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Effect of methionine sulfoxide reductase B1 silencing on high-glucose-induced apoptosis of human lens epithelial cells

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ABSTRACT

Aims: To determine roles of methionine sulfoxide reductase B1 (MsrB1) in protecting lens mitochondria against oxidative damage, the influences of MsrB1 gene silencing on high-glucose-induced apoptosis in human lens epithelial (HLE) cells were studied.
Main methods: Our study used four groups of cells: normal control, MsrB1 gene silenced, high glucose (30 mM) exposed and MsrB1 gene silenced cells followed with high glucose exposure. In all cases we detected cell viability, cell apoptosis rate, intracellular reactive oxygen species (ROS) and malondialdehyde (MDA) levels, alteration of mitochondrial membrane potential, release of mitochondrial cytochrome c as well as an increase in activity of caspase-3.
Key findings: The results showed that MsrB1 gene silencing by short interfering RNA (siRNA) in HLE cells clearly resulted in oxidative stress, decrease in mitochondrial membrane potential and release of mitochondrial cytochrome c as well as an increase in activity of caspase-3 and the percentage of apoptotic cells. When MsrB1-silenced HLE cells were exposed to high glucose, characteristic of high-glucose-induced mitochondrial dysfunctions were further exacerbated.
Significance: MsrB1 plays important roles in protecting HLE cell mitochondria against oxidative damage and inhibits oxidative stress-induced apoptosis in diabetic cataracts by scavenging ROS.

Introduction

Diabetic cataract is one of the most serious complications of diabetes (McCarthy and Taylor, 1996). High glucose-induced oxidative stress is believed to play a major role in diabetic cataract formation. It was reported that high glucose exposure caused protein oxidation and aggregation in lens cells and led to lens opacity (Jain et al., 2002). After being incubated with high glucose for 24 h, human lens epithelial (HLE) cells underwent apoptosis and exhibited signs of oxidative stress (Wu et al., 2008; Zhang et al., 2010; Kim et al., 2011). It was reported that apoptosis of HLE cells was implicated in the pathogenesis of cataract formation (Wride et al., 2006; Andersson et al., 2000).

In previous a study, we found that in diabetic mouse lenses, levels of MDA, protein carbonyl (PC) groups and methionine sulfoxide (MetO) were increased, and level of total sulfhydryl groups (TSH) was decreased; expression levels of the methionine sulfoxide reductases (Msrs) were decreased (Li et al., 2011). Methionine (Met) is one of the most easily oxidized amino acids and undergoes reversible oxidation, leading to the formation of MetO (Vogt, 1995). In mammals, there are MsrA and MsrB, which can catalyze MetO to Met (Kim and Gladyshev, 2007). Among all Msrs, MsrB1 is the only selenoprotein, known as selenoprotein R (SelR), localized in the cytosol and nucleus, suggesting that it plays specialized roles in repairing damaged proteins in these cellular compartments (Marchetti et al., 2005). It has been showed that MsrA is localized in all the organelles and repair of mitochondrial cytochrome c by MsrA could protect cataract formation (Brennan et al., 2009). However, no result has been reported about the role of MsrB1 in the maintenance of mitochondrial function yet in HLE cells.

In this study, the role of MsrB1 in high-glucose-exposed HLE cells was investigated. Oxidative stress and mitochondrial dysfunctions associated with cell apoptosis were detected. Our results suggest that MsrB1, although not localized in mitochondria, plays important roles in protecting mitochondria against oxidative damage and inhibiting oxidative-stress-induced cell apoptosis.

Materials and methods

Cell culture

Human lens epithelial cells (SRA01/04; HLE cells) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM

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glucose supplemented with 10% newborn calf serum and antibiotics (penicillin G 100 U/ml, streptomycin 100 μg/ml) at 37.0 °C in the presence of 5% CO_2. All reagents were from Gibco, Gaithersburg, USA.

MsrB1 gene silencing and cell treatments

Double-stranded short interfering RNA (siRNA) specific for MsrB1 was manufactured by Gene Pharma Inc. (Shanghai, China). The siRNA sequences for MsrB1 were the same as those used in a previous study (Marchetti et al., 2005). HLE cells were divided into four groups: normal control cells (NC) cultured in DMEM containing 25 mM glucose, high-glucose-exposed cells (HG), MsrB1-gene-silenced cells (Si) and MsrB1-gene-silenced cells followed with high-glucose exposure for 24 h (Si + HG). HLE cells of the Si and Si + HG groups were transfected (Table 1) with siRNA as described in previous works (Zeng et al., 2008; Du et al., 2010). After 24 h, all groups were treated with fresh serum free media with or without high glucose (30 mM) for 24 h. HLE cells were then harvested for further analysis. A random siRNA sequence was used as a nontargeting RNA as negative control (N.Si) and had no homology with any genes (Du et al., 2010). Additionally, ROS and MDA production were measured over time in HLE cells. Cells were observed at 36 and 48 h after the MsrB1 gene silencing transfection.

Detection of cell viability

Measurements of cellular viability of HLE cells were performed in triplicate in each 24-well cell culture plate (~2×10^5 cells per well) and averaged using an MTT [3-(4, 5 dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; Sigma, USA] colorimetric assay that measures mitochondrial metabolism. Briefly, MTT (dissolved in serum-free DMEM at 0.5 mg MTT/ml) was added to each culture plate well (0.5 ml/well) and incubated for 4 h at 37 °C to allow mitochondrial dehydrogenase within viable cells to metabolize MTT to insoluble formazan. At the end of the incubation, the medium was removed, the cells were solubilized with 1.0 ml dimethyl sulfoxide per well, and absorbance was measured at 570 nm using a spectrophotometer (Cecil, Model No. CE7200). Absorbance was expressed as percent of the absorbance in untreated control cells in parallel wells (Xu et al., 2011).

Measurement of intracellular ROS

The levels of intracellular ROS were estimated using a membrane-permeable fluorescent probe, dihydrodichlorofluorescein diacetate (H_2-DCFDA; Beyotime Inst. Biotech, Haimen, China). Briefly, as the manufacturer's instruction suggested, HLE cells cultured in 6-well plates (~1×10^6 cells in each well), were washed with PBS, 3 ml trypsin was added and incubated at 37 °C until cells detach. Then the trypsin was removed and serum was added into the cells to inhibit remaining tryptic activity. The cells were collected by centrifugation (4 °C, 600 g) for 5 min, and washed twice by PBS (centrifugation at 4 °C, 600 g for 5 min). The harvested cells were incubated for 30 min with the cell-permeable fluorescent ROS probe, after which the cells were washed in serum-free DMEM and collected. Cellular fluorescence derived from oxidized probes was measured using a fluorescence spectrometer (RF-5301, Shimadzu) with excitation and emission wavelengths of 485 nm and 530 nm (Bejma and Ji, 1999). Data are presented as the mean fluorescence intensity and are normalized to the normal control value. In addition, the morphology of treated HLE cells was observed using the same fluorescent probe (H_2-DCFDA) according to the manufacturer's instructions with a fluorescence microscope (IX71, Olympus, Tokyo, Japan) at 200×. Briefly, after applying the previously described treatments, HLE cells were rinsed twice with PBS and incubated in 100 μM H_2-DCFDA with serum-free medium. After incubation at 37 °C for 30 min, the HLE cells were washed twice with serum-free medium and observed under a microscope.

Table 1
Volumes of dilution medium in RNAi transfection.

Culture vessel	Plating medium	Dilution medium	siRNA	Lipofectamine 2000
24-well	500 μl	2×50 μl	1 μl	1 μl
6-well	2 ml	2×250 μl	5 μl	5 μl
60-mm	5 ml	2×0.5 ml	10 μl	10 μl

Detection of MDA contents

HLE cells were cultured in 60 mm Petri dishes. Lipid peroxidation in HLE cells (~1×107 cells per sample) was determined by measurement of MDA formation. MDA content was determined by the TBA (thiobarbituric acid) assay using an MDA measurement kit (Jiancheng Inst. Biotech, Nanjing, China) according to the manufacturer's instructions.

Isolation of total RNA and real-time PCR

Total RNA was isolated from the HLE cells using TRIzol (Invitrogen, USA) according to the manufacturer's instruction. Complementary DNA (cDNA) was prepared from the RNA using M-MLV reverse transcriptase, deoxyribonucleotides (dNTPs) and oligo-dT primer in Tris (hydroxymethyl) amino methane hydrochloride (Tris–HCl) buffer (50 mM, pH 8.3). All reagents were from Toyobo (Japan). The reaction mixture was incubated at 42 °C for 40 min and stopped by heating at 95 °C for 10 min (Zeng et al., 2008).

Real-time PCR was performed with the DNA Engine Opticon 2 (MJ Research, Watertown, MA), using the SYBR Green PCR Master Mix kit (Toyobo, Japan) according to the vendor's protocol. Primers for MsrB1 and the internal control housekeeping gene GAPDH are described in (Marchetti et al., 2005; Zeng et al., 2008), respectively. Primers were synthesized by Sun-Biotechnology Co. (Beijing, China). The thermal cycle conditions were as follows: for MsrB1, 95 °C for 10 min followed by 40 cycles of 15 s denaturation at 95 °C and 1 min at 60 °C; for GAPDH, 95 °C for 5 min followed by 25 cycles of denaturation at 95 °C for 30 s, 60 °C for 1 min and 72 °C for 1 min, with a final extension at 72 °C for 10 min. The expression levels of the target genes were related to the expression level of GAPDH. Relative expression levels were calculated using $2^{-\Delta\Delta Ct}$ rules (Zeng et al., 2008).

Western blot analysis

Western blots were processed as described in previous work (Li et al., 2011; De Luca et al., 2007). Briefly, HLE cells (~1×10^7 cells per sample) were lysed on ice for 10 min in RIPA lysis buffer and protein extracts were obtained by centrifugation (4 °C, 10,000 g for 15 min). Protein concentration was determined using the Bradford assay, and BSA was used as a protein standard. Total protein (40 μg) was analyzed using SDS-PAGE according to previously described methods (Laemmli, 1970). Briefly, the protein extract was mixed with 4× sample buffer (0.25 M Tris–HCl, pH 6.8, 40% (v/v) glycerol, 0.8 g SDS, 1% (w/v) Bromphenol Blue and 0.617 g DTT) and boiled for 5 min. The boiled mixture was electrophoresed on 15% (w/v) polyacrylamide gels. Proteins were transferred electrophoretically to polyvinylidene fluoride (PVDF) membranes (60 min at 0.25 A for MsrB1 and GAPDH) using an immunoblot transfer apparatus (Bio-Rad). After transfer, the membranes were blocked for 120 min at room temperature in 5% (w/v) non-fat milk in Tris-buffered saline containing Tween-20 (TBST; 500 mM NaCl and 20 mM Tris–HCl, pH 7.5, supplemented with 0.05% (v/v) Tween 20) to block non-specific binding. The blots was incubated overnight at 4 °C with 5% non-fat milk in TBST containing antibodies for MsrB1 (Santa Cruz Inc., USA) or the housekeeping protein GAPDH (Santa Cruz Inc., USA) as a