402 cells was successfully induced by PA (Fig. 2B and C). Moreover, the relative ratio of TG increase was lower in HSS-transfected cells compared with other two groups (Fig. 2C), indicating that HSS could decrease hepatic lipid accumulation. Interestingly, the decrease in TG level could not be observed in the mutant-HSS-transfected cells.

PA treatment also resulted in severe cell damage. As shown in Fig. 3, A and B, the exposure to PA for 24 h caused cell...
mortality, as measured by the MTT assay, in all three groups of cells, and the PA toxicity increased in a dose- and time-dependent manner. Below a concentration of 100 μM PA for 24 h, the apoptotic rate of BEL-7402 was ~30–40%; however, the PA toxicity in the HSS-transfected cells was significantly reduced compared with the wild-type or vector-transfected cells. Hence, 100 μM PA for 24 h were applied in the following experiments. Figure 3 clearly shows that the transfection of the HSS gene was able to increase the resistance of the cells to PA injury.

**ROS produced by peroxidation and the effect of HSS on the alteration of ROS.** DCFH-DA, a ratiometric ROS indicator dye, is widely used to display the intracellular ROS status. Figure 4 shows that the peroxidation of PA was significantly and profoundly enhanced, resulting in a marked elevation of the intracellular ROS levels in the BEL-7402 cells. As shown in Fig. 4A, after treatment with PA for 30 min, the intracellular ROS levels varied in a dose-dependent manner; they were significantly increased in the wild-type and vector-transfected cells (PA: 50 and 100 μM); however, the ROS level in the HSS-transfected cells was significantly decreased. To verify these observations of the HSS inhibition of ROS production, FCM was applied to evaluate the status of the intracellular ROS. Figure 4B displays a remarkable peak-shift: immediately after the PA (100 μM) treatment of the cells for 30 min, the fluorescence intensities in the vector-transfected and mutant-HSS-transfected cells were comparatively high, 490 ± 19.3 and 483 ± 17.2, respectively. However, the fluorescence intensity was 392 ± 16.4 in the HSS-transfected group, which was significantly reduced, compared with the vector or mutant transfection, *P* < 0.05 (Fig. 4C). Furthermore, the pretreatment of the cells with NAC and Na-DMPS was shown to suppress the elevation of ROS potently.

**PA-induced ER stress and the inhibition of HSS on ER stress.** ROS is believed as one the major leading causes of ER stress. Since our result above confirmed that HSS was able to localize to the ER in the BEL-7402 cells (Fig. 1), we then investigated whether HSS could protect the ER from PA-induced ER stress and apoptosis. In the non-HSS-transfected cells, the treatment of the cells with PA increased the expression of several ER stress marker proteins, including GRP-78, p-PERK, p-eIF2α, and cleaved fragment of SREBP-1 [nominated as SREBP-1(C), since it translocates in the nucleus after being cleaved], reflecting the occurrence of ER stress, ultimately, expression of CHOP protein, which is considered to be the critical mediator of ER stress-induced apoptosis was up-regulated as expected. (Fig. 5A). However, the PA-induced increases in the protein expression levels were downregulated in the HSS-transfected cells. Meanwhile, caspase-4 enzymatic activity, an important mark of ER stress (19), was quantified by the caspase-4 colorimetric assay kit. In the wild-type or the vector-transfected cells, caspase-4 activity was significantly increased after PA treatment (Fig. 5B), while enzymatic activity was largely reduced in HSS-transfected cells (*P* < 0.05). Interestingly, if a mutant HSS gene, in which the codon for C62-X-X-C65 was altered, was transfected the cells, the down-regulation of GRP-78, SREBP-1(C), p-PERK, p-eIF2α, and CHOP expression did not occur, indicating that the protective effect against ER stress might rely on the C62-X-X-C65 motif within the HSS polypeptide.

To convince the HSS inhibition on ER stress, morphology of ER in the HSS-transfected or nontransfected cells after PA treatment was also observed. Figure 5C shows that the PA treatment (100 μM) of the wild-type cells for 24 h induced marked ER changes, such as the dilation of the ER cisternae and ribosome drop-down. However, in the HSS-transfected cells, these morphological changes in the ER were scarcely detected. The statistical evaluation of the ER cisternae dilation...
in the HSS-transfected cells and wild-type cells is shown in Fig. 5D. It was clear that the PA treatment (100 μM H9262) of the wild-type cells for 24 h induced remarkable ER dilation, while the morphology of ER in the HSS-transfected cells was visibly improved.

**ER calcium disequilibrium.** The endoplasmic reticulum is believed to be the largest storage compartment of cytosolic calcium, and there is growing evidence that calcium disequilibrium within the endoplasmic reticulum plays a crucial role during the early stages of ER stress. In this study, we used Fluo-3AM, a ratiometric calcium indicator dye, to investigate the alterations in intracellular calcium storage and release. As shown in Fig. 6A, the treatment of the cells with PA for 30 min increased the concentration of resting cytosolic calcium in both the vector-transfected (as a control) and the mutant-HSS-transfected cells. However, the increase in the cytosolic Ca^{2+} concentration in the HSS-transfected cells was less notable than that of the vector-transfected cells, indicating that HSS transfection might desensitize the cells to PA-induced calcium disequilibrium during ER stress and minimize the calcium overload in the cytosol. Because NAC and Na-DMPS are well known to maintain intracellular calcium equilibrium, the pro-

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**Fig. 5. Evaluation of PA-induced ER stress.** A: Western blot assay of ER stress-related proteins. A total of 50 μg of proteins from each of the vector-transfected cells, mutant-HSS-transfected cells, and HSS-transfected cells were separated by 6% SDS-PAGE for Western blot of anti-glucose-regulated protein 78 (GRP-78), anti-phospho-PRK-like ER kinase (p-PERK), and anti-PERK antibodies; 12% SDS-PAGE for Western blot against anti-eukaryotic initiation factor 2α (eIF2α), anti-p-eIF2α, and anti-C/EBP homologous protein (CHOP) antibodies. After hybridization, anti-GAPDH antibody was used to normalize the loading controls. A total of 50 μg of nucleus proteins extracted from the vector-transfected cells, mutant-HSS-transfected cells and HSS-transfected cells were separated by 5–15% SDS-gradient gel for anti-sterol regulatory element-binding protein-1 (SREBP-1) antibody. SREBP-1(C) represents the cleaved form of SREBP-1 (~68 kD), and anti-histone H3 antibody was used to normalize the loading controls. B: caspase-4 activity was determined by colorimetric assay kit. C: TEM images of ER alteration in cells. All micrographs were taken at the same magnification; scale bar = 100 nm. D: statistical evaluation of the ER cisternae, cross-sectional area per 1,000 nm.

**Fig. 6. PA injury results in alterations in hepatocyte calcium homeostasis.** A: representative Fluo-3AM ratio images of cytosolic calcium in hepatocytes after 30 min with PA and KCl; NAC and Na-DMPS were added to the cells for 1 h before the PA treatment. B: single cell imaging of the depletion of the ER calcium stores with KCl in vector-transfected and mutant-HSS transfected hepatocytes (black), HSS-transfected hepatocytes (red), and hepatocytes pretreated with NAC and Na-DMPS (dash line). Each trace represents an average of 20 hepatocytes from a single experiment. C: quantification of the KCl-releasable stores in the hepatocytes pooled from 3 separate experiments. The peak release is quantified as the change in the ratio (ΔR) divided by the initial ratio (R). D: measurement of the sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) activity in hepatocytes with different pretreatments at 30 min after PA injury. *P < 0.05, compared with the vector transfection.
Protective effect of HSS against calcium disequilibrium, when exercised simultaneously with NAC and Na-DMPS, could be more convincing (Fig. 6A, lanes 5/6). To evaluate whether the elevation in the cytosolic calcium was associated with the depletion of the calcium storage of the endoplasmic reticulum, we utilized potassium chloride (KCl, a chemical that can induce the depletion of the ER calcium stores; Refs. 17, 32). The treatment of the cells with KCl (10 mM) after PA could uncover a calcium leak from the ER, and the amplitude of the peak of calcium release after the addition of KCl reflects the calcium-loading state of the ER. As expected, the KCl-sensitive calcium stores were significantly lower in the vector-transfected and mutant-HSS-transfected cells vs. the cells pretreated with NAC or Na-DMPS and HSS-transfection, suggesting that the elevated calcium concentrations in the cytosol of hepatocytes after PA treatment occurred as the result of the ER leakage and that HSS, together with NAC and Na-DMPS, provides effective protection from the depletion of the ER calcium stores (Fig. 6, B and C). Due to the critical role of the SERCA in the maintenance of the calcium equilibrium of the endoplasmic reticulum, we wanted to test the SERCA activity to investigate whether HSS has a potential preservative function toward SERCAs. Figure 6D shows that PA treatment for 1 h led to a severe impairment of the SERCA activity and that this SERCA inactivation was partially inhibited in the HSS-transfected cells. Similar to the results shown in Fig. 6A, the effect of HSS in rescuing SERCA activity was identical to those of NAC and Na-DMPS. PA treatment did not increase intracellular HSS contents. With regard to the question that whether endogenous HSS, instead of extra-delivered HSS, could participate in cell response to PA insult, we have observed the endogenous HSS alternation during PA intoxication. The results of real-time PCR and Western blot showed that the expression of endogenous HSS was downregulated when wild-type cells exposed to different dosages of PA for 24 h as well as 100 μM PA for different periods of time. As shown in Fig. 7, when dosage of PA was >50 μM for 24 h, or 100 μM PA treatment was >8 h, the expression of HSS, both at the mRNA level (Fig. 7, A and C) and the protein level (Fig. 7, B and D), was significantly reduced compared with control cells without PA injury. ER-induced cell apoptosis and antiapoptotic activity by HSS. ER stress can ultimately cause apoptosis and cell death. Lastly, we wished to ascertain whether PA-induced ER stress is related to cell death and whether this cell death could be prevented by HSS transfection. As shown in Fig. 8, A and B, the treatment of hepatoma cells with 100 μM PA for 24 h induced profound changes in their nuclear morphology. As detected using Hoechst 33342 staining, the wild-type and vector-transfected cells underwent typical apoptotic changes, including cell shrinkage and the formation of apoptotic bodies. However, the apoptotic rate was significantly decreased following the PA treatment in the HSS-expressing cells. Next, the apoptotic rate was analyzed using flow cytometry. In Fig. 8, C and D, the apoptotic rates in the wild-type and the vector-
transfected cells were comparatively high, 54.8 ± 0.9 and 51.1 ± 2.2%, respectively; however, the number of apoptotic cells was significantly reduced in the HSS-transfected group in which 27.8 ± 3.4% of the cells were apoptotic (P < 0.05 vs. the wild-type and vector-transfected cells). Furthermore, a reduction in the ATP content, which is another indicator of apoptosis, was found in the PA-treated cells. Figure 8E shows that the cellular ATP levels in the three cell types were notably reduced after the exposure to PA for 24 h. The amount of ATP in the HSS-transfected cells was still greater than the levels in the wild-type and vector-transfected cells, by 70.8 ± 1.5 and 72.0 ± 2.3%, respectively. These results indicate that HSS exerts an antiapoptotic effect in BEL-7402 cells.

DISCUSSION

The ER regulates protein synthesis, protein folding, and trafficking, cellular responses to stress and intracellular calcium (Ca^{2+}) levels. Alterations in Ca^{2+} homeostasis and the accumulation of misfolded proteins in the ER cause ER stress, which can partially ameliorate disruptions to homeostasis. However, when the protective response is insufficient, a set of responses leads to apoptosis, and the promotion of lipid synthesis and proinflammatory responses are coupled with the latter. Apoptosis is a form of cell death that involves the concerted action of a number of intracellular signaling pathways, including members of the caspase family of cysteine proteases. The two main apoptotic pathways, the death receptor (extrinsic) and mitochondrial (intrinsic) pathways, are activated by caspase-8 and caspase-9, respectively, both of which are found in the cytoplasm. Recent studies point to the ER as a third subcellular compartment implicated in apoptotic execution (40). Indeed, the disruption of ER function has been linked to the development of several disorders, including Alzheimer’s, type-1 diabetes mellitus, and hepatic steatosis. Although a causative role has yet to be clearly demonstrated in most cases, possible mechanisms by which ER dysfunction could initiate or promote a disease state have been proposed. Several independent studies have demonstrated that ER stress can lead to the activation and dysregulation of the sterol responsive element-binding proteins (particularly SREBP-1), transcription factors that control lipid biosynthesis and uptake, often resulting in lipid accumulation (46). The potential role of ER stress in the pathogenesis of hepatic steatosis supports the therapeutic relevance of strategies that either block calcium disequilibrium in the ER or alleviate the burden of unfolded proteins in the ER to reduce ER stress.

It has recently been reported that the protective effect of HSS is likely associated with mitochondrial protection (50, 51). However, the ER is the most abundant of the cytosolic organelles that govern the synthesis of proteins and lipids. To date, there are increasing numbers of studies addressing the roles of ER stress during the pathogenesis of NAFLD. The addition of palmitate to cell cultures could alter the lipid composition in the ER membrane toward an increased degree of saturation, which preceded apoptosis and was possibly induced by the calcium flux from the ER to the cytoplasm and the subsequent ER stress (3, 22, 30). These findings suggest that the induction of ER stress may significantly contribute to the pathogenesis of NAFLD (1). Therefore, we surmised that it would be of interest to explore whether HSS, when overex-

Fig. 8. An assessment of PA-induced apoptosis. A and B: wild-type cells, vector-transfected cells, and HSS-expressing cells were analyzed after Hoechst 33342 staining. The cells treated with PA have undergone chromatin condensation and margination. C and D: apoptosis was analyzed using FCM. The PA treatment significantly increased the apoptotic rate in the vector-transfected and wild-type cells. However, apoptosis was markedly inhibited in the HSS-expressing cells. E: intracellular ATP levels. The ATP level was measured as described in MATERIALS AND METHODS. The statistical evaluation of the apoptotic rate was performed based on 3 independent experiments. *P < 0.05, compared with the control.
pressed within cells, could preserve the ER from such stress injury. First, our results indicated that HSS was able to localize to the ER after plasmid DNA transfection. This result also encouraged us to determine the functional role of the presence of HSS in the ER. Next, we established a model of hepatocyte steatosis in vitro using PA lipotoxicity in liver cells that resembles NAFLD in humans. The present study demonstrated that, after incubation with PA for 24 h, the cells display remarkable increases in lipid droplet accumulation and the activation of several ER stress-related proteins, such as GRP-78, SREBP-1(C), p-PERK, p-eIF2α, and CHOP, indicating that ER stress is actually involved in the pathogenesis and development of hepatocyte lipotoxicity. By showing PA participation in ER stress, our results were also in agreement with findings from both human studies in patients with NAFLD (10) and animal models induced by feeding a high-fat diet (8, 47).

We hypothesize that saturated fatty acids in the steatotic liver induce a stress in the ER that exceeds the capacity of the UPR, resulting in apoptosis and liver injury. Third, a set of overexpressed proteins as GRP-78, SREBP-1(C), p-PERK, p-eIF2α, and CHOP is a good index of ER stress occurred in the cells. Our results indicated clearly that these protein expressions were significantly inhibited in the HSS-transfected cells compared with the nontransfected or mutant-HSS-transfected cells (Fig. 5A), implying that HSS was able to suppress ER stress, which would help the cells resist steatosis injury and apoptosis. HSS was widely described as a stimulator that promotes liver regeneration. Therapeutically, the administration of exogenous HSS protein stimulated hepatocyte proliferation (54) and reversed experimental hepatic fibrosis (16). These results indicate that HSS acts as a growth-promoting factor that induces hepatocyte proliferation and also as a potential therapeutic agent that protects the liver. In our study, the treatment with PA resulted in severe ER stress and apoptosis in the wild-type, vector-transfected, and mutant-HSS transduced cells, but the HSS-transfected cells appear to be more resistant to PA injury, as shown by their decrease in cell mortality (Fig. 3), their response to ER stress (Fig. 5), and their apoptotic rate (Fig. 8). In addition, we found that the overexpression of HSS through plasmid DNA transfection is able to protect cells from PA damage, as determined by morphological assessment, such as the dilation of the ER cisternae and ribosome drop-down (Fig. 5C). These lipotoxic effects of PA were largely prevented by HSS, suggesting that HSS is able to help the cells minimize or even become desensitized to the insult of ER stress, lipid peroxidation, and apoptosis in a cellular model of NAFLD.

The FFA present in cells provides a perpetuating and propagating mechanism for oxidative stress via the process of lipid peroxidation and produces large amounts of ROS and free radicals that cause secondary damage to cellular membranes and key organelles, including the ER; additionally, the SERCAs in the membrane of the ER are highly sensitive to ROS (6, 52). In our study, we observed distinct alterations in the cytosolic calcium homeostasis after the exposure of the hepatocytes to PA (Fig. 6A), whereas there was little change in the calcium homeostasis in the cells that were transfected with HSS or pretreated with NAC/Na-DMPS. These results indicated that HSS, NAC and Na-DMPS all have protective functions in hepatocytes and are able to ameliorate imbalances in calcium homeostasis. It is well accepted that the SERCAs are essential molecules that regulate the movement of calcium across the ER membrane, thereby playing a vital role in maintaining calcium homeostasis within cells. Next, we focus on the identification of the activity of SERCAs in the cells subjected to PA treatment, and we reveal that HSS protection functions by maintaining the SERCA activity. The results shown in Fig. 4 imply that HSS might decrease the cellular concentrations of ROS and alleviate the damage induced by free radicals.

In this context, HSS serves as a scavenger, identical in function to NAC/Na-DMPS, to remove free radicals. Although the mechanism of free radical scavenging by HSS remains unclear, the abundance of thiol and disulfide bonds within HSS crystal structure enable this protein to capture certain unstable substances, such as superoxide species, to prevent their potential damage to the ER membrane and thereby protect the SERCAs from any direct or indirect insult. HSS was found located in the mitochondrial intermembrane system as well as in other subcellular locations such as ER and nucleus. HSS located in intermembrane system was involved in passing its electrons on to molecular oxygen via interaction with cytochrome c and cytochrome c oxidase. This connection to the respiratory chain efficiently prevents the formation of toxic hydrogen peroxide (9). More recently, the data confirmed that HSS plays the crucial function as an antioxidative agent sustaining the levels of reducing agents such as clusterin (38). A recent work in our laboratory demonstrated that HSS transfection decreases hypoxia and reoxygenation-induced mitochondrial ROS production and preserves mitochondrial respiratory chain complex activities (20). Hence, we propose that HSS might function as a free radical scavenger, thereby alleviating hepatic injury and promoting the survival of hepatocytes after HSS transfection.

However, more precise investigations will be required to elucidate the molecular mechanism underlying the HSS alleviation of ER stress; in particular, attention should be directed to exploring how HSS targets the SERCA in the ER membrane and the mechanisms by which HSS functions. Nevertheless, we provide the first evidence that HSS is a suppressor of ER stress that functions by scavenging free radicals in a manner that is equivalent to NAC/Na-DMPS.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS

Author contributions: I.Z., Y.L., S.J., and H.Y. performed experiments; W.A. edited and revised manuscript; W.A. approved final version of manuscript.

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