Transfection of hepatic stimulator substance gene desensitizes hepatoma cells to H$_2$O$_2$-induced cell apoptosis via preservation of mitochondria

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Abstract

Hepatic stimulator substance (HSS) protects liver cells from various toxins. However, the mechanism by which HSS protects hepatocytes remains unclear. In this study, we report that the HSS gene, after transfection into BEL-7402 hepatoma cells, is stably expressed in the mitochondria. Hydrogen peroxide (H$_2$O$_2$)-induced cell apoptosis in the HSS-transfected cells is reduced, as shown by morphologic analysis. In the HSS-transfected cells, disruption of mitochondrial transmembrane potential (MTP) and cytochrome c leakage are reduced. The anti-apoptotic gene Bcl-2 is also highly expressed. In addition, ATP levels in the HSS-transfected cells are maintained.

In conclusion, in hepatoma cells, HSS gene expression protects cells against H$_2$O$_2$ injury, and this effect is likely to be associated with preservation of mitochondria.

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Keywords: Hepatic stimulator substance (HSS); Mitochondria; Apoptosis

Hepatic stimulator substance (HSS) was initially identified in the cytosol of a newborn rat liver [1] and was reported to stimulate remnant liver regeneration after partial hepatectomy [2,3]. Partial purification of the HSS protein indicated that the molecular size of HSS is 15–18 kDa with characterization of heat and pH resistance, but protease-sensitivity [4]. Biological function of HSS protein is believed to stimulate cellular DNA synthesis and cell mitosis [5]. In contrast to other non-liver-specific growth factors such as epidermal growth factor (EGF) [6], transforming growth factor-α (TGF-α) [7] and hepatocyte growth factor (HGF) [8], the action of HSS is featured with the tissue-specificity, i.e., only hepatocytes or liver-derived tumor cell lines are responsive to HSS stimulation. Therapeutically, HSS has been shown to protect the liver from acute injury caused by carbon tetrachloride (CCl$_4$) and D-galactosamine in several animal models [9–11]. Interestingly, several in vitro studies later demonstrated that, although HSS stimulates cell growth in dividing hepatocytes, it is unable to stimulate proliferation in resting-state adult liver. For instance, addition of HSS to primary cultured hepatocytes had minimal effects on cell growth; instead, it augmented the mitogenic effects of other growth factors.
such as epidermal growth factor (EGF). This property of HSS had been observed and the protein responsible for the growth-augmenting activity has been named augmenter of liver regeneration (ALR). In 1994, ALR cDNA gene cloning was first succeeded in rat [12] and accordingly, later in the following year, the human ALR gene was identified, and mapped to the chromosome 16 [13]. However, in contrast to the previous beliefs, the ALR gene was not specifically expressed in the liver tissue, but presented in other tissues, including the stomach, pancreas, kidney, lung, heart and testis [13]. Subsequently, it is demonstrated that ALR occurs constitutively within hepatocytes in an inactive form, and when released from the cells it is converted to an active form in response to partial hepatectomy, as well as to other circumstances involved in liver maturation, thus, revealing its potential role in liver homeostasis or other tissues [14]. With respect to the effects of ALR stimulation on liver regeneration, it has previously been reported that ALR can inhibit natural killer (NK) cell cytotoxic activity in a population of mononuclear leukocytes (MNL) in the liver, thereby reducing the effects of NK cells on hepatocytes during regeneration. Recently, more attention has focused on functional elucidation of ALR enzymatic activity. In 2001, Lisowsky et al. first reported that mammalian ALR is a sulphhydryl oxidase with a CXXC active motif in the carboxy-terminal domain [15]. The X-ray crystal structure of ALR protein was reported in 2003 and the result indicates that ALR is a flavin-dependent sulphhydryl oxidase [16]. Although ALR was first reported to localize primarily in the mitochondria [17], it has been also found in the cytosol [18], the nucleus [19], and even as a secreted growth factor [20]. The multiple cellular localizations of correspond with other examples of sulphhydryl oxidase distribution. Hence, ALR is likely to have multiple roles in regulation of cell growth and cell protection.

It has recently been reported that the activity of cytochrome c as an electron acceptor for ALR is about 100-fold higher than that of oxygen when diithiothreitol (DTT) is the reducing substrate [21]. This in vitro result indicated that ALR might be involved in the respiratory chain pathway via mediation of cytochrome c. Evidence for ALR association with the mitochondrial redox process had been previously noted in vivo when observing the resistance of hepatocytes to CCl₄-induced toxicity in regenerating rat liver. It was proposed that the regenerating liver is protected, at least in part, by HSS-induced increases in mitochondrial respiratory activity and plasma membrane fluidity [22]. As an alternative supporting data, it has been also found that administration of ALR protein induces an increase in the mitochondrial gene expression and enhances cytochrome c content and oxidative phosphorylation capacity of liver mitochondria [23]. Taking together, it could be predicted that HSS or namely ALR plays a key role in regulation of biogenesis at mitochondrial level. Mitochondrion seems to be the central organelle not only because of its importance in generation of energy, but also in the execution of the cell programmed death. A substantial volume of experimental evidence points to the central role of mitochondria in apoptosis [24–26]. This includes the changes in opening of the permeability transition pore (PTP) and the release of apoptosis-stimulating compounds from mitochondria (such as cytochrome c) and activation of pro-caspase [27,28]. Permeabilization of the outer mitochondrial membrane occurs through non-specific injury to the membrane lipids. It can also occur by specific mechanism that induce opening of the mitochondrial transition pore (MTP) [29]. Increased mitochondrial permeability leads to the release of pro-apoptotic factors such as cytochrome c from all regions of the intermembrane space, leading to disruption of mitochondrial membrane potential [30].

It is not clear yet whether HSS, as a hepatrophic growth factor and a promising liver-specific therapeutic drug in the treatment of liver injury, is involved in the regulation of liver-cell apoptosis. Therefore, this investigation aimed to explore possibility of HSS involvement in hydrogen peroxide (H₂O₂)-induced cell apoptosis. The results of the present study demonstrated for the first time that, after transfection into hepatoma cells, the hHSS gene could desensitize the cells to H₂O₂-induced cell death. Meanwhile, several mitochondrial parameters are apparently improved in the cells subjected to hHSS transfection, and expression of the anti-apoptotic gene Bcl-2 expression is upregulated, indicating that the function of hHSS gene in liver cells may be related to its anti-apoptotic effect, which is presumably due to stabilized mitochondrial membrane permeability.

Materials and methods

Reagents

Dulbecco’s modified Eagle’s medium (DMEM) and total RNA extraction kit (TRIzol) are from Gibco BRL. Fetal calf serum is from Hyclone. DNA plasmid transfection Lipofectin kit is from Boehringer Mannheim. SuperScript™ III first-strand synthesis system for reverse transcription polymerase chain reaction (RT-PCR) is from Invitrogen, the QIAamp RNA purification kit is from QIAGEN, and the Power SYBR Green PCR Master kit was purchased from Applied Biosystems. The MitoProbe™ DiICl(5) assay kit for flow cytometry was from Molecular Probes. Cytosol and mitochondrial fractionation kit (ApoAlert) was purchased from Clontech. Bicinchoninic acid (BCA) kit was from Pierce. The Annexin V FITC kit is from Roche. The ENLITEN total ATP rapid detection kit is from Promega. Anti-cytochrome c and anti-Bcl-2 antibody were from Santa Cruz Biotechnology. Peroxidase-conjugated AffiniPure goat anti-mouse immunoglobulin (IgG) is from Cell Signaling Technology. Bisbenzimide Hoechst 33342, MitoTracker Red 580 and LDH activity assay kit are from Sigma.

Cell culture and plasmid transfection

BEL-7402 hepatoma cells were cultivated at 37°C in DMEM containing 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin in a 5% CO₂ humidified-atmosphere incubator. A total of 2 x 10⁶ BEL-7402 cells were seeded and allowed to grow to 50–70% confluence. The cells were transfected with 5 µg of HSS-pCDNA 3.1 or pCDNA 3.1 alone with liposome DOTAP, following the manufacturer’s manual. HSS-cDNA was cloned in our lab and the sequences had been proved with alignment of
RNA extraction and real-time polymerase chain reaction (RT-PCR)

Total RNAs from the HSS-transfected, vector-transfected and wild-type cells were extracted using the QIAamp RNA purification kit. Extracted RNA was transcribed into cDNA using the SuperScript™ III first-strand synthesis system. cDNA was synthesized from 3 μg of total RNA in a 20 μl reaction mixture. Real-time PCR was performed using the Power SYBER Green Master kit according to the manufacturer’s protocol. The hHSS gene was amplified using the ABI Prism 7300 Sequence Detection System (Applied Biosystems) using specific oligonucleotide primers. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified as an internal standard. Primers were designed using the primer design software Primer Express (Applied Biosystems).

Isolation of cytosol and mitochondrial fractions

Isolation of cytosol and mitochondrial fractions was performed according to the ApoAlert kit. After H2O2 treatment the cells plated in 75 cm² flasks were harvested by trypsinization. 5 x 10⁶ cells were centrifuged at 600g for 5 min at 4 °C. The pellet was resuspended in 0.8 ml of ice-cold fractionation BufferMix, incubated on ice for 10 min, and homogenized with a Dounce tissue grinder on ice. The homogenate was then transferred to a microcentrifuge tube and centrifuged at 700 g of each diluted sample were mixed with 450 μl of fractionation BufferMix. Protein concentration was determined by BCA method using bovine serum albumin as standard. Cytochrome c and Bel-2 in cytosol and mitochondrial fraction were analysed by using Western blot.

Flow cytometric analysis (FCM)

Cells were seeded in 100 mm culture dishes. After attachment, the cells were incubated with 0.6 mM of H2O2 for 6 h. After being washed twice with PBS, cells were resuspended in binding buffer [10 mM Hepes/NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.5% NP-40, with proteinase inhibitor cocktail freshly added]. Extracts were incubated 30 min on ice and centrifuged at 12,000 rpm at 4 °C, and the supernatants were recovered. For electrophoresis, 25 μg of mitochondrial/cytosolic proteins or 50 μg of total cellular proteins were separated by 12% SDS–PAGE and electrotransferred onto a nitrocellulose membrane. The blots were blocked with 5% non-fat milk at 4 °C overnight. The next day, the membranes were briefly washed with Tris-buffered saline containing 0.05% Tween 20 (TBS-T), incubated with corresponding primary antibodies (anti-cytochrome c antibody 1:1000 and anti-Bcl-2 antibody 1:500) for 2-4 h and subsequently stained with goat-anti-mouse IgG secondary antibodies (1:1000). The membranes were then developed with enhanced chemiluminescence (ECL) reagents.

Confocal microscopy analysis

To observe the subcellular localization of the hHSS gene after transfection, the hepatoma cells transfected with pEGFP-N1-hHSS vector, in which hHSS cDNA was ligated to the N-terminal of the pEGFP-N1- vector, were seeded in 35 mm culture dishes. After incubation for 24–96 h, the cells were stained with 250 nM MitoTracker Red580 dye at 37 °C. The dyed cells were analysed using a Leica NT fluorescent confocal microscope (Leica).

H2O2-induced apoptosis

The cells were cultured in 60-mm diameter dishes at a density of 1 x 10⁶ cells/dish and allowed to grow to confluence. After cell attachment, the serum-free culture medium was replaced and the cells were grown for 12 h. The cells were then pretreated with final concentration of 0.4, 0.6, 0.8, 1.0 mM H2O2 for 2-8 h, respectively. H2O2-induced toxicity was evaluated by the MTT method, as described previously [32]. According to dose and time dependency of H2O2 toxicity, administration of 0.6 mM of H2O2 for 6 h was applied for induction of apoptosis in the following experiments.

Morphological assessment of apoptosis

Cell apoptosis was morphologically evaluated by staining cells with either hematoxylin-eosin (HE) [33] or Hoechst 33342 [34] stains by standard procedures. Transmission electron microscopy (TEM) was used for further characterization of the ultrastructure of apoptotic cells. The cells treated with 0.6 mM of H2O2 for 6 h were harvested and fixed in 2.5% glutaraldehyde in 0.1 M PBS, pH 7.4. The cells were then post-fixed in 1% osmium tetroxide in PBS and dehydrated with a graded ethanol series followed by propylene oxide. After samples were embedded in epoxy resin, ultrathin sections were prepared and examined at 80 kV with a JEM-1200EX electron microscope (Jeol).

LDH assay

In order to confirm whether the integrity of cell membrane was maintained during 0.6 mM H2O2-induced cell damage for 6 h, the leakage of intracellular lactate dehydrogenase (LDH) was determined. The cells were cultured on 24-well plates in serum-free DMEM. After exposure 0.6 mM of H2O2 for 6 h, LDH released into the medium was measured by using the in vitro toxicity assay kit according to the manufacturer’s instructions. Briefly, the cells were centrifuged at 2000g for 4 min. The LDH in the supernatants converted a tetrazolium dye, which could be measured spectrophotometrically at 490 nm. The LDH activity is expressed as percentage of total LDH activity of the lysed cells.

Measurement of mitochondrial membrane potential (ΔΨm)

DiIC1(5) is a mitochondria-specific dye for evaluation of mitochondrial membrane potential. The dye intensity is reduced when cells are treated with reagents that disrupt mitochondrial membrane potential [35]. After exposure to 0.6 mM of H2O2 for 6 h, cells were assessed by flow cytometry using the mitochondria-sensitive dye MitoProbe™ DiIC1(5) assay kit. Following H2O2 treatment, the cultured cells were maintained in a CO2 incubator with a final concentration of 50 mM prewarmed DiIC1(5) at 37 °C for 30 min. Then, ΔΨm was measured by flow cytometry, as described previously [35].

Determination of cellular ATP contents

Cellular ATP content was analysed using an ATP assay kit. After exposure to 0.6 mM of H2O2 for 6 h, the cells were lysed with 400 μl of lysis buffer, which was supplemented with 1.5% trichloroacetic acid (an ATPase inhibitor) just before use. The cell lysate was diluted 1:100 using Tris buffer. A total of 50 μg of each diluted sample were mixed with 450 μl

ALR gene [31]. After transfection for 8 h, the cells were subjected to screening with the gentamycin analogue G418 (400 μg/ml) for 14 days. The cells resistant to G418 were used for further study.

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of reconstituted luciferase solution and the ATP concentrations were immediately measured with a Glomax™ 96 microplate luminometer (Promega). For normalization of samples, total cellular protein from each cell lysate sample was determined with the BCA method.

Data analysis

All values were expressed as means ± standard deviation (SD). Statistical significance was determined by one-way ANOVA. P values <0.05 were considered significant.

Results

hHSS expression and localization in the cells

Real-time RT-PCR amplification indicated that hHSS RNA was expressed at greater levels in the transfected cells than in the vector-transfected (pCDNA3.1 alone) or the wild-type cells (Fig. 1a).

Mitochondrial expressions of hHSS in three types of cells (wild-type, vector-transfected cells and hHSS-transfected) were detected by Western-blot analysis. As shown in Fig. 1c, a 15 kDa band was clearly seen in the hHSS-transfected cells. This 15 kDa protein was of the same size as the positive control produced by the anti-hHSS antibody. By contrast, little HSS expression was detected in the wild-type and vector-transfected cells. After transfection in BEL-7402 cells, the green signal produced by hHSS-EGFP in cytoplasmic compartments was localized to the same regions as the red signal generated by Mito-tracker Red 580 (Fig. 1b), indicating that hHSS is mainly localized in the mitochondria of living hepatoma cells. The localization of hHSS-EGFP signal in the wild-type or the vector-transfected BEL-7402 cells was universally distributed in the cytosol, but not specifically in the mitochondria (data not shown).

H2O2 toxicity on the cells

H2O2 toxicity was evaluated using the MTT assay. As shown in Fig. 2a and b, exposure to H2O2 caused cell mortality within 8 h in the three groups of cells. The toxicity increased in a dose- and time-dependent manner. However, the H2O2 toxicity in the hHSS-transfected cells was significantly reduced compared with wild-type or vector-transfected cells.

![Fig. 1. Expression and localization of the hHSS gene. Levels of hHSS RNA from three types of cells were quantified by real-time RT-PCR and expressed as genomic equivalents per culture. Expression of hHSS in the hHSS-transfected cells is clearly greater than that in the wild-type and vector-transfected cells (a). Immunofluorescence labeling of cells was performed to detect hHSS-EGFP and MitoTracker Red 580 in the BEL-7402 hepatoma after 48 h. B1, B2 and B3 represent hHSS-EGFP, MitoTracker Red 580 and merged images, respectively. Green: hHSS-EGFP; Red: MitoTracker Red 580; Yellow: co-localization image (b). (c) The different expression levels of hHSS in three types of cells. Twenty five micro grams of mitochondrial protein from each of the cell cultures were subjected to 12% SDS–PAGE and electrotransferred on nitrocellulose membrane and analysed by Western blot with anti-hHSS serum. COX IV was used for normalization of loading controls. In order to exclude the possibility that mitochondrial fraction was coherent with cytosol protein, the blot was rehybridized with β-tubulin antibody. Similarly, mitochondrial integrity was also monitored by analyzing mitochondrial cytochrome c level in cytosolic fraction.](image-url)
cells, indicating that hHSS gene expression can increase resistance to H₂O₂ injury. It has been reported that type of cell death (apoptosis and necrosis) induced by H₂O₂ mainly depends upon concentration of the oxidant used and incubation period. In case of cell necrosis one of the significant changes is loss of cell membrane stabilization. Therefore, we determined LDH leakage as a parameter of integrity of cell membrane. As shown in Fig. 2c, LDH leakage was increased in the cells subjected to 0.6 mM H₂O₂ for 6 h, and the increased LDH level showed no significant difference between hHSS-transfected and wild-type cells. However, if concentration of H₂O₂ was increased to 1.0 mM for 6 h, massive release of intracellular LDH could be observed, thus indicating that the cell mortality shown in Fig. 2a and b is not mainly caused by cell necrosis.

Morphological evidences of anti-apoptosis by hHSS gene

Fig. 2a and b shows that transfection of hHSS gene protects the cells from 0.6 mM H₂O₂-induced damage. To further confirm that this protection is related to anti-apoptotic effect, the cellular morphologies were assessed using florescence microscopy. As shown in Fig. 3, the treatment of wild-type and vector-transfected cells with 0.6 mM of H₂O₂ for 6 h induced marked nuclear changes such as chromatin condensation and fragmentation under light microscopy (a) and florescence microscopy (b). In addition, the cell lesions caused by H₂O₂ was also assessed by electron microscopy. H₂O₂ administration resulted in a typical apoptotic appearance [36], including the compaction and marginalization of chromatins and degradation of nuclear membranes in the non-hHSS-transfected cells. However, apoptosis was markedly reduced in the hHSS-transfected cells.

The apoptosis evaluated by flow cytometry

As shown in Fig. 4a and b, apoptotic ratio in the wild-type and the vector-transfected cells were comparatively high, 55.64 ± 4.32% and 55.81 ± 3.70%, respectively. However, the number of apoptotic cells was significantly reduced in the hHSS-transfected group, 20.79 ± 3.33% of cells were apoptotic (P < 0.05 vs wild-type and vector-transfected cells).
Effect of hHSS gene on alternation of $\Delta \psi_m$ and cytochrome c leakage

Alternation of $\Delta \psi_m$ is known to be an early event in the apoptosis-signaling process [37]. It is well known that the hHSS gene is localized in the mitochondria of hepatoma cells, so we investigated whether the gene has an important role in protection of mitochondria from H$_2$O$_2$-induced damage and apoptosis. As shown in Fig. 4c and d, exposure of the cells to H$_2$O$_2$ (0.6 mM for 6 h) resulted in a marked loss of the $\Delta \psi_m$ leading to severe impairment of the mitochondrial membrane and marked leakage of cytochrome c. In the hHSS-transfected cells, loss of $\Delta \psi_m$ was markedly reduced after H$_2$O$_2$ treatment compared with wild-type and vector-transfected cells. In addition, H$_2$O$_2$ exposure led to considerable leakage of cytochrome c into the cytosol of the wild-type and vector-transfected cells, but this phenomenon was markedly reduced in the hHSS-transfected cells. Furthermore, we observed an anti-apoptotic function of hHSS gene. Interestingly, Bcl-2 expression was markedly increased in the HSS-transfected cells, providing additional evidence of hHSS gene involvement in the regulation of apoptosis (Fig. 5e).

Effect of hHSS on ATP content

Furthermore, reduced ATP content, which is an indicator of energy production of mitochondria, was found in H$_2$O$_2$-toxicated cells. Fig. 6 shows that the cellular ATP levels in the three cell types are greatly reduced after exposure to H$_2$O$_2$ for 6 h. Although the ATP content of the hHSS-transfected cells reduced to a low level, the amount of ATP in the hHSS-transfected cells is still greater than the levels in the wild-type and vector-transfected cells, 39.4% and 40.25% greater, respectively. This indicates that although energy metabolism is reduced with H$_2$O$_2$, the magnitude of this reduction is less after hHSS-transfection.

Discussion

The electron transport chain of mitochondria is known to be a primary source of reactive oxygen species (ROS) formation in most cells [38]. It has been reported that H$_2$O$_2$ induces a reduction in GSH levels and an increase in oxidative stress [39]. As a type of ROS, H$_2$O$_2$ acts as an early messenger molecule in signaling cascades activated by several external and developmental stimuli [40]. It has been well documented that H$_2$O$_2$ primarily targets the mitochondria. Through an, as yet, not fully understood mechanism, H$_2$O$_2$ has an important role in regulation and execution of the hypersensitive cell death program and is widely used as a potent in vitro apoptosis inducer [7].

In this study, treatment with H$_2$O$_2$ resulted in severe damage to the wild-type and vector-transfected cells, but the hHSS-transfected cells seem to be more resistant to H$_2$O$_2$ injury, as shown by cell mortality (Fig. 2a and b) and the apoptotic ratio (Fig. 4a and b), and which is consistent with our recent report focused on characterization of recombinant HSS (article in press, Protein Journal). It was reported that administration of ALR protein enhances cytochrome content in liver [30], however, our data shows that overexpression of HSS gene in hepatoma cells do not influence mitochondrial cytochrome c level (Fig. 5a and b).
In addition, we also found that high expression of hHSS gene is able to prevent the cells from H$_2$O$_2$ damage by morphological assessment, such as shrinkage, condensation and marginalization of chromatin and degradation of nuclear membrane (Fig. 3).

Mitochondria have a pivotal role in apoptosis. It is well known that exposure of the cells to H$_2$O$_2$ induces apoptosis through pathways that involve disruption of normal mitochondrial function. One key mechanism seems to involve opening of mitochondrial permeability transition pores (PTP) that allow passage of some small pro-apoptotic molecules into the cytoplasm [41], such as cytochrome c [29]. It has been reported that the PTP opening increases when the mitochondrial membrane potential decreases [31]. As shown in our study, the exposure of hepatoma cells to H$_2$O$_2$ impairs mitochondrial function, leading to a marked reduction in the depolarization rate of the mitochondrial membrane [42].

The involvement of mitochondria in H$_2$O$_2$-induced apoptosis was further confirmed by the release of some key effector proteins into the cytosol. Of those released proteins, cytochrome c is considered most important because it could trigger a critical step in the activation of the mitochondrial pathway [36]. Our results show that H$_2$O$_2$ injury causes an increase in leakage of cytochrome c into the cytosol; however, the amount of cytochrome c leakage in the hHSS-transfected cells is significantly reduced compared with wild-type and vector-transfected cells. Although it has been widely reported that following cytochrome c leakage several components of the pro-caspase as Caspase-3 and Caspase-9 are activated in apoptotic cells [43], we are unable to confirm this fact at 6 h of H$_2$O$_2$ injury. However, as an alternative supporting document, Bcl-2, a dominant anti-apoptotic gene, is highly expressed in the HSS-transfected cells subjected to H$_2$O$_2$, indicating that a possible role of the hHSS gene in desensitizing hepatoma cells to H$_2$O$_2$.
H₂O₂-induced cell death is probably associated with mitochondrial protection.

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References


Fig. 5. Western-blot analysis of the cytosolic and mitochondrial cytochrome c. Twenty five micro grams of proteins from each of wild-type cells, vector-transfected cells, and hHSS-transfected cells were separated by 12% SDS–PAGE and hybridized with anti-cytochrome c antibody and rehybridized with β-actin and COX IV for normalization of loading controls. A significant reduction in cytochrome c leakage was found in hHSS-transfected cells compared in wild-type cells, P < 0.05. At the same time, cytochrome c accumulated in the cytosol of wild-type cells and vector-transfected cells, and was markedly more abundant than in the cytosol of hHSS-transfected cells (lane 6 from the left in panel a and c). Densitometric analysis of cytochrome c leakage is shown in b and d. Meanwhile, 50 μg of total protein was run on 12% SDS–PAGE and stained with anti-Bcl-2 antibody. Bcl-2 expression was markedly upregulated in the hHSS-transfected cells, as shown by Western-blot analysis (panel e).

Fig. 6. Cellular ATP contents. Cells with or without hHSS transfection were exposed to 0.6 mM of H₂O₂ and the cellular ATP contents were measured. *P < 0.05 by comparison with ATP content in wild-type cells.

H₂O₂-induced cell death is probably associated with mitochondrial protection.


