Hepatic stimulator substance inhibits calcium overflow through the mitochondria-associated membrane compartment during nonalcoholic steatohepatitis

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Nonalcoholic fatty liver disease is considered a disorder of the endoplasmic reticulum (ER) and mitochondria. Recent studies have shown that the ER and mitochondrial membranes overlap by 15–20%, a region referred to as the 'mitochondria-associated ER membrane' (MAM). Some proteins, including sarco/ER calcium ATPase (SERCA), are located in the MAM and have an important role in Ca^{2+} signaling and homeostasis between the ER and the mitochondria. Our previous study showed that hepatic stimulator substance (HSS) inhibits the ER stress induced by reactive oxygen species, thus reducing mitochondrial damage. However, the mechanism underlying the protective effect of HSS on the ER and ER-mitochondrial interaction remains unclear. In this study, we confirmed that the exogenous expression of HSS protected the liver from steatosis in mice with nonalcoholic steatohepatitis. More importantly, the protection provided by HSS allowed SERCA in the MAM compartment to function well, preventing the extensive influx of cytosolic free Ca^{2+} to the mitochondria, thus preserving the mitochondrial functions from calcium overload and relieving palmitic-acid-induced hepatocyte steatosis. Our results suggest that the protective effect of HSS on SERCA expression is associated with the maintenance of calcium homeostasis within the MAM, thus ameliorating the disordered Ca^{2+} communication between the ER and mitochondria.

Nonalcoholic fatty liver disease (NAFLD) is a spectrum of diseases ranging from simple steatosis to nonalcoholic steatohepatitis (NASH), which involves more severe hepatic lesions and marked inflammation, than fibrosis, and also end-stage liver disease, such as cirrhosis.[1] NAFLD is now recognized as one of the most common causes of liver disease in the world.[2] The endoplasmic reticulum (ER), one of the largest cellular organelles, is responsible for the proper assembly and posttranslational modification of proteins destined for intracellular organelles and the cell surface. The ER is also the main storage site for cytosolic calcium. The ER Ca^{2+} levels are controlled by the balance between calcium release and calcium uptake, which has an important role in the regulation of ER homeostasis. The disruption of ER homeostasis, often called 'ER stress', has been observed in the livers of humans with NAFLD and also in the experimental rodents.[3] Therefore, it is strongly believed that ER stress and the subsequent response to it, called the 'unfolded protein response', have important roles in the pathogenesis of NAFLD and may be critical factors in the progression of NAFLD to NASH.[4] Therefore, the mechanisms that disrupt the Ca^{2+} equilibrium of the ER and disturb ER-mediated Ca^{2+} signaling, which may exacerbate the progression of NAFLD, have become topics of intense investigation. It also appears that the ER and mitochondria interact both physically and functionally via a special structure known as the 'mitochondria-associated membrane' (MAM),[5] which allows the ER-mediated transfer of calcium to the mitochondria, altering the mitochondrial functions and oxidative stress in NAFLD. So far, a large number of proteins have been have identified in the MAM compartment, including the mitochondrial voltage-dependent anion channel and sarco/ER calcium ATPase (SERCA), which regulate the Ca^{2+} flow between the ER and mitochondria.[6] During ER stress, the transient overflow of calcium transmitted from the ER to the adjacent mitochondrial network via the MAM pathway is particularly harmful to

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the mitochondria because this Ca^{2+} wave, or eventual Ca^{2+} overload if sustained, can impair oxidative phosphorylation and ATP production, and even trigger mitochondrial apoptosis.[7] Knowledge of the MAM-Ca^{2+} signaling pathway and its effect on the mitochondria is essential for understanding the development of NAFLD.

Hepatic stimulator substance (HSS) was first described in the liver cytosol of a weanling rat by LaBrecque and Pesch[8] in 1975. HSS stimulates hepatic DNA synthesis and therefore promotes liver regeneration after partial hepatectomy.[9] The effect of HSS on hepatocytes is believed to be a liver-specific and non-species-specific phenomenon.[10] HSS was subsequently shown to protect hepatocytes from toxic agents, such as CCl_4,[11] D-galactosamine,[12] and H_2O_2.[13] HSS also confers resistance to hepatic ischemia–reperfusion injury by protecting mitochondrial functions,[14] and the protective effects of HSS on liver functions may be related to its improvement of mitochondrial functions.[15,16] Despite the HSS nomenclature, an earlier study showed that HSS has little effect on stimulating the growth of primary cultured hepatocytes, but can augment the proliferative effects of other mitogens, such as epidermal growth factor. Based on this property, HSS is also known as augmenter of liver regeneration (ALR).[17] The conserved C_{62}–X–X–C_{65} (CxxC) motif within the C-terminal region of the ALR protein molecule is required for its role in mitochondrial protection.[18] In a recent study, we also demonstrated that HSS inhibits ER stress and protects SERCA activity against injury by reactive oxygen species (ROS), thus preventing cell damage.[19] The molecular features of ALR have been extensively investigated and the crystal structure of the ALR protein indicates that it is a sulfhydryl oxidase with a FAD-binding site and a conserved proximal CxxC motif.[20] Because mammalian *ALR* is highly homologous to the yeast *Saccharomyces cerevisiae ERV*1 gene, originally called 'essential for respiration and viability',[21] it is also known as 'growth factor, ERV1 homolog of *S. cerevisiae*' (GFER). In mammals, ALR messenger RNA is expressed in multiple organs,[22] and the protein is mainly located in the intermembrane space of the mitochondria,[23] where it seems to be associated with the electron transfer in the respiratory chain.[24] Its association with the mitochondria and the regulation of ATP synthesis make ALR critically important in the survival of hepatocytes and the inhibition of its expression causes cell death.[25]

We have previously demonstrated that the inhibition of ER stress by HSS may protect the mitochondrial. However, the mechanisms involved remain unclear. We speculate that the ER protection provided by HSS could be associated with the blockage of Ca^{2+} overflow from the ER to the mitochondria, stimulating the mitochondrial metabolism. If this is the case, the regulation of this activity, particularly during the progression of NAFLD, is an important area of research.

Here, we demonstrate that ER stress occurs during the progression of NAFLD, when SERCA activity at the MAM is severely impaired and mitochondria are damaged and become dysfunctional. Parallel with this effect on SERCA, mitochondrial Ca^{2+} overload occurs, and the mitochondrial functions are further impaired by lipotoxicity. However, the exogenous expression of HSS restored SERCA activity and prevented mitochondrial Ca^{2+} overload, possibly improving intracellular Ca^{2+} homeostasis and alleviating hepatic steatosis.

MATERIALS AND METHODS
Reagents
Dulbecco's modified Eagle's medium and fetal bovine serum were purchased from Gibco (Paisley, UK) and Hyclone (Victoria, Australia), respectively. Antibodies directed against glucose-regulated protein 78 (GRP78, 78 kDa, Cat. No. 3183), B-cell leukemia/lymphoma-2 (BCL-2, 26 kDa, Cat. No. 15071) were purchased from Cell Signaling Technology (Danvers, MA, USA). Acyl-CoA synthetase 4 (FACL-4, 79 kDa, Cat. No. ab155282) was purchased from Cell Signaling Technology (Cambridge, UK). Antibodies directed against HSS fragment (23 kDa, Cat. No. sc-365886), SERCA2 (100 kDa, Cat. No. sc-8094), C/EBP homologous protein (CHOP, 27 kDa, Cat. No. sc-7351), β-actin (42 kDa, Cat. No. sc-47778), protein disulfide isomerase (PDI, 55 kDa, Cat. No. sc-74551), cytochrome c (Cytc, 14 kDa, Cat. No. sc-13156), and BAX (20 kDa, Cat. No. sc-23959) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). A triglyceride (TG) quantification kit was purchased from Applygen (Beijing, China). A mitochondrion isolation kit was purchased from Applygen. A mitochondrial membrane potential (MMP) detection kit, mitochondrial permeability transition (MPT) and complex V (ATPase) activity detection kit were purchased from Genmed Company (Shanghai, China). A CellTiter-Glo Luminescent Cell Viability Assay kit was purchased from Promega (Madison, WI, USA). An endoplasmic reticulum isolation kit was purchased from Sigma-Aldrich (St. Louis, MO, USA). A Ca^{2+}-ATPase assay kit was purchased from Jiancheng (Nanjing, China). 1, 2-Bis (2-aminophenoxy) ethane-N, N, N′, N′-tetraacetic acid acetoxymethyl ester (BAPTA-AM) was purchased from Sigma-Aldrich. Methionine/choline-deficient (MCD) diet was purchased from Huafukang Company (Beijing, China).

Preparation of HSS-Bearing Adenovirus
Human *HSS* cDNA tagged with *FLAG* was used to construct a replication-deficient adenoviral vector, pAdxsi (Chinese National Human Genome Centre, Beijing, China), as described previously.[14] Successive steps in its construction were routinely checked with viral titration and the final product was designated 'Ad-HSS'. The adenoviral vector, pAdxsi, lacking the *HSS* gene, was designated 'Ad-Null'.

Animal Studies
Male C57BL/6 mice (6–7 weeks old) were supplied by the Experimental Animal Centre, Capital Medical University, Beijing, China. Totally, 48 animals were applied in the study and they were divided into 4 groups. Each group contained 12

animals. The mice in the group one were the control and fed with a regular diet, and mice in other three groups (designated as the *HSS*-transfected, vector-transfected and control-transfected group) were fed with a methionine and choline-deficient (MCD) diet. The mice were transfected with 500 μl of either the Ad-*HSS* or Ad-Null virus (1×10^9 plaque-forming units) via the tail vein. The control-transfection was subjected to normal saline injection. The mice in every group were killed either at the end of the first week ($n = 6$) or the second week ($n = 6$). All the animals received humane care in compliance with the Guidelines of the Committee for Institutional Animal Care and Use of Capital Medical University.

Biochemical and Histological Analyses
The serum concentrations of alanine aminotransferase (ALT), aspartate aminotransferase (AST), TG, and cholesterol were measured. The liver tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and stained with hematoxylin and eosin. A portion of each liver sample was used to prepare frozen tissue sections, which were stained with oil red O. The rest of the liver tissue was stored at −80 °C for further analysis.

Cell Culture
Hepatocellular carcinoma cell line, BEL-7402, was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The HepG2 cell was obtained from the American Type Culture Collection (Manassas, VA, USA). The BEL-7402 or HepG2 cells were transfected with an *HSS*-containing plasmid (designated 'HSS-Tr/7402' or 'HSS-Tr/HepG2') and the control cells were transfected with empty plasmid (designated 'vector-Tr/7402' or 'vector-Tr/HepG2'), which are maintained in our laboratory. Untransfected BEL-7402 or HepG2 cells were considered wild-type cells. The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, and the stably *HSS*-transfected cells were screened with gentamicin (G418, 300 μg/ml for 2 months). The resistant clones were isolated, and the relevant cells were cultured at 37 °C in an incubator under a humidified atmosphere of 5% CO_2.

Isolation of Mitochondria
Approximately 3×10^7 cells were homogenized in mitochondrial isolation buffer (10 mM Tris (pH 7.4), 250 mM sucrose, 0.15 mM $MgCl_2$). The homogenate was centrifuged at $800 \times g$ at 4 °C for 5 min, and the supernatant was centrifuged at 10 000 g for 100 min to obtain a mitochondria-enriched pellet. The pellets were then centrifuged at 12 000 g for 10 min, pooled, washed, and resuspended in isolation buffer for activity measurements.

Assay of Complex V (ATPase) Activity
This assay linked ATPase activity to nicotinamide adenine dinucleotide (NADH) oxidation via the conversion of phosphoenolpyruvate to pyruvate by pyruvate kinase, and

then from pyruvate to lactate by lactate dehydrogenase. The reaction buffer consisted of 250 mM sucrose, 5 mM $MgCl_2$, 2 mM KCN, 50 mM KCl, and 20 mM Tris-HCl (pH 7.5). Before the assay, 0.25 mM NADH, 1 mM phosphoenol pyruvate, 2.5 U/ml lactate dehydrogenase, and 2 U/ml pyruvate kinase were added to the reaction buffer. The reaction was initiated by the addition of 10 μg of mitochondria, and the changes in absorbance were recorded at 340 nm for 5 min.

Determination Of Intracellular ATP Levels
The intracellular ATP concentrations were determined with the CellTiter-Glo Luminescent Cell Viability Assay. Cells were plated in 96-well plates at 2.5×10^4 cells/well, treated with 300 μM palmitic acid (PA) for 24 h, and then lysed with 100 μl of lysis buffer. After the lysates were vortexed and centrifuged, the ATP contents were measured with a Glomax 96 Microplate Luminometer (Promega).

Determination of MMP (ΔΨm) and MPT
Membrane-permeable JC-1 dye has advantages over other cationic dyes in that it can selectively enter mitochondria and reversibly changes color (from green to red) as the membrane potential increases. In healthy cells with high ΔΨm, JC-1 spontaneously forms complexes with red fluorescence, whereas in unhealthy cells with low ΔΨm, JC-1 remains in the monomeric form, displaying only green fluorescence. Reduced fluorescence intensity indicates that the mitochondrial membrane structure is impaired. For the MPT assay, cells were treated with 1.0 μM calcein-AM and 1.0 μM $CoCl_2$ to selectively load the mitochondria with calcein by quenching the cytosolic calcein signal with $CoCl_2$. The loss of mitochondrial calcein fluorescence under basal conditions was then used as a measure of nonpathological MPT.

Assay Of SERCA Activity
ER was extracted according to the manufacturer's instructions. In brief, after centrifugation at 600 g for 5 min, the cells were harvested and then homogenized in the Isotonic Extraction Buffer using a Dounce homogenizer. After centrifugation at 1,000 g at 4 °C for 10 min, the supernatant was collected, and centrifuged at 12 000 g at 4 °C for 15 min. Subsequently, the supernatant was carefully transferred to a fresh tube and centrifuged at 100 000 g at 4 °C for 60 min. The pellet, which contained the ER compartment, was kept on ice before the experiments. SERCA activity was measured with a Ca^{2+}-ATPase assay kit (Jiancheng Bioengineering Institute, Nanjing, China) and normalized to the protein concentration. This approach was the colorimetric measurement of Pi using the malachite green assay. An aliquot of extracted ER was added to the malachite green reagent and allowed to incubate at 37 °C for 10 min. The amount of Pi was determined at 636 nm against a standard curve. Pi generated during the reaction period was then converted to ATPase activity.

Preparation Of MAM Fractions From Cells And Mouse Livers

The protocol described by Wieckowski *et al*[26] was used to prepare the MAM from cultured cells and tissues. During each step of preparation, the mitochondrial and ER fractions were saved to assay their purity. The MAM fraction was finally collected and suspended in mitochondrion resuspension buffer (MRB) for further analysis. To prepare the MAM from liver tissues, all the procedures were similar to those described for the cellular preparation, except the tissue homogenization and centrifugation steps. The crude mitochondrial pellet was resuspended in IBliver-3 buffer and centrifuged at 10 000 g for 10 min to isolate the MAM. The MAM fraction was suspended in mitochondrion resuspension buffer for further analysis.

Western Blotting

Protein concentrations were determined with a bicinchoninic acid protein quantitation kit. The aliquoted proteins (100 μg) were separated with 12% SDS–PAGE, and after they were blotted onto membrane, primary antibodies were used to detect specific proteins. The primary antibodies were then detected with horseradish-peroxidase-labeled anti-IgG secondary antibodies. The blots were visualized with the Odyssey Two-Color Infrared Imaging System (Li-Cor, Lincoln, NE, USA).

Calcium Measurement

Cells (1×10^6) were washed with D-Hank's solution (Sigma) for three times and incubated at 37 °C with Fura3-AM or Rhod-2-AM (1 μM; Molecular Probes) for 1 h. Then the cells were washed with Hank's solution (Sigma) for 3 times and balanced at room temperature at least for 20 min. The cells were then pretreated with BAPTA-AM (100 μM) for 10 min to chelate the cytosolic Ca^{2+}. The cells were then incubated with PA (300 μM) and the Ca^{2+} fluorescence intensity was measured with a Leica NT fluorescence confocal microscope (Leica, Wetzlar, Germany). To measure cytosolic Ca^{2+}, the cells were treated with Fura3-AM and their fluorescence (488 nm excitation; 515 nm emission) was recorded for 5 min. To measure mitochondrial Ca^{2+}, the cells were treated with Rhod-2AM and their fluorescence (559 nm excitation; 567 nm emission) was measured for 5 min.

Statistical Analysis

Results are expressed as means ± s.e. GraphPad (version 5.03, GraphPad Software Inc., CA, USA) was used to analyze the data. Comparisons of two groups were made with Student's t test, and $P < 0.05$ was considered statistically significant. All experiments were repeated at least twice.

RESULTS

HSS Attenuates Steatosis and Liver Injury in a Mouse Model of NASH

C57BL/6 mice that were fed an MCD diet for up to 2 weeks displayed clear manifestations of NASH and were used in the subsequent experiments. The bodyweights of the mice decreased after they were fed the MCD diet for 1 week ($P < 0.05$) or 2 weeks ($P < 0.01$), and the liver wet weights were reduced in the mice that were fed the MCD diet for 2 weeks ($P < 0.01$). The administration of Ad-HSS (1×10^9 plaque-forming units, 500 μl) to the MCD mice did not improve their bodyweights or liver wet weights compared with those of the Ad-Null group (Figure 1a and b).

The serum AST and ALT levels of the MCD mice increased after 1 or 2 weeks compared with those of the Ad-Null group ($P < 0.05$). The exogenous expression of Ad-HSS reduced the serum ALT levels in the MCD mice at 1 and 2 weeks ($P < 0.05$), but only reduced their serum AST levels at 2 weeks compared with those of the control (Ad-Null) group (Figure 1c and d); $P < 0.05$. The serum TG levels increased in the MCD mice after 2 weeks compared with those in the control group ($P < 0.01$), but HSS transfection partly reduced the serum TG levels compared with those of the control mice ($P < 0.05$; Figure 1e). The hepatic TG levels increased in the MCD mice after 1 and 2 weeks compared with those of the control group ($P < 0.05$ and $P < 0.01$, respectively). However, the exogenous expression of HSS reduced the hepatic TG levels in the MCD mice after 1 and 2 weeks ($P < 0.05$ and $P < 0.01$, respectively; Figure 1f).

A hepatic morphological analysis showed that the liver tissues of the MCD mice were filled with large numbers of fat vacuoles (Figure 1g). Oil red O staining showed that large amounts of lipid droplets were present in the hepatic tissues. The exogenous expression of HSS reduced the fat vacuoles and lipid accumulation within the liver tissues (Figure 1h), suggesting that HSS protects the liver from steatosis in mice with NASH.

To verify location and expressions of exogenously delivered HSS and their relation with attenuation of hepatic steatosis, the mice were injected with 500 μl of the Ad-HSS (1×10^7, 10^8 and 10^9 plaque-forming units) via tail vein and fed with an MCD diet for 2 weeks. It was found that exogenous HSS mainly localized in hepatocytes. More importantly, the exogenous HSS expression by adenoviral injection was apparently associated with attenuation of hepatic lipid deposition in a dose-related pattern ($P < 0.05$; Figure 1i).

HSS Suppresses ER Stress and Protects Mitochondria from Lipotoxicity

After the mice were fed the MCD diet for 1 or 2 weeks, hepatic ER stress, manifesting as the elevated expression of GRP78 and CHOP, was induced and HSS expression was downregulated. However, the exogenous expression of HSS inhibited this ER stress (Figure 2a), and cell apoptosis, detected as the elevated expression of BAX and BCL-2, also
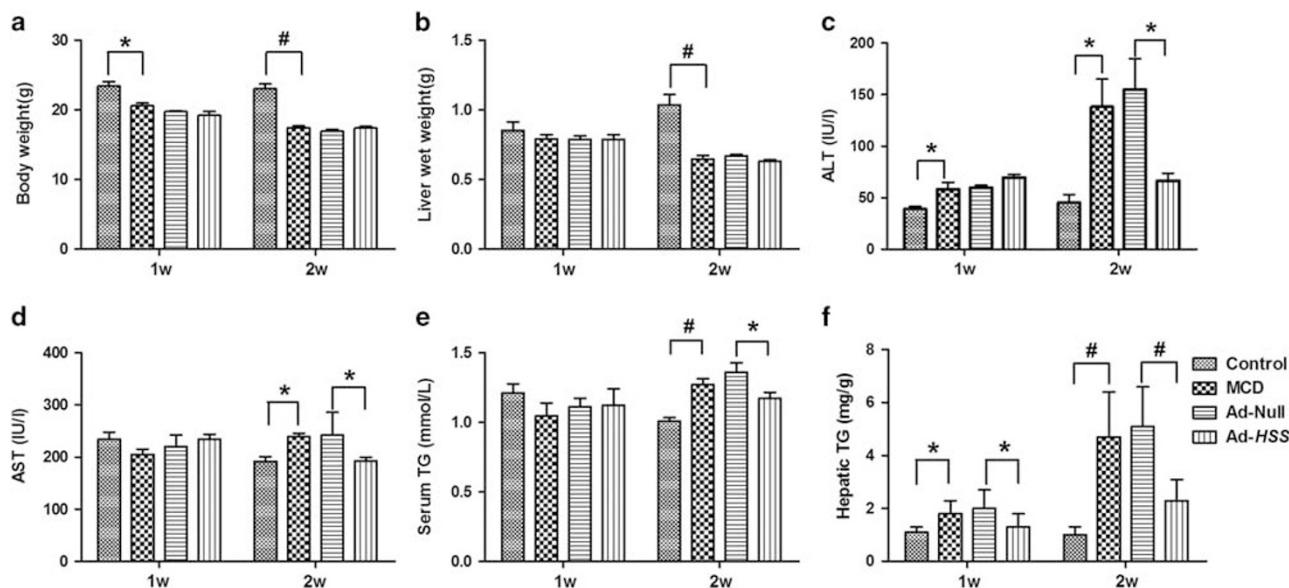
Figure 1 Hepatic stimulator substance (HSS) attenuates steatotic injury. C57BL/6 mice were fed an methionine and choline-deficient (MCD) diet for 2 weeks to establish a mouse model of nonalcoholic steatohepatitis (NASH). (**a**) Bodyweight. (**b**) Liver wet weight. (**c**) Serum alanine aminotransferase (ALT). (**d**) Serum aspartate aminotransferase (AST). (**e**) Serum triglyceride (TG). (**f**) Hepatic TG. (**g**) Liver tissues stained with hematoxylin and eosin. (**h**) Liver tissues stained with oil red O. (**i**) Topographical relationship between steatosis area and HSS expression. Data are presented as mean ± s.e. ($n = 12$ per group).*$P < 0.05$, #$P < 0.01$.

decreased (Figure 2b). The exogenous expression of HSS effectively reduced liver apoptosis levels in the MCD mice, suggesting that HSS protects the mitochondria from lipid-induced apoptosis.

HSS Enhances SERCA Expression in MAMs of Mice with NASH

SERCA is an ER-associated calcium pump, responsible for the transport of Ca^{2+} from the cytosol to the ER, helping to maintain the intracellular Ca^{2+} concentrations at physiological levels. To study the mechanism underlying the protective effects of HSS in terms of SERCA, the MAM fractions were extracted from the livers of mice with NASH, and the liver cells were subjected to lipid intoxication. Western blotting results indicated that hepatic SERCA expression was reduced in the MCD mice relative to that in the control mice. However, SERCA expression in the mice expressing exogenous HSS was restored to levels similar to those of the control mice, indicating that HSS maintains the expression of SERCA from ER stress (Figure 3). Furthermore, the protective effects of HSS on SERCA occurred in both the ER and MAM compartments (Figure 3). These results clearly indicate that the exogenous expression of the *HSS* gene increases SERCA expression in the MAM compartment.

Inhibition of ER Stress by HSS Attenuates Steatosis

To determine whether the increased SERCA activity induced by the exogenous expression of HSS improves hepatocyte resistance to lipid injury, cells were treated with PA (300 μM) for 24 h. A western blotting analysis indicated that the PA

treatment induced ER stress, based on the expression and upregulation of several ER-stress-related markers, such as GRP78 and CHOP. ER stress was alleviated in the *HSS*-Tr/7402 cells (Figure 4a). To convince our results, another cell line HepG2 was employed in the experiment. The results were identical to those of BEL-7402 cells, indicating that ER stress was decreased in the HSS-Tr/HepG2 cells (Figure 4a). The PA-induced collapse of the mitochondrial membrane structure (ie, impaired MPT and MMP) was also prevented in the *HSS*-Tr/7402 cells (Figure 4b and c), resulting in improved respiratory chain complex V activity and ATP levels relative to those in the BEL-7402 cells transfected with vector-Tr (Figure 4d and e). The TG levels were also reduced in the *HSS*-Tr/7402 cells (Figure 4f). In the vector-Tr/7402 cells, PA treatment caused morphological abnormalities in the mitochondrial ultrastructure, including the loss of mitochondria, the swelling and vacuolization of the organelle, and swollen/deficient cristae. However, in the *HSS*-expressing/7402 cells, the structure of the mitochondria was markedly improved, with abundant mitochondria and healthy cristae, and dilatation of the cristae structure was rarely observed. (Figure 4g). Like the vector-Tr/7402 cells, PA treatment could induce the abnormalities of mitochondrial ultrastructure in vector-Tr/HepG2 cells. However, the transfection of HSS could improve the structure of the mitochondria (Figure 4g).

HSS Rescues SERCA Expression to Inhibit ER Stress

The reduced SERCA expression induced by PA (300 μM) was slightly ameliorated in the *HSS*-Tr/7402 cells compared with
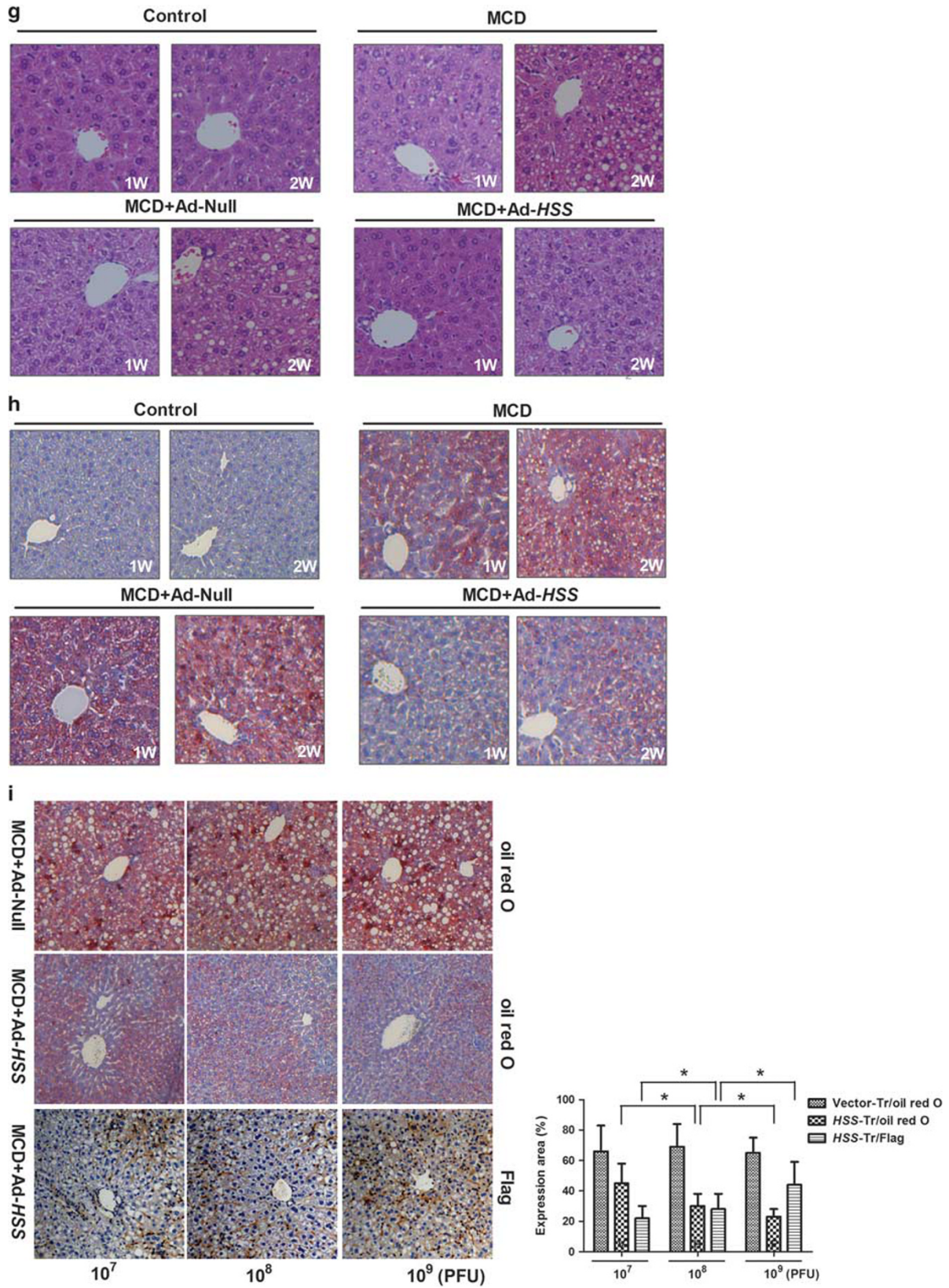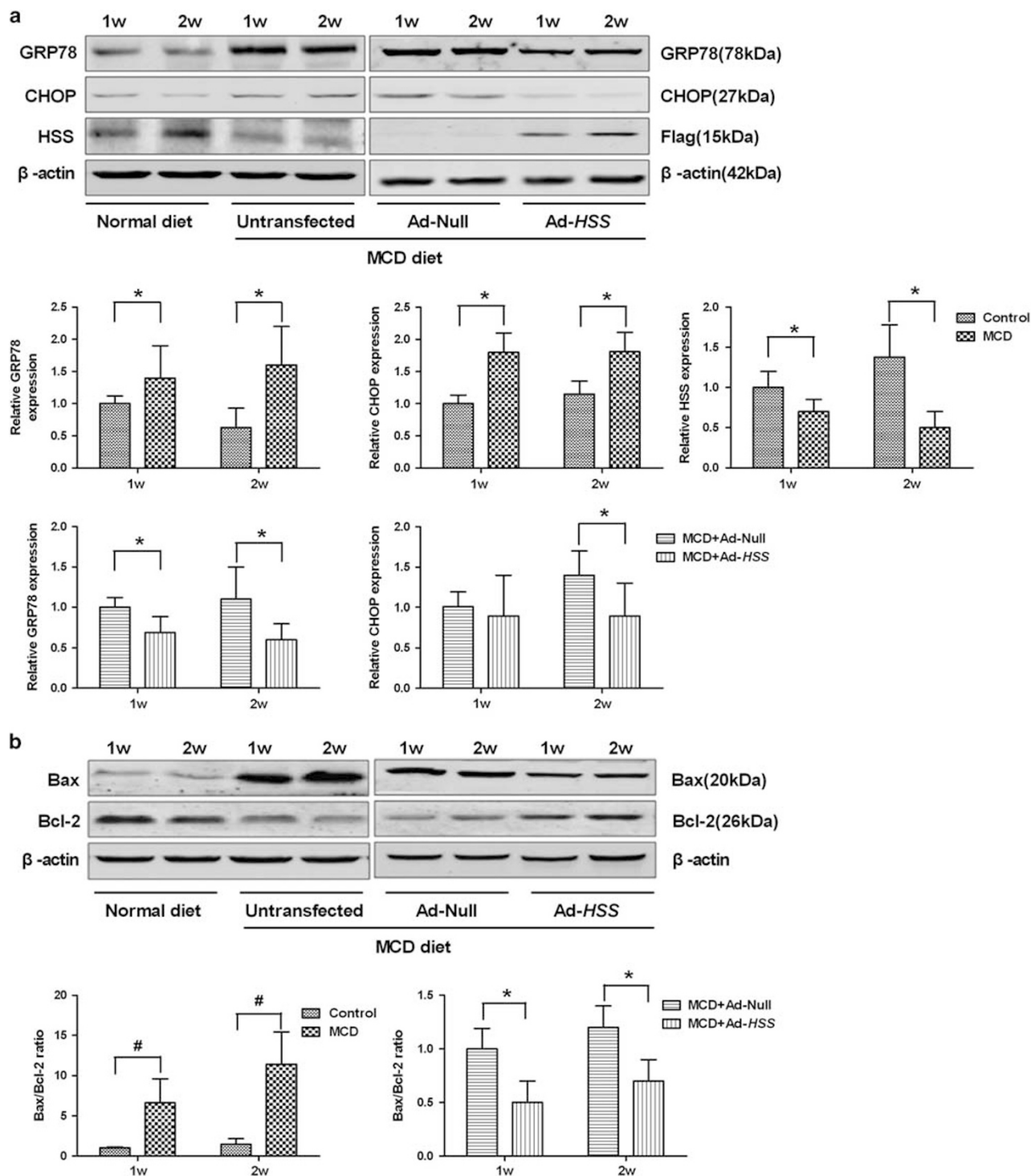
Figure 1 Continued

Figure 2 Hepatic stimulator substance (HSS) suppresses endoplasmic reticulum (ER) stress and mitochondrial apoptosis in mice with nonalcoholic steatohepatitis (NASH). (**a**) Levels of ER stress markers and HSS were determined with western blotting and normalized to β-actin. (**b**) Levels of apoptotic indices BAX and BCL-2 were determined with western blotting and normalized to β-actin. Densitometry results are shown. Data are presented as mean $\pm$ s.e. ($n = 3$ per group). *$P < 0.05$, #$P < 0.01$.

that in the vector-Tr/7402 cells (Figure 5a). We also used the HSS-Tr/HepG2 cells to convince the results above. As we expected, the decreased SERCA expression promoted by PA was also inhibited in the HSS-Tr/HepG2 cells (Figure 5a). SERCA activity was also preserved in the HSS-Tr/7402 cells (Figure 5b). Moreover, SERCA rescue occurred not only in
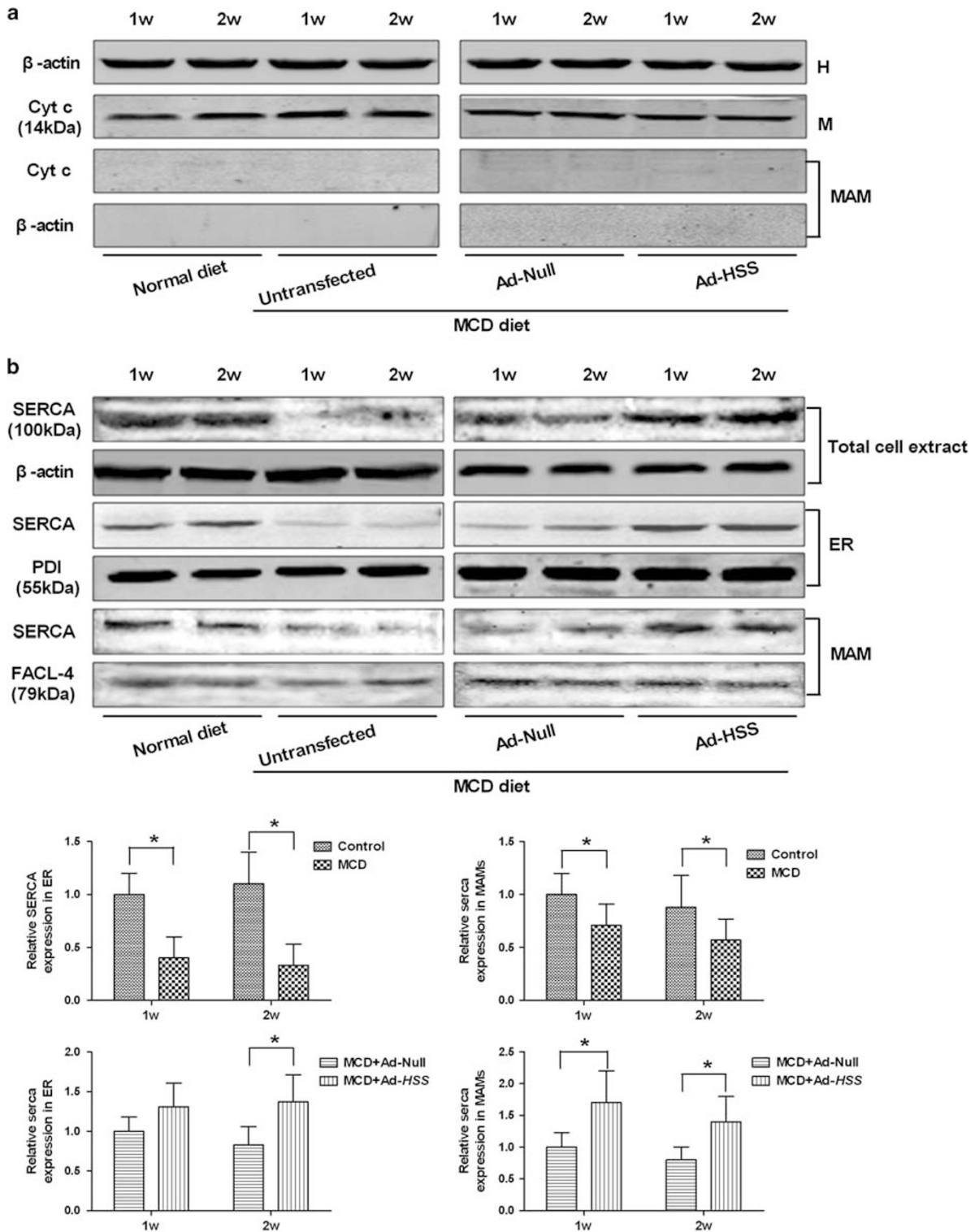
Figure 3 Hepatic stimulator substance (HSS) enhances sarco/ER calcium ATPase (SERCA) expression in mitochondria-associated membrane (MAM) in mice with nonalcoholic steatohepatitis (NASH). Proteins were extracted from the cell cytosol, endoplasmic reticulum (ER), and MAM. Western blotting was used to determine the purity of MAM (**a**) SERCA expression in the cytosolic, ER, and MAM extracts (**b**). As an ER marker, protein disulfide isomerase (PDI) should be enriched in the ER fraction, and as a MAM marker, FACL-4 should also be enriched in this fraction. Cytochrome c localizes to the inner mitochondrial membrane and should be heavily expressed in the mitochondrial fraction, but not in the MAM fraction. β-actin should be absent from the mitochondrial, MAM, and ER compartments. Densitometry results are shown. Data are presented as mean ± s.e. ($n = 3$ per group). *$P < 0.05$.
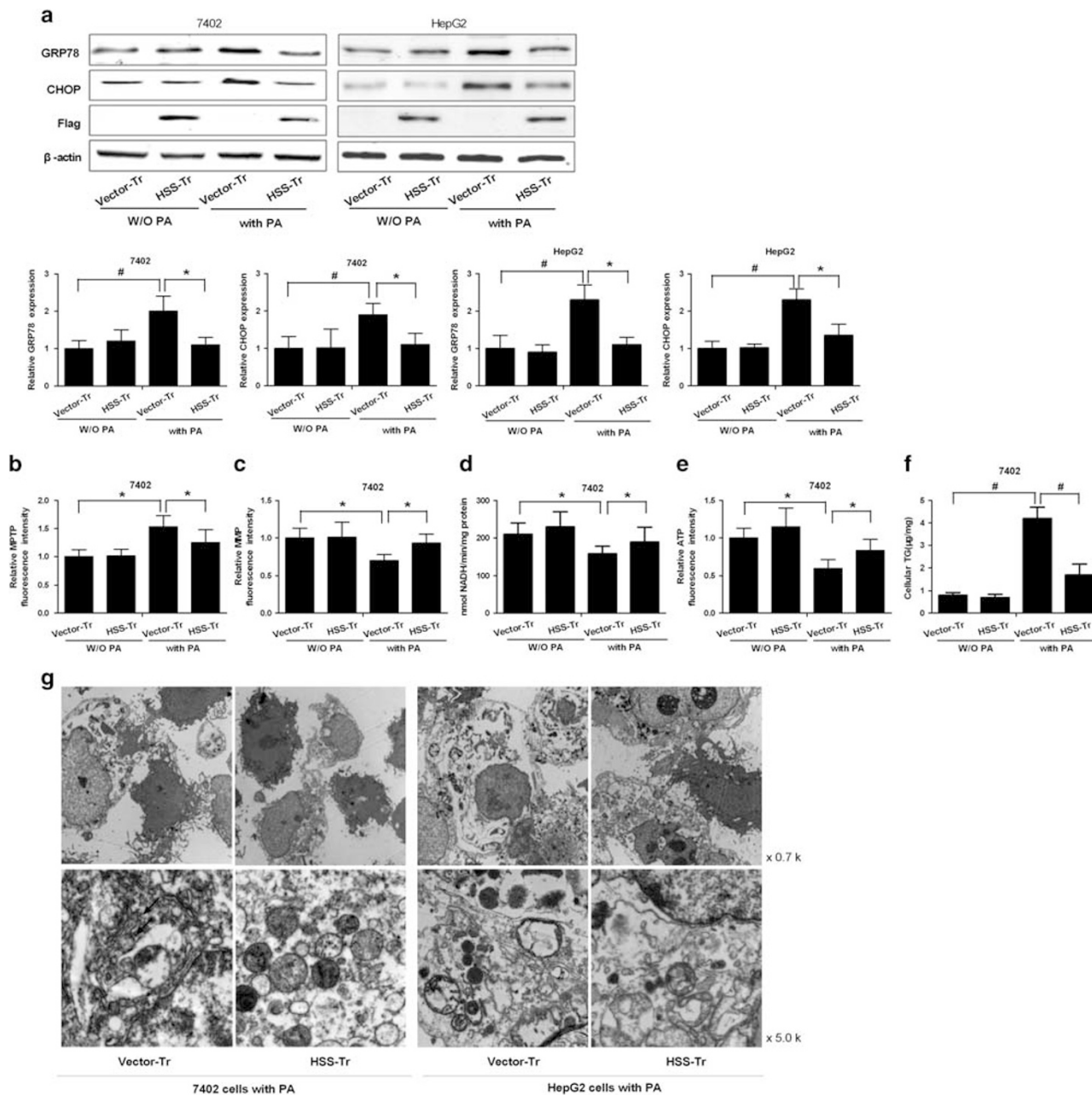
Figure 4 Hepatic stimulator substance (HSS) expression inhibits endoplasmic reticulum (ER) stress and attenuates mitochondrial damage. Cells were treated with palmitic acid (PA; 300 μM) for 24 h. (**a**) Levels of ER stress markers were determined in the *HSS*-transfected (*HSS*-Tr) and control cells with western blotting. Densitometry results are shown. Relative MTP (**b**) and MMP (**c**) fluorescence intensities; mitochondrial complex V activity (**d**); relative ATP contents (**e**); and cellular TG levels (**f**). (**g**) Electron microscopic image of mitochondria. Palmitic acid (PA) treatment caused marked disintegration of the mitochondrial ultrastructure, including the loss in mitochondria, mitochondrial swelling, and vacuolization) in most hepatocytes. HSS transfection markedly attenuated the morphological injuries to the mitochondria induced by PA. Data are presented as mean ± s.e. (*n* = 3 per group). *$P < 0.05$, #$P < 0.01$.

the ER membrane, but also in the MAM compartment in the *HSS*-Tr/7402 cells (Figure 5c).

HSS Protects Mitochondria from Ca^{2+} Overload
It has been reported that the mitochondrial Ca^{2+} uptake from the cytosol requires the direct contact between the mitochondria and the ER membrane *via* the MAM. Therefore, we

investigated whether the protective effect of HSS on SERCA expression during ER stress protects the mitochondria from Ca^{2+} overflow *via* the MAM compartment. To address this question, ER stress was induced with PA, and the cytosolic and mitochondrial Ca^{2+} oscillations were monitored after *HSS* transfection. The results indicate that the release of Ca^{2+} into the cytosol and mitochondria increased immediately
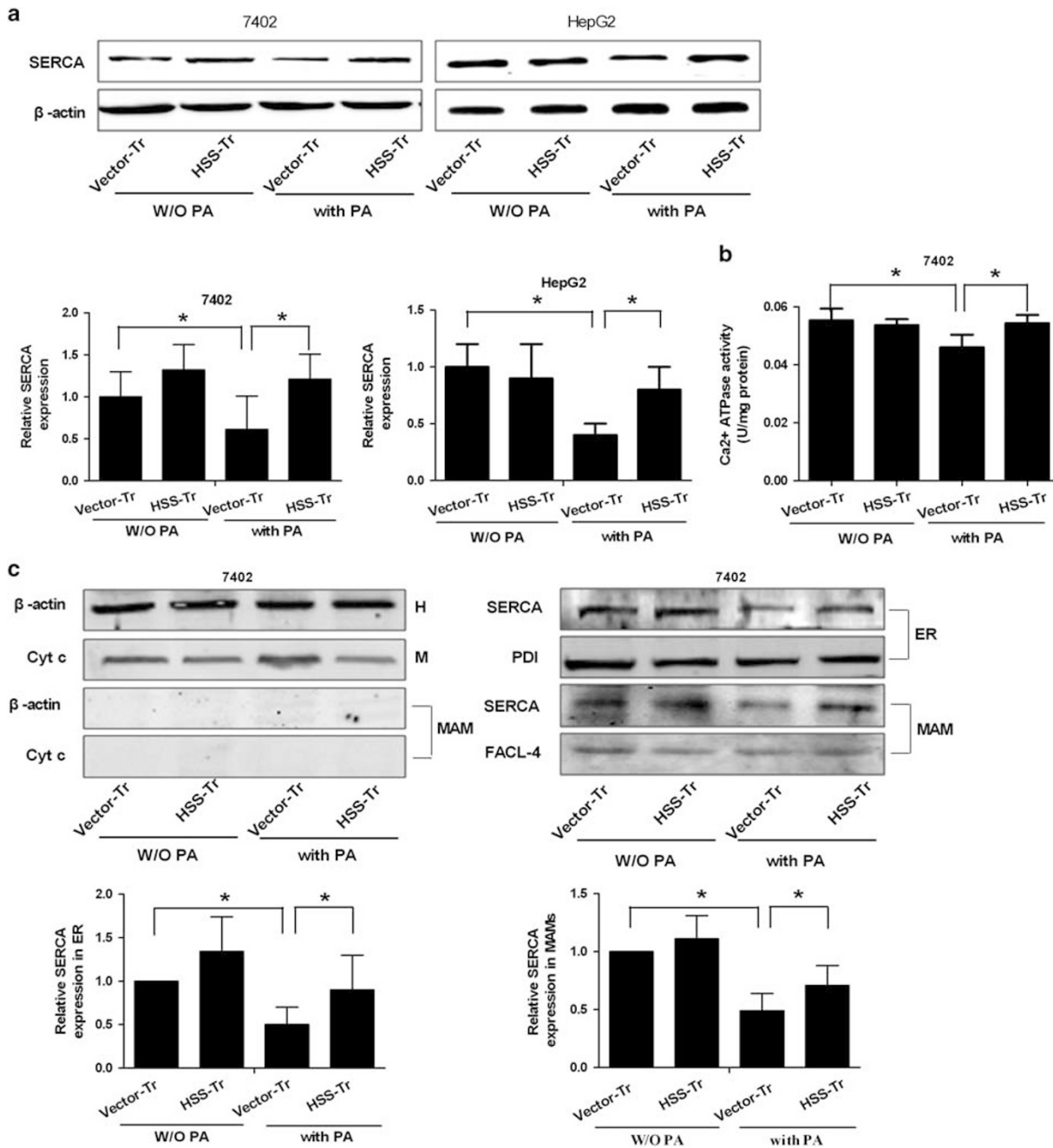
Figure 5 HSS promotes SERCA expression at MAM. Cells were treated with PA (300 μM) for 24 h. (**a**) Levels of SERCA were determined with western blotting. Densitometry results are shown. (**b**) SERCA activity was analyzed. (**c**) SERCA expression was measured in the ER and MAM compartments. Densitometry results are shown. *$P < 0.05$.

after the addition of PA to vector-Tr cells, whereas the Ca^{2+} levels in these two compartments decreased in the *HSS*-Tr cells (Figure 6). As it is hard to measure SERCA activity in the MAM compartment, we used the calcium-specific chelator BAPTA-AM (100 μM) for 10 min to abolish the oscillations in free cytosolic Ca^{2+}. This strategy excluded any possibility that the free intracellular Ca^{2+} would move into the mitochondria, except through the MAM compartment. Under these

conditions, the mitochondrial Ca^{2+} levels are partly dependent on the SERCA activity in the MAM. As shown in Figure 6a, the PA-induced increase in Ca^{2+} release was abolished in the cytosolic compartment. A reduction in the mitochondrial Ca^{2+} concentration was also observed in the BAPTA-AM-pretreated cells (Figure 6b), suggesting that ER stress induces an increase in cytosolic Ca^{2+} as a result of the inactivation of SERCA, permitting the influx of free Ca^{2+} into
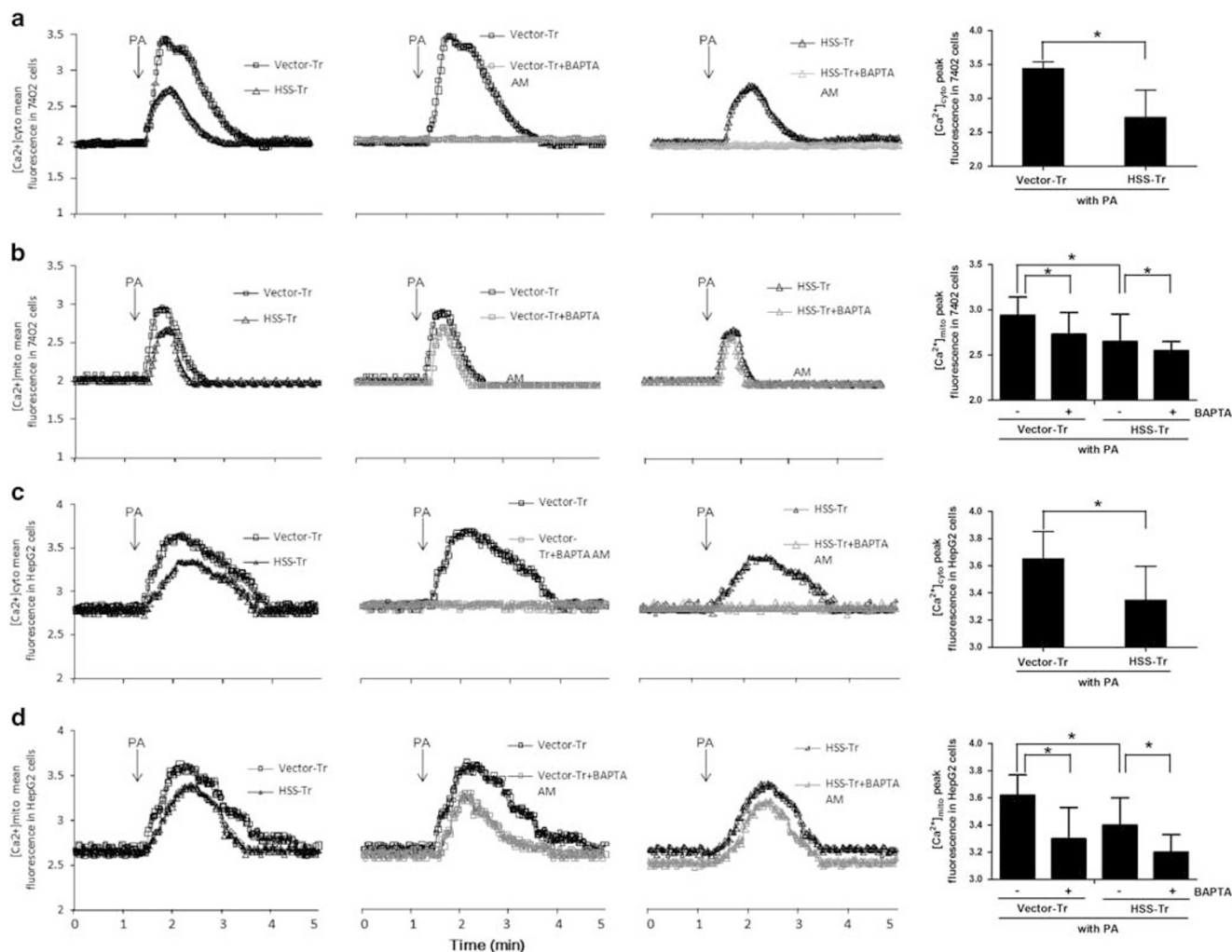
Figure 6 Hepatic stimulator substance (HSS) protects sarco/ER calcium ATPase (SERCA) in the MAM compartment to maintain intracellular Ca^{2+} homeostasis. Ca^{2+} fluorescence intensity was measured with confocal microscopy. Cells were stimulated with palmitic acid (PA; 300 μM). Fluorescence results of (**a**) cytosolic and (**b**) mitochondrial Ca^{2+} in 7402 cells. Fluorescence results of (**c**) cytosolic and (**d**) mitochondrial Ca^{2+} in HepG2 cells.

the mitochondria through the MAM compartment. Consequently, mitochondrial Ca^{2+} levels increased sharply after PA treatment. However, the mitochondrial Ca^{2+} levels were lower in the *HSS*-Tr cells than in the control (vector-Tr) cells, suggesting that HSS protects SERCA in the MAM compartment (Figure 6b). The above results were also convinced in the HepG2 cells (Figure 6c and d).

DISCUSSION
As mentioned above, ER stress is one of the major contributors to the pathogenesis of NASH. As a primary regulator of Ca^{2+}, SERCA2b inhibits the sustained release of Ca^{2+} through the ER lumen, maintains the homeostasis of intracellular calcium, and alleviates ER stress. Therefore, increased SERCA2b concentrations have been used as pilot treatments of type II diabetes and NASH.[27] Supporting this use, we have confirmed that the exogenous expression of HSS attenuated hepatic steatosis by inhibiting ER stress and

preserving the mitochondrial functions, which appeared to be associated with the regulation of SERCA activity.

Steatosis is accompanied by increasing ROS generation, which may lead to further liver damage.[28] The mitochondrial respiratory chain is the primary source of ROS, which can damage the mitochondrial DNA, proteins, and lipids.[29] Altering the ROS levels occurred under ER stress can also affect the intracellular redox status and calcium homostasis, which has a critical role in the regulation of SERCA activity by promoting multiple redox-dependent modifications of SERCA Cys674.[30] SERCA is a Ca^{2+} ATPase that is responsible for transferring Ca^{2+} from the cytosol into the ER lumen at the expense of ATP hydrolysis. Three isoforms (SERCA1-3) have been identified and are encoded by three different genes (*ATP2A*1–3) in mammals. SERCA2b is the primary isoform of SERCA2 and predominantly occurs in the liver,[31] where it is an important regulator of ER stress and lipid homeostasis. The inhibition of SERCA activity can result in the release of

Ca^{2+} from the ER lumen and the disruption of ER homeostasis.[32]

Our previous study also demonstrated that HSS reduces ROS concentrations, protecting SERCA activity. Here, we demonstrated that NASH is accompanied by reduced SERCA expression in the MAM compartment and the onset of mitochondrial Ca^{2+} overload. Simultaneously with the inactivation of SERCA by PA, the HSS content also decreased, indicating that the depletion of HSS protein correlated with the progression of ER stress. Furthermore, when HSS was introduced at this time, SERCA expression was restored and ER stress was alleviated. Therefore, we propose that the HSS-induced inhibition of ER stress is attributable to the preservation of SERCA expression by HSS, particularly in the MAM compartment, during free-fatty-acid-induced lipotoxicity.

MAM is a structure conserved across all eukaryotic phyla, and is a key determinant of cell survival or death by regulating the transfer of Ca^{2+} and other metabolites. SERCA, a MAM-enriched protein,[33] is responsible for pumping cytoplasmic Ca^{2+} back into the ER lumen. The proper maintenance of the MAM and the Ca^{2+} flux is essential for mitochondrial function and metabolic homeostasis.[34] During ER stress, the extent of the MAM increases and the ER and mitochondria move closer together, probably causing a change in the MAM proteome.[35] The integrity of the ER/mitochondrial Ca^{2+} cross-talk is impaired under ER stress, possibly by variations in the extent and composition of the MAM. In our experiment, the expression of SERCA in the MAM region was reduced during ER stress, weakening the cytoplasmic Ca^{2+} influx and particularly the MAM transport of Ca^{2+} into the ER lumen.

The depletion of ER calcium inhibits oxidative protein folding, which may be attributed to another key regulator of calcium homeostasis and signaling, for example, calnexin. Calnexin depalmitoylation during ER stress impairs the association between calnexin and SERCA2b,[36] and the blockade of the interaction between calnexin and SERCA could inhibit SERCA activity and accelerate the mitochondrial Ca^{2+} overload. Therefore, the ER chaperones and oxidoreductases in the MAM compartment should be screened for potential interactive partner with SERCA to determine the extent to which they regulate calcium homeostasis.

Aberrant Ca^{2+} regulation in the ER is a primary cause of hepatic ER dysfunction and stress.[37] Altered Ca^{2+} signaling through the MAM plays a key role in connecting ER stress to mitochondrial dysfunction. The proportion of the ER in close contact with the mitochondria compared with the total ER content is higher in the livers of high-fat-diet mice than in those of the controls.[38] In the present study, we demonstrated that SERCA expression in the MAM regulated the calcium homeostasis between the ER and the mitochondria. We speculate that an important reason for this is that SERCA-regulated ER stress causes the ER and mitochondria to move closer together, resulting in more rapid Ca^{2+} transfer from the

ER to the mitochondria through the MAM. Higher rates of Ca^{2+} accumulation in the mitochondria impair the mitochondrial oxidative capacity, increase ROS generation, and cause cellular stress and abnormal metabolism.

Thus far, the contribution of the MAM-localized protein SERCA to the development of NASH remains unclear. This study provides the first demonstration of the protective effect of HSS on SERCA expression in the MAM during lipotoxic injury, and shows that this protection reduces ER stress and helps to maintain the intracellular Ca^{2+} concentrations at physiological levels, thus effectively preventing hepatic steatosis. These results provide novel insight into the interactions between HSS and SERCA, and their potential application in the treatment of NASH.

DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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