Over-expression of GRP75 inhibits liver injury induced by oxidative damage

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Introduction

Heat shock proteins (HSPs) have been implicated in the molecular pathogenesis of neurodegenerative diseases considering their pivotal role as cellular sensors of various types of stress-related events, including mitochondrial-related neurodegeneration [1], their conformation and folding chaperone activity on newly formed proteins [2], trafficking of target proteins across membranes [3], escort of misfolded proteins to the proteasome [4,5], α-synuclein fibril and oligomer assembly [6], and maintenance of neuronal homeostasis after mitochondrial insults [7].

Mortalin, one of the members of the HSP70 protein family (also called stress 70 protein, HSP75, or glucose-regulated protein 75 kDa), is a multifaceted protein with a major role in the import and folding of mitochondrial proteins [8]. It has been postulated that GRP75 is a cytoprotective factor against various stresses.

It has been reported that GRP75 is predominantly located in mitochondria, but a growing body of evidence has demonstrated that it is also present in other cellular compartments, including the endoplasmic reticulum, cytoplasmic vesicles, and cytosol [9]. GRP75 can inhibit reactive oxygen species (ROS) accumulation, a mechanism that may be involved in the cytoprotective effect of GRP75 over-expression on glucose deprivation [10].

Mitochondria are the sources of energy in eukaryotic cells. In recent years, it has become clear that mitochondria also play a key role in cell death pathways [11]. A previous investigation demonstrated that GRP75 over-expression provides significant protection against metabolic stress induced by glucose deprivation [12].

It has been reported that normal liver tissue has very low constitutive GRP75 expression [13]. Liver fibrosis is a result of chronic hepatocellular damage due to a variety of liver diseases, and cell oxidative damage is an important process in liver fibrosis. Whether Grp75 can inhibit mitochondrial oxidative damage of hepatocyte and protect liver...
cell from apoptosis in the process of liver fibrosis? In this study, Grp75 gene was transferred into HL-7702 cells and rats to investigate its anti-oxidant effect of inhibition in liver injury.

Materials and Methods

Cell culture
The HL-7702 cell line was purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China) and stable transfection of HL-7702 cells with EGFP-N2-GRP75 was performed as described below. Cells were grown routinely in Dulbecco’s modified Eagle’s medium containing 10% Solcoseryl (Hyclone, Logan, USA), 50 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified incubator in 5% CO2. The cells were fed every 2 days and were passaged twice a week.

The EGFP-N2 empty vector or EGFP-N2 containing full-length GRP75 cDNA was transfected into HL-7702 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, USA). A sample of 2 μg of plasmid DNA per 5 × 106 cells was used for transfection. Neomycin-resistant colonies were isolated in medium supplemented with 0.6 mg/ml G418 and were expanded in tissue culture flasks. GRP75 protein expression was assayed by western blotting. To study the effects of GRP75, H2O2 (400 μM) was added to the medium for both GRP75-overexpressing and normal HL-7702 cells for 2 h to induce cell injury.

MTT reduction assay
Cell viability was determined by measuring the methyl thiazolyl tetrazolium (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide) (MTT) reduction ability of cells according to the method of Mosmann [14]. MTT, which reacts with dehydrogenases and cofactors of the respiratory chain, is an indicator of mitochondrial activity [15]. In brief, MTT was dissolved in phosphate-buffered saline (PBS) at 5 mg/ml and was added to the culture medium at the end of the incubated time to yield a final concentration of 0.5 mg/ml. After a further 3 h of incubation at 37°C, 0.1 ml of isopropanol–HCl was added to each well, and the absorbance of the solubilized MTT formazan products was measured at 570 nm. Results are expressed as percentage of MTT reduction relative to control cells.

Cytochrome c measurement
Cells were lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% sodium dodecyl sulfate (SDS), and 50 mM Tris-HCl, pH 8.0) supplemented with 1 g/ml aprotinin and 100 g/ml phenylmethylsulfonyl fluoride. The cell suspension was incubated on ice for 30 min and then centrifuged at 20,000 g for 15 min at 4°C. The supernatant was collected for further analysis. Protein concentrations in the samples were determined by Bradford assay. A total of 20 μg of protein was separated on a 10% or 15% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane (Amersham Pharmacia Biotech, Little Chalfont, UK). Membranes were blocked with 5% (w/v) fat-free dry milk in TBS-T buffer (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.05% Tween-20) and incubated overnight at 4°C with mouse anti-cytochrome c (CST, Beverly, USA) and rabbit anti-GADPH (Santa Cruz, California, USA) as primary antibodies, followed by washing and incubation with appropriate horseradish peroxidase-conjugated polyclonal goat IgG as secondary antibody (Santa Cruz).

ROS measurement
To measure ROS production in HL-7702 cells treated with H2O2, we used the DCFH-DA method. DCFH-DA is membrane-permeable and is converted to highly fluorescent 2’,7’-dichlorofluorescein (DCF) in the presence of ROS. After treatment, HL-7702 cells (10⁴ cells/well in a 96-well plate) were incubated with DCFH-DA (final concentration 100 μM) for 30 min, and the fluorescence was monitored on a CytoFluor multi-well plate reader using excitation and emission wavelengths of 485 and 530 nm, respectively. Results are expressed as a percentage relative to DCF fluorescence in control cells.

ATP quantification
Intracellular ATP was determined by the luciferin–luciferase method [16] using an ATP bioluminescence assay kit (Beyotime, Shanghai, China). Cells supplemented with glucose or under low-glucose conditions were washed with cold PBS and lysed with 100 μl of cell lysis buffer. Then 1 μl of the lysate was diluted to 100 μl with water and mixed with 100 μl of luciferase–luciferin reagent. After 10 s, the light emitted was recorded using a luminometer at 562 nm and integrated over 5 s.

Matrix metalloproteinase measurement
Intracellular matrix metalloproteinase (MMP) was evaluated using the fluorescent, lipophilic, and cationic probe 5,5‘,6,6’-tetrachloro-1,1‘,3‘,3‘-iodide (JC-1; Beyotime) according to the manufacturer’s instructions. In brief, cells were incubated with JC-1 staining solution for 20 min at 37°C and washed three times with JC-1 staining buffer. The fluorescence intensity was measured on a multi-well plate reader using excitation and emission wavelengths of 514 and 529 nm for the green fluorescence of the monomer form, and 585 and 590 nm, respectively, for the red fluorescence of the aggregate form. Cellular MMP in each group was evaluated as the red/green fluorescence ratio observed under a fluorescence microscope. Data were expressed as a percentage of the control.
Rat model of liver fibrosis and experimental design
Adult male Wistar rats (150–200 g; SLAC Laboratory Animal, Shanghai, China) had free access to sterilized food and autoclaved water at 20–22°C under a 12-h light–dark cycle. All studies were conducted in accordance with the principles and procedures outlined by the Animal Experimentation Ethics Committee of Fudan University (Shanghai, China).

Animals were divided into four groups (n = 5–6/group). Group 1: animals were treated by intraperitoneal injection of saline only. Group 2: the EGFP-N2 vector containing full-length GRP75 cDNA was transfected into rats using in vivo jetPEI (Polyplus, France) via tail vein injection. The vector dose was 200 μg/kg. Group 3: liver fibrosis was induced by intraperitoneal injection of carbon tetrachloride (CCl4) (1:1 in corn oil) at a dose of 0.1 ml/100 g body weight twice weekly for 8 weeks. Group 4: animals were injected with CCl4 for 8 weeks and were transfected with the GRP75 vector.

Animals were sacrificed at the end of the 11th week and all livers were removed. Blood was obtained by cardiac puncture and serum samples were distributed into aliquots and stored at −80°C until required for analysis. Serum alanine transaminase (ALT) was measured using an automated technique. Portions of liver were fixed in 10% buffered formalin overnight before embedding in paraffin for histopathological and immunohistochemical examination. The remaining portion of the liver was used to extract protein for assay of cytochrome c by Western blotting.

Immunohistochemistry
Sections were stained with hematoxylin and eosin to assess liver histology. Immunostaining was performed on liver sections (4 μm) after de-paraffinization. Peroxidase activity was quenched using 3% H2O2 in PBS. Sections were then washed in water and preblocked with normal rabbit serum for 10 min. The primary GRP75 antibody (1:100; Santa Cruz) was added and incubated for 2 h at room temperature. After rinsing with Tris-buffered saline (TBS) for 10 min, samples were incubated with a secondary mouse peroxidase antibody (Dako, Carpinteria, USA) for 30 min at room temperature. The sections were rinsed again in TBS and then incubated with diaminobenzidine for 5 min. Section areas showing positive staining were quantified on a computerized morphometry system to determine the amount of GRP75.

Statistical analysis
Data were expressed as mean ± SD. Statistical analysis was performed using one-way analysis of variance. Bonferroni and Dunnett two-sided post hoc tests were performed for multiple comparisons. An independent sample t-test was used for two-group comparisons. P < 0.05 was considered statistically significant.

Results
GRP75 inhibits H2O2-induced cell injury
HL-7702 cells were stably transfected with EGFP-N2-GRP75. Results showed that GRP75 expression was significantly greater in HL-7702 cells transfected with EGFP-N2-GRP75 than in cells transfected with the empty EGFP-N2 vector, representing endogenous GRP75 protein expression [Fig. 1(A)] and that GRP75 over-expression protected
HL-7702 cells against H2O2-induced damage [Fig. 1(B)]. ATP concentrations were significantly higher (P < 0.05) in GRP75-overexpressing HL-7702 cells treated with H2O2 than in control cells treated with H2O2 (Table 1), indicating that GRP75 over-expression can affect changes in ATP concentrations induced by H2O2 treatment.

To further investigate the effect of GRP75 on HL-7702 cells under glucose deprivation, GRP75-overexpressing and control cells were probed with JC-1. After 2 h of H2O2 treatment, red fluorescence was hardly observed for HL-7702 cells [Fig. 1(C)], indicating MMP loss. In comparison, red fluorescence in GRP75-overexpressing cells treated with H2O2 was much greater, indicating the protective effect of GRP75 on MMP.

The effect of GRP75 on ROS accumulation following H2O2 exposure was also monitored. Table 1 shows that ROS accumulation did not significantly differ between GRP75-overexpressing cells and the control. Control cells exhibited an immediate and rapid increase in ROS accumulation under H2O2 exposure, while this response was significantly lower in GRP75-overexpressing cells (P < 0.05). As shown in Fig. 1(D), cytochrome c expression was significantly higher in H2O2-treated cells than that in GRP75-overexpressing cells treated with H2O2.

**GRP75 inhibits CCl4-induced liver fibrosis**

Rats treated with CCl4 showed significantly higher serum ALT and aspartate aminotransferase (AST) compared with control rats (Table 2), indicating that hepatic injury was induced by CCl4. ALT and AST concentrations in GRP75-overexpressing rats treated with CCl4 were much lower than in the CCl4-only group (P < 0.05). As shown in Fig. 2, fibrous bands were evident in liver sections from rats. The severity of liver fibrosis was obviously less in GRP75-overexpressing rats.

We further investigated GRP75 expression in each group of rats. The immunohistochemistry data showed that GRP75 expression was hardly detected in control rats [Fig. 3(A)] and similar in the CCl4-treated and the GRP75-overexpressing groups [Fig. 3(B,C)], and reached the highest level in GRP75-overexpressing rats injected with CCl4 [Fig. 3(D)].

Western blotting was performed to assess cytochrome c levels. The results in [Fig. 4(B)] demonstrate that cytochrome c expression was significantly higher in the CCl4-only group when compared with the GRP75-overexpressing group treated with CCl4.

**Discussion**

GRP75 is a highly conserved member of the HSP70 family of proteins and is found in multiple subcellular sites. The protein is involved in multiple physiological functions, including mitochondrial import, antigen processing, and control of cell proliferation and differentiation [17].

Previous studies in our laboratory demonstrated that glucose deprivation can induce cell damage under hypoglycemic conditions, including both necrosis and apoptosis [18], and this phenomenon was confirmed in HL-7702 cells in the present study.

There are different animal models of liver fibrosis, including hepatotoxin-induced injury (CCl4, dimethylnitrosamine, and thioacetamide), nutritional derangement (alcohol...
and a high-fat, low-choline diet), and cholestasis induced by bile duct ligation [19].

We observed that GRP75 inhibits liver injury induced by oxidative damage, both in vitro and in vivo. Concentrations of MTT, AST, and ALT were measured to identify the protective effect of GRP75 against H2O2-induced damage in HL-7702 cells. Mitochondrial functions were detected under H2O2 exposure because GRP75 resides predominantly in mitochondria, and it was conceivable that GRP75 exerts its protective effect in this organelle.

We measured intracellular ATP in HL-7702 cells to identify whether GRP75 over-expression could ameliorate ATP depletion during H2O2 treatment. Our data show that GRP75 over-expression affected the changes in intracellular ATP induced by H2O2. HL-7702 cells over-expressing GRP75 showed moderate cell damage compared with control cells, and ATP concentrations also exhibited a significant discrepancy. We observed a similar effect of GRP75 over-expression on the MMP of HL-7702 cells. H2O2-induced progressive MMP depolarization and GRP75 over-expression prevented this decrease in MMP.

It is widely accepted that ROS are produced predominantly in mitochondria as a by-product of oxidative phosphorylation [20]. Our data demonstrate that GRP75 over-expression in HL-7702 cells significantly inhibited ROS accumulation induced by H2O2 exposure.

It has been reported that the release of cytochrome c may induce cell apoptosis [21,22]. We measured cytochrome c in the cytoplasm to identify whether GRP75 can regulate its release. Cytochrome c expression on H2O2 exposure was obviously less in GRP75-overexpressing HL-7702 cells than in control cells.

GRP75 ameliorated CCl4-induced hepatic inflammation in rats, as evidenced by improved histological findings and lower ALT and AST concentrations [23]. We also analyzed ATP and cytochrome c concentrations to indentify the mechanism of inhibition and data showed a similar trend as for cell cultures.

In conclusion, GRP75 reduced hepatic inflammation induced by CCl4. It prevented oxidative damage both in vitro and in vivo via amelioration of mitochondrial function such as an increase in ATP and inhibition of ROS.
accumulation and the release of cytochrome c. This study provides useful information for future therapies for liver fibrosis.

References