Archives of Biochemistry and Biophysics 502 (2010) 137–143

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi

SelK is a novel ER stress-regulated protein and protects HepG2 cells from ER stress agent-induced apoptosis

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ARTICLE INFO

Article history:
Received 28 May 2010
and in revised form 31 July 2010
Available online 6 August 2010

Keywords:
Selenoprotein K
Endoplasmic reticulum stress
siRNA
Apoptosis

ABSTRACT

Selenoprotein K (SelK), an endoplasmic reticulum (ER) resident protein, its biological function has been less-well studied. To investigate the role of SelK in the ER stress response, effects of SelK gene silence and ER stress agents on expression of SelK and cell apoptosis in HepG2 cells were studied. The results showed that SelK was regulated by ER stress agents, Tunicamycin (Tm) and β-Mercaptoethanol (β-ME), in HepG2 cells. Moreover, the SelK gene silence by RNA interference could significantly aggravate HepG2 cell death and apoptosis induced by the ER stress agents. These results suggest that SelK is an ER stress-regulated protein and plays an important role in protecting HepG2 cells from ER stress agent-induced apoptosis.

Introduction

Selenium is an essential micronutrient in mammals [1]. The major biological form of this micronutrient is selenocysteine that is present in the active sites of selenoenzymes [2–5]. The human selenoproteome consists of 25 selenoproteins [6]. Known functions of selenoproteins include antioxidant protection and cellular redox balance (attributed to the glutathione peroxidases, thioredoxin reductases, methionine sulfoxide reductases and selenoprotein P), thyroid hormone activation and inactivation (catalyzed by the iodothyronine deiodinases) and selenium transport to peripheral tissues (through plasma selenoprotein P) [7–13]. However, many of them with partially characterized biological functions or unknown functions have been less-well studied. In these selenoproteins, there are seven selenoproteins have been identified as residents of the endoplasmic reticulum (ER)[1], including the 15-kDa selenoprotein (Sep15), type 2 iodothyronine deiodinase and selenoproteins K, M, N, S, and T. Recent studies have suggested that their roles may be involved in the quality control of protein folding in the ER (e.g., Sep15), retro-translocation of misfolded proteins from the ER to cytosol (e.g., SelS) or regulation of calcium homeostasis (e.g., SelT and SelN) [5]. However, there is only one report that linked the function of SelK to cellular redox homeostasis [14]. Thus, it is interesting to investigate the role of SelK in the ER stress response, and effect of SelK gene silence on cell survival.

Selenoprotein K (SelK) is a newly identified selenoprotein and located in ER and plasma membrane [6,15,16]. Previous studies which measured SelK mRNA levels in mouse and human tissues suggested the ubiquitous expression of this protein, such as heart, skeletal muscle, pancreas, testes and liver [14,17]. A SelK gene silence assay suggested that *Drosophila* SelK might be required for normal development but did not reveal changes in total antioxidant status of embryos and cells [18]. However, Lu et al. found that overexpression of SelK attenuated the intracellular reactive oxygen species level and protected cells from oxidative stress-induced toxicity in cardiomyocytes [14]. The discrepancy between two reports may be due to the fundamental differences existing between the antioxidant defense systems in insects and mammalian, since *Drosophila* selenoproteins are not critical for protection against oxidative stress [19]. Nevertheless, the biochemical or biological functions of mammalian SelK in ER need to be elucidated by further experiment.

The efficient functioning of the ER is essential for proper cellular activities and survival. The discrepancy between the demand for and the capacities of the ER functions leads to ER stress [20]. The ER stress response is characterized by changes in specific proteins, causing translational attenuation, induction of ER chaperones and degradation of misfolded proteins [21]. Prolonged and/or severe ER stress may induce apoptosis [22,23]. The apoptotic pathways

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[1] *Abbreviations used*: ER, endoplasmic reticulum; Sep15, 15-kDa selenoprotein; SelK, Selenoprotein K; GADD153, growth arrest- and DNA damage-inducible gene 153; Tm, Tunicamycin; β-ME, β-Mercaptoethanol; CCTCC, China Center for Type Culture Collection; DMEM, Dulbecco's Modified Eagle's Medium; NCS, newborn calf serum; N.C., negative control; PCR, polymerase chain reaction; PMSF, phenylmethane sulfonate fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gels; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PI, propidium iodide; GRP78, glucose-regulated protein 78; Tg, thapsigargin; ERAD, endoplasmic reticulum-associated protein degradation.

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doi:10.1016/j.abb.2010.08.001

in ER-stressed cells include induction of CHOP/GADD153 and activation of JNK kinase and caspase-12. All the apoptotic pathways eventually lead to the activation of caspase-3 [24–26]. However, the protective mechanisms against ER stress-induced apoptosis have not yet been fully understood.

In this paper, the effects of ER stress agents and SelK gene silence on SelK expression and cell apoptosis in HepG2 cells were investigated. The results suggest that SelK is an ER stress-regulated protein and plays an important role in protecting HepG2 cells from ER stress agent-induced apoptosis.

Materials and methods

Materials and cell culture

Tunicamycin (Tm) was purchased from Sigma. β-Mercaptoethanol (β-ME) was purchased from Amresco. HepG2 cells were obtained from China Center for Type Culture Collection (CCTCC) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 10% (v/v) heat-inactivated newborn calf serum (NCS) and antibiotics (penicillin, 100 U/mL; streptomycin, 100 μg/mL) in 5% CO_2 at 37 °C. Exponentially growing cells were used throughout the study.

SelK RNA interference

The target sequence used for knockdown of human SelK (GenBank accession number NM_021237) was AATGGGTAGAATCAATCATCT (219–239) and is specific for hSelK based on BLAST search (NCBI database). A random siRNA sequence (sense 5′-UUCUCCGAACUG GUCACGUTT-3′; antisense 5′-ACGUGACACGGCGUUAGAATT-3′) was used as a negative control (N.C.) and had no homology with any genes. siRNAs were synthesized by Shanghai GenePharma (Shanghai, China).

HepG2 cells were plated in 6-well plates to 50% confluence and transfected with 5 μL of 20 μM siRNA and 5 μL of Lipofectamine 2000 (Invitrogen) in 2 mL of DMEM in the absence of serum and antibiotics. After transfection for approximately 24 h, the cells were treated with the fresh serum free media containing indicated concentrations of β-ME or Tm for indicated time and harvested for analysis.

Real-time polymerase chain reaction (Real-time PCR)

Total RNAs were extracted from the cells using Trizol reagent (invitrogen) according to the manufacturer's instructions. The levels of mRNA for SelK were examined by Real-time PCR analysis. Complementary DNA (cDNA) was prepared by incubation of the mRNA with M-MLV reverse transcriptase (100 U, Toyobo), 10 mM dNTP (Toyobo), and oligo $(dT)_{15}$ (500 ng, Toyobo) at 42 °C for 40 min in storage buffer (Toyobo). Following inactivation of the enzyme by incubation at 95 °C for 5 min, cDNA was submitted to polymerase chain reaction (PCR). The following primer sequences were used: SelK: 5′-TGCCCACCTTCGTCCTCGTC-3′ (forward) and 5′-CTGTC GGTTTCTGTATCTCCCT-3′ (reverse); GAPDH: 5′-CCATGTTCGTCAT GG GTGTGAACCA-3′ (forward) and 5′-GCCAGTAGAGGCAGGGATGATGTTC-3′ (reverse); SelS: 5′-GTTGCGTTGAATGATGTCTTCCT-3′ (forward) and 5′-AGAAACAAACCCCATC AACTGT-3′ (reverse) [27]; GPx-1: 5′-CGCTTCCAGACCATTGACATC-3′ (forward) and 5′-CGAGG TGGTATTTTCTGTAAGATCA-3′ (reverse) [28]. PCR conditions for SelK and SelS were 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min; for GAPDH and Gpx-1 were 94 °C for 5 min followed by 25 cycles of 94 °C for 30 s, 60 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 10 min [29]. Real-time PCR was performed using a DNA Engine Opticon 2 (MJ Research, Boston, MA), and the SYBR Green PCR Master Mix kit (Toyobo), according to the vendor's protocol.

Western blot assay

Cells were lysed in cell lysate (100 mM Tris–HCl, pH 8.0, containing 0.15 mM NaCl, 1 mM EDTA, 1 mM phenylmethane sulfonate fluoride (PMSF), 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1% Tween-20) for 20 min on ice. The protein content was determined by Bradford assay. Proteins were separated on 10% sodium dodecyl sulfate–polyacrylamide gels (SDS–PAGE) and then were transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat milk T-TBS solution and then immunodetected using primary antibodies and goat anti-ribbit IgG-horseradish peroxidase conjugate (Pierce) by using an enhanced chemiluminescence detection kit (Pierce). The primary antibodies used in this study were as follow: anti-SelK antibody (Sigma), anti-GAPDH (Santa Cruz Biotechnology), anti-GRP78 and anti-CHOP (Beijing Biosynthesis Biotechnology Co., Ltd.).

Cell viability and caspase-3 activity assays

After treatment with different concentrations of β-ME or Tm for indicated time, cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Briefly, cells were incubated with MTT (0.5 mg/mL) at 37 °C for 4 h; the solution was then removed and formazan salts dissolved with dimethyl sulphoxide, and the absorbance at 570 nm of the resulting solution was measured. Caspase 3 activity in cell lysates was determined using a colorimetric Caspase 3 Activity Assay Kit (Beyotime), according to the vendor's protocol.

Measurement of cell apoptosis by morphology examination

HepG2 cells transfected with SelK and negative control siRNAs were challenged with indicated concentrations of β-ME or Tm for 24 h. Cells were harvested and washed twice with PBS, then stained with Hoechst 33,258 (Sigma) for 30 min at room temperature. After washing with PBS, nuclear morphology was observed under a fluorescence microscope (IX71, Olympus, Tokyo, Japan) at 200×.

Measurement of cell apoptosis by flow cytometry

Quantitative analysis of cell apoptosis was carried out by flow cytometry using an Annexin-V-FITC apoptosis detection kit (Beyotime) according to the manufacturer's protocol [30]. Briefly, the cells treated with Tm or β-ME were harvested and 5×10^5 cells were stained with Annexin-V-FITC for 10 min at room temperature. The cells were centrifuged at 1000*g* for 5 min at room temperature. After washed once with PBS, the cells were stained with propidium iodide (PI). The percentage of apoptotic cells was analyzed by BD™ LSR II flow cytometer immediately. The data were analyzed using WinMDI 2.9 software.

Statistical analysis

The experiments were repeated at least three times. All data are expressed as means ± SD. Comparisons between experimental conditions were done using Student's *t*-test. Differences were considered significant at $P < 0.05$.