1. Title page

Administration of Naked Plasmid Encoding Hepatic Stimulator Substance by
Hydrodynamic Tail Vein Injection Protects Mice from Hepatic Failure by Suppressing the
Mitochondrial Permeability Transition

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2. Running title page

Running title: hepatic stimulator substance and liver mitochondria

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Abbreviations:

ALT, alanine aminotransferase; ALR, augmenter of liver regeneration; AST, aspartate aminotransferase; CCl₄, carbon tetrachloride; COX-IV, cytochrome c oxidase subunit IV; CsA, cyclosporin A; D-gal, D-galactosamine; EM, electron microscopy; EGFP, enhanced green fluorescent protein; FHF, fulminant hepatic failure; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; HE, hematoxylin and eosin; hHSS, human hepatic stimulator substance; HSS, hepatic stimulator substance; HTV, hydrodynamic tail vein; ig, injection gastric; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; JC-1, 5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazolocarbocyanine iodide; MPT, mitochondrial permeability transition; MTP, mitochondrial transmembrane potential; NIM811, N-methyl-4-isoleucine-cyclosporin; pCMV, cytomegalovirus promoter; PMSF, phenylmethyl sulfonyl fluoride; SDS, sodium dodecyl sulfate; TAA, thioacetamide; Tris, tris(hydroxymethyl)aminomethane; Tween-20, polyoxyethylene-(20)-sorbitan; VDAC, voltage-dependent anion channel.

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Abstract

Acute liver failure is a devastating illness of various causes with considerable mortality. Hepatic stimulator substance (HSS) has been suggested for use as a protective agent against acute hepatic injury induced by chemical poisons because it has a variety of biological activities. However, the mechanism whereby HSS protects against hepatotoxins is poorly understood. In this study, we established a hepatic gene transfer system via hydrodynamic tail vein (HTV) injection to deliver a naked plasmid containing the $hHSS$ gene and analyzed HSS-mediated protection of the liver during fulminant hepatic failure (FHF) induced by D-galactosamine (D-gal) and lipopolysaccharide (LPS). The results showed that the reporter gene, enhanced green fluorescent protein (EGFP), was efficiently expressed in the liver of BALB/c mice. Hydrodynamic-based transfection of $hHSS$ yielded a 70% survival rate compared with 36.7% for the control group at 24 hr after D-gal/LPS treatment. Additionally, $hHSS$ expression preserved liver morphology and function. Importantly, $hHSS$ hydrodynamic-based transfer ameliorated indices of the mitochondrial permeability transition (MPT) resulting from the toxic effects of D-gal/LPS on the liver such as mitochondrial swelling, mitochondrial transmembrane potential (MTP) disruption and cytochrome $c$ translocation. Furthermore, mitochondrial morphology and ATP levels were maintained in $hHSS$-administered mice. HSS-mediated protection was similar to that observed with the MPT inhibitor $N$-methyl-4-isoleucine-cyclosporin (NIM811), indicating a possible role for HSS in the regulation of MPT. In conclusion, a single dose of $hHSS$ plasmid protected mice from FHF, and this hepatoprotective effect appeared to correlate with the inhibition of MPT.
Introduction

Hepatic stimulator substance (HSS) is a hepatotrophic protein that was initially identified in the liver cytosol of weanling rats (LaBrecque and Pesch, 1975). As a unique growth factor that can specifically activate hepatic origin cells to grow regardless of animal species, HSS is able to stimulate the proliferation of hepatocytes and hepatoma cells \textit{in vitro} and \textit{in vivo}, promote liver regeneration after partial hepatectomy, and protect against acute liver failure induced by chemical poisons such as CCl$_4$, D-galactosamine, cadmium, acetaminophen, thioacetamide, and ethanol (LaBrecque, 1991; Theocharis, et al., 1998; Margeli, et al., 2003; Lü, et al., 2004; Webber, et al., 1993; Liatsos, et al., 2003). Although it has been widely accepted that the HSS protection against acute liver injury induced by chemical poisons is associated with its ability to stimulate DNA synthesis and the proliferation of hepatocytes, the precise mechanism involved has yet to be fully clarified (Liatsos, et al., 2003; Maruyama, et al., 2002; Mei, et al., 1993; Ho, et al., 2002; Tzirogiannis, et al., 2004). With regard to HSS, the majority of experiments have used partially purified HSS prepared from the livers of newborn animals or adult rats subjected to partial hepatectomy. As such, impurities in the HSS samples used in these experiments become a major obstacle when attempting to unmask the mechanism by which HSS interacts with hepatocytes. Recently, HSS has been supplied in the form of a recombinant protein (Sheng, et al., 2007; Gao, et al., 2009). However, due to the inconvenience of sophisticated facilities and expensive reagents, \textit{in vivo} studies which are aimed at understanding HSS actions by administration of recombinant HSS protein have rarely been conducted.

With the tremendous growth of biotechnology, the need for information concerning gene expression, protein levels, subcellular localization, and function in the liver has stimulated
interest in the development of efficient methods of transferring genes specifically to the liver with minimal toxicity. Naked DNA is considered to be the simplest and safest gene delivery system (Hickman, et al., 1994; Yoshida, et al., 1997; Heller R, et al., 1996; Suzuki, et al., 1998). Recently, it has been found that a high level of gene expression in hepatocytes can be readily obtained by simple tail vein injection of plasmid DNA in a large volume of solution within a short time, which is known as hydrodynamic-based transfection (Maruyama, et al., 2002; Liu, et al., 1999; Zhang, et al., 1999; Yang, et al., 2001; Herweijer, et al., 2001). Hydrodynamic delivery of the HSS gene may increase the feasibility of HSS application and the probability of HSS actions’ elucidation. Although applying viral vectors could enhance gene transfer to the liver, there might be some safety concerns.

Therefore, in this study we attempted to achieve hepatic-enriched gene transfer with a simple and effective HSS gene delivery strategy. We employed a naked eukaryotic expression plasmid encoding human hepatic stimulator substance (pcDNA3.0-hHSS) that was previously constructed in our laboratory (Li, et al., 2010), and we investigated the effect of pcDNA3.0-hHSS injected hydrodynamically via the tail vein on D-gal/LPS-induced fulminant hepatic failure (FHF). We found that HSS expression in the liver was enhanced by hydrodynamic tail vein injection of pcDNA3.0-hHSS plasmid and that HSS overexpression ameliorated fulminant hepatic failure in mice subjected to D-gal/LPS. Meanwhile, we analyzed possible mechanisms of HSS protection on mitochondria. The results showed that, after efficient delivery and expression of the HSS gene to the liver, mitochondrial swelling, mitochondrial membrane potential, intracellular ATP levels, and cytochrome c translocation from the mitochondrial intermembrane space to the cytosol were improved as compared to vector control delivery. In order to confirm HSS-mediated protection on
mitochondria, N-methyl-4-isoleucine-cyclosporin (NIM811), a well-known inhibitor of the mitochondrial permeability transition (MPT), was used. The results demonstrated that the preservation of mitochondria mediated by HSS is identical to that exerted by NIM811, suggesting that HSS-mediated protection during acute liver injury may, at least in part, be related to its suppression of MPT.
Methods

Animals

Male BALB/c mice weighing 18-20 g were purchased from the Academy of Military Medical Sciences (Beijing, China) and maintained at constant room temperature (22–25ºC) on a 12:12-hr light–dark cycle. All animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health.

Gene Delivery

To investigate transgene expression by HTV injection in mice, a cytomegalovirus (CMV) early-immediate promoter-driven plasmid encoding enhanced green fluorescent protein (pCMV-EGFP-NI) was used. GFP expression was observed under a fluorescence microscope. For the preventive study, a naked eukaryotic expression plasmid encoding human HSS (pcDNA3.0-hHSS) previously constructed in our laboratory (Li, et al., 2010) was adopted.

Injection procedures were performed by hand through a 27 gauge, 0.45 inch needle. In an attempt to achieve hydrodynamic-based gene transfer to the liver, various doses of HSS plasmid (80, 150 and 300 μg) dissolved in a large volume (from 2.25 to 2.5 ml) of normal saline (12.5% of the body weight of a mouse) was injected into the mice via the tail vein in 5 s. The animals were usually placed into a restraining device and injected without anesthesia. The final amount of injected solution was 125 ml/kg body weight.

hHSS expression achieved 24 hr after HTV injection of 150 μg pcDNA3.0-hHSS was determined in different internal organs (the liver, heart, spleen, lungs, and kidneys) and the effect of time course (a 48 hr time frame) on hHSS expression levels in the liver was investigated.
GFP Assay

After pCMV-EGFP-NI was injected, the mice were killed and the liver was divided into 3 sections composed of median, left and right lobes. Liver pieces were collected from each lobe and frozen in Tissue-Tek OCT compound (Electron Microscopy Sciences, Hatfield, PA, USA) for cryo-sectioning. Other tissue specimens such as heart, spleen, kidney, and lung were also isolated. Ten-micrometer thick sections were prepared, examined, and photographed with a Leica fluorescence microscope (Leica DM 5000B, Leica Microsystems CMS, GmbH, Germany). To assure the accuracy of the observations, tissues were prepared from two animals and blindly assessed by two individuals.

Induction of Fulminant Hepatic Failure in Mice

Mice were pre-sensitized with D-gal (Sigma, St. Louis, MO) and treated with low doses of LPS (Sigma, St. Louis, MO). D-gal depletes uridine triphosphate (UTP) in hepatocytes and inhibits RNA synthesis. This metabolic arrest increases the sensitivity of mice to the lethal effects of LPS by 100,000-fold (Zhuge and Cederbaum, 2009). D-gal/LPS-treated mice developed severe fulminant hepatic failure comparable to that in humans.

Twenty-four hours following the injection of HSS plasmid or pcDNA3.0 vector (Invitrogen, Grand Island, NY) dissolved in saline, D-gal (350 mg/kg) and LPS (100 μg/kg) were given intraperitoneally. Survival was monitored for 48 hr after D-gal/LPS administration.

Histological Observation and Biochemical Measurement

One specimen each from the median, left and right lobes of the liver was fixed in 10% neutral-buffered formalin and embedded in paraffin. Five-micrometer thick sections were stained with hematoxylin-eosin (HE) for histological examination by light microscopy (Leica DM 5000B,
Leica Microsystems CMS, GmbH, Germany).

The ultrastructural morphology of mitochondria was determined by electron microscopy. Liver specimens were fixed with 4% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 4 hr at 4°C. After fixation and an overnight wash in sodium cacodylate buffer, the specimens were postfixed with 1% osmium tetroxide in sodium cacodylate buffer at 4 °C for 1 hr, dehydrated in alcohol and embedded in araldite resin. Semi-thin sections (1 μm) were removed for localization under the optical microscope and ultra-thin ones (50 nm) for ultrastructural observation (HITACHI H-800, Japan).

For biochemical assessment of liver injury, blood taken from the inferior vena cava was centrifuged at 1,600 g for 10 min at room temperature to obtain serum. Serological activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined using an autoanalyzer (HITACHI 7600-020, Japan) in the Clinical Chemistry Laboratory of Youan Hospital, Capital Medical University, Beijing. Serological LDH activity was examined according to the manufacturer’s instructions for the Mouse LDH ELISA Kit (Jiancheng Bioengineering, Nanjing, China).

**NIM811 Administration**

In an attempt to evaluate the role of MPT in FHF induced by D-gal/LPS, BALB/c mice (as described above) were gavaged with NIM811 (60 mg/kg) (a generous gift of Novartis, Basel, Switzerland) along with 500 ml olive oil as the vehicle daily for two days before the exposure to D-gal/LPS. A final treatment with NIM811 (60 mg/kg, *ig*) was given along with D-gal (350 mg/kg, *ip*) and LPS (100 μg/kg, *ip*) on the third day. Twenty-four hours after D-gal/LPS exposure, cytosol and mitochondrial fractions were prepared.
Isolation of Total Proteins

The tissues from different organs (liver, heart, spleen, lung, and kidney) were harvested and homogenized with lysis buffer (0.5% sodium deoxycholate, 0.5% Triton X-100, 50 mM Tris, 150 mM NaCl, 62.5 mM sucrose, 5 mM EDTA, 1 mM PMSF). The homogenate was centrifuged at 12,000 \textit{g} for 15 min at 4 °C and the supernatant was collected as the total protein sample for western blot analysis.

Isolation of Cytosol and Mitochondrial Fractions

The isolation of cytosol and mitochondrial fractions was performed according to the manufacturer’s instructions for the Cytosol/Mitochondria Isolation Kit (Applygen Technologies, Beijing, China). Fresh tissues were quickly removed, chopped into small pieces (1 x 1 mm) and placed in ice-cold Mitochondrial Isolation Buffer. After homogenization, the homogenate was centrifuged at 800 \times \textit{g} for 5 min at 4°C. Next, the supernatant was collected and further centrifuged at 800 \times \textit{g} for 10 min at 4°C. The pellet was gently resuspended in Isolation Buffer and centrifuged at 12,000 \times \textit{g} for 10 min at 4°C. Consequently, the cytosolic fraction in the supernatant was harvested and the pellet was resuspended in Isolation Buffer and centrifuged at 12,000 \times \textit{g} for 10 minutes at 4°C.

The mitochondrial pellet was resuspended in the same Isolation Buffer and the protein concentration was determined using the bicinchoninic acid (BCA) method with bovine serum albumin (BSA) (Thermo Scientific Pierce, Rockford, IL) as a standard. The freshly prepared mitochondria were used to determine the levels of ATP, mitochondrial swelling, and mitochondrial transmembrane potential (MTP). Cytochrome \textit{c} levels in cytosol and mitochondrial fractions were analyzed by western blot.
Western Blotting

For electrophoresis, 30 μg of proteins was separated by 12% SDS-PAGE and electrotransfered onto a nitrocellulose membrane. The blots were blocked with 5% non-fat milk for 1-2 hr, then the membranes were briefly washed with Tris-buffered saline containing 0.05% Tween-20 (TBS-T) and incubated with anti-cytochrome c antibody (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-FLAG antibody (1:7000) (Sigma-Aldrich, St Louis, MO) at 4°C overnight. The membranes were stained with goat-anti-mouse IgG secondary antibodies (1:10000) (Cell Signaling Technology, Beverly, MA) and then developed with enhanced chemiluminescence (ECL) reagents (Santa Cruz Biotechnology, Santa Cruz, CA).

Assay of Mitochondrial Swelling

Aliquot of mitochondrial sample (equivalent to 100 μg protein) was incubated in buffer containing 150 mM KCl, 10 mM Tris–MOPS, 5 mM glutamate, 2.5 mM malate, and 1 mM KH₂PO₄. Mitochondrial swelling was monitored by the decrease in absorbance at 540 nm after adding 100 μM CaCl₂.

Measurement of Mitochondrial Transmembrane Potential (ψ₀)

JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide) is a lipophilic fluorochrome that is used to evaluate the status of MTP. For the measurement of MTP (ψ₀), a cuvette was maintained at 37°C by an external water bath. Two milliliters of respiratory buffer was introduced into the cuvette followed by 10 μl of isolated liver mitochondrial extract (containing between 0.1 and 0.15 mg of protein) and 10 μl of 20 μM JC-1 (Sigma, St. Louis, MO). The contents in the cuvette were continuously stirred with a magnet. The wavelength-dependent monitoring mode was adopted as it was suitable for capturing rapid changes within a few minutes
by continuously scanning the green fluorescence of the monomers and the red fluorescence of the J-aggregates from 520 to 620 nm. Subsequent changes were recorded at 1-min intervals. MTP was measured on a luminescence spectrometer (Perkin Elmer Life Sciences, Turku, Finland).

**Determination of Cellular ATP Content**

The ATP content of mitochondrial fractions was analyzed using the CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega, Madison, WI) according to the manufacturer’s instructions.

**Data Analysis**

Data are presented as means ± standard error unless otherwise noted. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Student-Newman-Keul post hoc analysis to determine the significance of differences between groups. A \( P \) value less than 0.05 denoted a statistically significant difference.

**Results**

**GFP and hHSS Gene Delivered Hydrodynamically via the Tail Vein Was Effectively Expressed in the Liver of BALB/c Mice**

The hydrodynamic-based GFP expression results showed that approximately 10% of hepatocytes were transfected at 24 hr following HTV injection of 150 \( \mu \)g pEGFP-N1 (Fig. 1E). It was reported in earlier studies that HTV injection of naked plasmid DNA resulted in transgene expression in the spleen, heart, lungs and kidneys at levels about 100-fold lower than that in the liver (Liu, et al., 1999). Our finding that GFP was sparsely expressed in the spleen is consistent with these previous studies (Fig. 1C). However, we were unable to confirm transgene expression in the heart (Fig. 1A), lungs (Fig. 1B), or kidneys (Fig. 1D) when EGFP was used as the reporter.
gene. Thus, our study showed a target gene transferred by HTV injection was predominantly expressed in the liver. With 150 μg of pcDNA3.0-hHSS delivered hydrodynamically via tail vein, hHSS gene, as measured by western blot analysis, was exclusively expressed in the liver at 24 hr, but not in the heart, spleen, lungs, and kidneys (Fig. 1G, H).

hHSS expression at different time points (0, 12, 24, 36 and 48 hr) following HTV injection of 150 μg pcDNA3.0-hHSS was assayed by the means of western blot analysis. As shown in Fig. 1I, K, the level of hydrodynamic-based hHSS expression reached the peak level in approximately 24 hr.

Additionally, we investigated the effects of pcDNA3.0-hHSS doses (80, 150 and 300 μg) on hHSS expression and found that transgene expression was dose-related (Fig. 1J, L). Although 300 μg plasmid yielded the obvious hHSS expression in mitochondria, it didn’t present a significant difference as compared with 150 μg plasmid.

**hHSS Expression Protected against FHF Produced by D-gal/LPS**

Mice were divided into four experimental groups: the mice in group 1 were intraperitoneally injected with saline alone; the mice in group 2 were treated with D-gal (350 mg/kg)/LPS (100 μg/kg) intraperitoneally alone; in groups 3 and 4, the control vector or pcDNA3.0-hHSS, respectively, was injected into the mice hydrodynamically via the tail vein at 24 hr prior to D-gal (350 mg/kg)/LPS (100 μg/kg).

hHSS expression in the liver protected mice from death resulting from FHF (Fig. 2A). As shown in Fig. 2A, it seemed that the range from 18 to 42 hr was the death window because mice treated with D-gal/LPS began to die at 18 hr and all death occurred within 42 hr. In addition, the death peak fell at 24 hr, considering that approximately 90% of mice exposed to D-gal/LPS died.
D-gal/LPS treatment of mice that did not undergo HTV injection yielded 30% survival, whereas 36.7% survival was observed in vector-treated mice at 24 hr following D-gal/LPS treatment (Fig. 2A). It is important to note that no significant difference was detected in terms of survival between the above two groups. In contrast, 70% and 66.7% of mice transfected with hHSS survived at 24 hr and 48 hr, respectively, indicating that hHSS transfection rescued mice with FHF from death (Fig. 2A).

In control vector-injected mice, D-gal/LPS treatment induced severe scattered necrosis, vacuolar degeneration and persistent inflammatory infiltrate distributed in a perisinusoidal, lobar and portal fashion on liver histology (Fig. 2D). hHSS expression preserved the structure of the hepatic lobule (Fig. 2E). The hepatic cords could be identified and the degree of hepatocyte necrosis was dramatically lessened in hHSS expressing mice. This result proves that hHSS expression helped mice resist hepatic damage caused by D-gal/LPS.

After 24 hr of D-gal/LPS poisoning, the levels of ALT and AST were determined (Fig. 2F). Although ALT and AST levels were more elevated in mice treated with the control vector than those in mice treated with D-gal/LPS alone, there was no statistical difference between these groups. However, the transfection of hHSS suppressed the elevation of ALT and AST induced by D-gal/LPS.

LDH is an enzyme found in many body tissues, including the liver. Elevated serum LDH indicates liver damage. We measured LDH leakage as a marker of hepatotoxicity. As shown in Fig. 2G, LDH leakage increased as a result of D-gal/LPS treatment, whereas hHSS transfection significantly inhibited LDH leakage.
hHSS Expression Protected Hepatic Mitochondrial Structure

Electron microscopic (EM) examination showed that liver cell mitochondria are regular in size, cristae-rich and feature a typical folded intermembrane space and dense matrix (Fig. 3A). D-gal/LPS intoxication elicited morphological abnormalities in the mitochondrial ultrastructure, including enlargement in volume and deficient or swollen cristae (Fig. 3B, D). As shown in Fig. 3C, the hHSS treatment prominently prevented the reduction in the number of cristae and the dilation in the structure of cristae. In addition, hHSS maintained the mitochondrial volume within its normal range.

NIM811, a new derivative of Cyclosporin A (CsA) that is a potent inhibitor of MPT, can duplicate the mitochondrial protection seen with CsA by attenuating the mitochondrial dysfunction and liver damage caused by storage/reperfusion injury or bile duct ligation (BDL) (Theruvath, et al., 2008; Rehman, et al., 2008). In order to obtain convincing data that HSS liver protection is related to mitochondria, we included NIM811 as a positive control. As shown in Fig. 3C, E, treatment with either NIM811 or hHSS prominently prevented the reduction in the number of cristae and the dilation in the structure of cristae.

hHSS Expression Protected Energy Storage in the Liver

Energy production by mitochondria was markedly lower in FHF mice without the NIM811 or pcDNA3.0-hHSS treatment (Fig. 4A). Although the ATP levels of HSS-expressing mice fell to a low level, the relative amount of ATP in HSS-expressing mice was approximately 62.1% higher than that in the vector-injected mice. Additionally, the decline in ATP levels resulting from D-gal/LPS poisoning was substantially inhibited in mice treated with NIM811 (Fig. 4A). Our results suggest that hHSS expression restored energy production in mitochondria, which is in line
with the protective effect of NIM811 on the maintenance of ATP levels.

**hHSS Expression Provided Protection from Mitochondrial Swelling and $\psi_m$ Alteration**

MPT is a sudden nonselective increase in the permeability of the inner mitochondrial membrane to solutes of molecular mass less than 1500 Da. MPT leads to a loss of mitochondrial transmembrane potential ($\psi_m$), mitochondrial swelling, rupture of the outer mitochondrial membrane (Juhaszova, et al., 2008; Hunter, et al., 1976), and consequently the release of proapoptotic proteins such as cytochrome c. As shown in Fig. 4B, D-gal/LPS toxicity caused a marked loss of $\psi_m$. NIM811, a well-established inhibitor of MPT, suppressed the collapse of $\psi_m$, indicating that MPT plays a role in the pathogenesis of FHF produced by D-gal/LPS. *hHSS* expression reduced the loss of $\psi_m$ by 50% compared with that detected in the vector-injected mice after D-gal/LPS treatment. Thus, *hHSS* appeared to alleviate D-gal/LPS-induced liver injury by suppressing MPT in an animal model.

Mitochondrial swelling was assessed in isolated mitochondria as a reflection of the occurrence of MPT (Gogvadze, et al., 2003). Large-amplitude swelling was found to occur in mitochondria isolated from D-gal/LPS-treated mice. In contrast, mitochondrial swelling was substantially counteracted by NIM811 administration, indicating a role of MPT in liver injury resulting from D-gal/LPS. Likewise, *hHSS* expression significantly repressed the D-gal/LPS-induced mitochondrial swelling, implying that the beneficial effect of *HSS* on FHF occurs through its regulation of MPT (Fig. 4C).

**hHSS Expression Inhibited Cytochrome c Leakage**

It has been shown that the onset of MPT can cause cytochrome c to be released from the mitochondrial intermembrane space into the cytosol (Gogvadze, et al., 2003; Kim, et al., 2003).
As shown in Fig. 5, D-gal/LPS intoxication led to the leakage of cytochrome c from the mitochondrial intermembrane space into the cytosol in mice that received neither NIM811 nor hHSS. In contrast, hHSS transfection into the liver significantly prevented the release of cytochrome c, comparable to the inhibitory effect of NIM811 on cytochrome c translocation. Densitometric analysis of the cytochrome c content in mitochondria demonstrated that hHSS preserved approximately twice as much cytochrome c in the intermembrane space as did the control vector treatment after D-gal/LPS poisoning, suggesting that hHSS is a potent agent for preserving mitochondrial membrane integrity.

Discussion

In in vivo studies that dealt with the protective effects of HSS, the form of HSS administered was mainly HSS extract from regenerating livers, and these studies focused on its regenerative function. The purpose of the current study was to evaluate the protection effect of pcDNA3.0-hHSS administered by HTV injection against FHF produced by D-gal/LPS toxicity, and to see if this effect was indeed related to mitochondria.

It has been shown that the therapeutic effect of gene transfer can be achieved in the liver simply by a fast injection of naked plasmid DNA in large volume solution via tail vein. (Wang, et al., 2004). Our assessment of pcDNA3.0-hHSS delivery indicated that enough hHSS protein was produced to exert protective effects on FHF in mice.

Previous studies have demonstrated that although HSS promotes cell growth in dividing hepatocytes, it is unable to stimulate the division of primary cultured or mature hepatocytes.
However, HSS augments the mitogenic effects of other growth factors, such as epidermal growth factor (Kiso, et al., 1995). Therefore, HSS crude extract was further purified and the fraction that was responsible for the growth-augmenting activity was referred to as augmenter of liver regeneration (ALR) (Francavilla, et al., 1987).

In 1996, the human ALR gene was identified and mapped to chromosome 16 in a region syntenic with mouse chromosome 17 (Giorda, et al., 1996). The cDNA of human ALR encoding 125 amino acids is reportedly 87.2% identical to mouse ALR, and the expression product of mouse ALR was also found to locate to the mitochondrial intermembrane space. Our study supports the species-nonspecific effects of HSS because the HSS gene of human origin was inserted into pcDNA3.0 and hHSS expressed in the liver attenuated FHF in BALB/c mice subjected to D-gal/LPS (Fig. 2).

Recent findings that revealed features of the molecular structure of HSS, its subcellular localization, and its functional linkage with mitochondria have given researchers better insight into its protective mechanism.

In 2001, Lisowsky et al. first reported that mammalian ALR is a sulfhydryl oxidase with a CXXC active motif in the carboxy-terminal domain that is highly conserved in yeast, mice, rats and humans (Giorda, et al., 1996; Lisowsky, et al., 2001). Our laboratory reported that the hHSS gene, after transfection into BEL-7402 hepatoma cells, was stably expressed in the mitochondria, which is consistent with the discovery that ALR is primarily localized in the mitochondria (Wu, et al., 2007; Lange, et al., 2001). Additionally, HSS has been shown to induce mitochondrial gene expression and interact with the respiratory chain via the modification of cytochrome c (Farrell, et al., 2005). More recently, an in vitro study demonstrated that after administration of carbonyl
cyanide $m$-chlorophenylhydrazone (CCCP), a specific agent that promotes MPT, MPT and cytochrome $c$ leakage were significantly inhibited in HSS-expressing cells compared with vector-transfected cells. Moreover, the protection provided by HSS was comparable to that observed with CsA, a known inhibitor of MPT (Wu, et al., 2010). Thus, an influence of HSS on mitochondria is clearly implicated in its mechanism of protection from liver injury caused by chemical intoxication.

In the last 10 years, it has also become apparent that mitochondria are the control centers for cell death. In our study, D-gal/LPS toxicity provoked leakage of cellular enzymes such as LDH and ALT/AST, severe inflammation as the result of leukocyte infiltration, and necrosis as assessed by HE staining (Fig. 2). HSS ameliorated hepatic injury as observed in histological manifestation, suggesting that it could protect the cells against liver injury at least at the level of cytoplasm membrane, probably due to autocytoprotection (Mei, et al., 1993). Apart from HSS autocytoprotection, a mitochondrial pathway involving MPT, which is caused by the opening of the nonselective highly conductive permeability transition pores in the mitochondrial intermembrane space, might also contribute to the protection of hepatocytes (Juhaszova, et al., 2008; Gogvadze, et al., 2003).

Permeability transition pores nonspecifically conduct low molecular weight solutes to cause mitochondrial depolarization, uncoupling of oxidative phosphorylation, and large-amplitude colloid osmotic swelling. ATP depletion after uncoupling produces necrotic cell killing, whereas swelling leads to outer membrane rupture and the release of proapoptotic proteins (such as cytochrome $c$) from the intermembrane space (Kim, et al., 2003).

We found that D-gal/LPS intoxication caused mitochondrial swelling, the collapse of $\psi_m$,
impairment of energy production, and an increase in the release of cytochrome c into the cytosol, suggesting the occurrence of MPT (Fig. 4). In addition, Bcl-2 family proteins play a critical role in regulating MPT. Our result showed the gene Bcl-2 was highly expressed in livers of mice transfected with hHSS, confirming a possible role of hHSS in protecting mitochondrial damage by blocking MPT (Supplemental Figure 1). NIM811, a new derivative of CsA, mimics the action of CsA in preventing the formation and opening of mitochondrial permeability transition pores, and it can fully duplicate the mitochondrial protective efficacy of CsA (Mbye, et al., 2008). It has been suggested that NIM811 binds to Cyclophilin D and blocks its association with the adenine nucleotide translocase (ANT), which in turn prevents the formation and opening of the mitochondrial permeability transition pores (Sullivan, et al., 1999).

In this study, hHSS exerted an effect similar to that of NIM811 on the indices regarding the onset of MPT. In this respect, our study lends support to a mechanism that the hepatoprotective effect of HSS occurs through its suppression of MPT. Further studies will be required to explore the precise protein-protein interactions of HSS within the mitochondria, to elucidate the molecular mechanism underlying HSS-mediated liver protection, and to identify candidate HSS-binding molecules.

In summary, a single dose of hHSS-encoding naked plasmid delivered hydrodynamically via the tail vein produced high-level expression of hHSS in the liver and protected mice from fulminant hepatic failure. The hepatoprotection provided by hHSS gene transfer appeared to be attributable to the restoration of mitochondrial respiratory function and the inhibition of MPT. Thus, MPT represents an attractive therapeutic target in the prevention and treatment of acute hepatic injury.
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**Authorship Contributions:**

Participated in research design: Wei An

Conducted experiments: Shenglan Li, Zuoqing Tang, Hao Yu, Wen Li, Ying Jiang

Shenglan Li contributed to Figs 1-5; Zuoqing Tang contributed to Figs 1-5; Hao Yu contributed to Figs 3-5; Wen Li provided technical support in mitochondrial isolation; Ying Jiang provided technical support in hydrodynamic tail vein injection.

Contributed new reagents or analytic tools:

Performed data analysis: Yutong Wang

Wrote or contributed to the writing of the manuscript: Wei An

Other:
References


Footnotes

*S Li and Tang contribute equally to this work*

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Legends for Figures

**Fig. 1** Transgene expressions of GFP and *hHSS* delivered hydrodynamically via tail vein. *GFP* gene was expressed at 24 hr following HTV injection of 150 μg pEGFP-N1 predominantly in the liver (E) while GFP was observable remotely in the spleen (C) and undetectable in the heart (A), lung (B) and kidney (D). Liver biopsies from saline-treated mice were used as controls (F). *hHSS*
expression examined from both the total protein (G) and the mitochondrial fraction (H) was determined in different organs at 24 hr after HTV injection of 150 μg pcDNA3.0-hHSS by means of Western blot analysis (G, H). hHSS gene was tagged with Flag and Anti-Flag Tag Monoclonal Antibody was used in order to exclusively detect exogenous HSS. hHSS expression in the liver at different time points (0, 12, 24, 36, 48 hr) following HTV injection of 150 μg pcDNA3.0-hHSS was assayed by Western blot analysis for the mitochondrial fraction (I). The effects of plasmid doses (80, 150, 300 μg) on hHSS expression was investigated when the mitochondrial fraction (J) were extracted from the liver at 24 hr after HTV injection of pcDNA3.0-hHSS. The mitochondrial fraction of hHSS contents was quantified by densitometric analysis (K, L). Antibodies against GAPDH and VDAC were used for normalization of loading controls. **P < 0.01 vs. 24 hr time point or 150 μg plasmid.

**Fig.2 Effects of hHSS expression in the liver on FHF induced by D-gal/LPS.** Serum activities of ALT, AST and LDH and tissue sections were obtained from mice killed at 24 hr after saline solution administration or D-gal/LPS poisoning. hHSS expression in the liver rescued mice from lethality produced by D-gal/LPS and its beneficial effect was the most prominent at 24 hr (A). Twenty to thirty mice were used per group. Hepatic histological findings revealed that the degree of D-gal/LPS induced hepatic damage could be remarkably attenuated by HTV injection of pcDNA3.0-hHSS. (B) control; (C) D-gal/LPS; (D) vector plus D-gal/LPS; (E) pcDNA3.0-hHSS plus D-gal/LPS. hHSS expression inhibited the increases of ALT and AST resulting from D-gal/LPS toxicity (F). LDH leakage resulting from D-gal/LPS was inhibited by hHSS expression in the liver (G). **P < 0.01 vs. vector plus D-gal/LPS. ##P < 0.01 vs. control

**Fig.3 Electron micrographs of mitochondria from mice with different treatments.** Liver samples were prepared from mice killed at 24 hr after saline solution administration or D-gal/LPS poisoning. Panel A: untreated; Panel B: vehicle (olive oil) plus D-gal/LPS; Panel C: NIM811 plus D-gal/LPS; Panel D: vector plus D-gal/LPS; Panel E: hHSS plus D-gal/LPS. The hepatocyte mitochondria manifested mild morphological changes in mice treated with NIM811 or HSS, whereas swollen cristae and giant mitochondria were observed in mice without the protection of the agents.
Fig. 4 hHSS expression preserved mitochondrial respiratory function and protected the permeability of mitochondrial membrane. Mitochondria freshly isolated at 24 hr following saline solution or D-gal/LPS treatment were used to measure ATP levels (A), mitochondrial swelling (B), and mitochondrial membrane potential ($\psi_m$) (C). Data from mice treated with vehicle (olive oil) plus G-gal/LPS were not shown, because the vehicle showed little protective effect on the three parameters and NIM811 is a well-established inhibitor of MPT. **$P < 0.01$ vs. vector plus D-gal/LPS. ## $P < 0.01$ vs. control.

Fig. 5 Western blot analysis of the cytosolic and mitochondrial cytochrome c. The cytosolic and mitochondrial fractions were prepared at 24 hr after saline solution or D-gal/LPS treatment. Representative blot for cytochrome c in mitochondria (A) and cytosol (B). Densitometric analysis of cytochrome c content in mitochondria (C) and cytosol (D). Antibodies against Cox-I and GAPDH were used for normalization of loading controls. * $P < 0.05$ vs. vector plus D-gal/LPS. # $P < 0.05$, ## $P < 0.01$ vs. control.
Figure 3
Figure 4
Figure 5