Biochemical and Biophysical Research Communications xxx (2017) 1–5

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

ABCB10 depletion reduces unfolded protein response in mitochondria

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ARTICLE INFO

Article history:
Received 9 March 2017
Accepted 14 March 2017
Available online xxx

Keywords:
ABCB10
Mitochondria
Redox
Mitochondria unfolded protein response
Signaling pathway

ABSTRACT

Mitochondria have many functions, including ATP generation. The electron transport chain (ETC) and the coupled ATP synthase generate ATP by consuming oxygen. Reactive oxygen species (ROS) are also produced by ETC, and ROS damage deoxyribonucleic acids, membrane lipids and proteins. Recent analysis indicate that mitochondrial unfolded protein response (UPR^{mt}), which enhances expression of mitochondrial chaperones and proteases to remove damaged proteins, is activated when damaged proteins accumulate in the mitochondria. In *Caenorhabditis elegans*, HAF-1, a putative ortholog of human ABCB10, plays an essential role in signal transduction from mitochondria to nuclei to enhance UPR^{mt}. Therefore, it is possible that ABCB10 has a role similar to that of HAF-1. However, it has not been reported whether ABCB10 is a factor in the signal transduction pathway to enhance UPR^{mt}. In this study, ABCB10 was depleted in HepG2 cells using small interfering RNA (siRNA), and the effect was examined. ABCB10 depletion upregulated ROS and the expression of ROS-detoxifying enzymes (SOD2, GSTA1, and GSTA2), and SESN3, a protein induced by ROS to protect the cell from oxidative stress. In addition, ABCB10 depletion significantly decreased expression of UPR^{mt}-related mitochondrial chaperones (HSPD1 and DNAJA3), and a mitochondrial protease (LONP1). However, the putative activity of ABCB10 to export peptides from mitochondria was not lost by ABCB10 depletion. Altogether, these data suggest that ABCB10 is involved in UPR^{mt} signaling pathway similar to that of HAF-1, although ABCB10 probably does not participate in peptide export from mitochondria.

1. Introduction

Mitochondria are organelles that having two distinct membranes—the outer membrane and the inner membrane—which divide the organelle into two distinct compartments: intermembrane space and matrix [1]. Although mitochondria produce 13 proteins from their own DNA, the organelles contain approximately 1200 proteins [2]. Most of the mitochondrial proteins are encoded by nuclear genomic DNA, and imported into mitochondria after translation of their mRNAs in the cytosol [1,3].

Mitochondria have many functions, including ATP generation, metabolisms and apoptosis. ATP generation is a particularly important role of mitochondria. The electron transport chain (ETC) coupled with ATP synthase generate ATP, accompanied with consumption of oxygen [4,5]. However, because ETC cannot transport electron with absolute efficiency, there may be some leakage of electron, which react with oxygen, and change into reactive oxygen species (ROS) [6]. ROS tends to react with and modify DNA, unsaturated lipids and proteins [7]. Therefore, ROS, majorly occurring in the mitochondria, tend to damage mitochondrial DNA, membrane lipids and proteins. Some ROS-detoxification enzymes are expressed to protect mitochondria from the damage [6]. In addition, mitochondrial unfolded protein response (UPR^{mt}) is activated when damaged or misfolded proteins accumulate in the mitochondria [8–10].

In UPR^{mt}, expression of mitochondrial chaperones and proteases is enhanced to remove damaged or misfolded proteins [11,12]. In *Caenorhabditis elegans*, HAF-1 plays an essential role in the signal transduction pathway to enhance UPR^{mt} [13]. HAF-1 is a

Abbreviations: ETC, electron transport chain; ROS, reactive oxygen species; UPR^{mt}, mitochondrial unfolded protein response; siRNA, small interfering RNA; ABCB10, ATP-binding cassette subfamily B member 10; MDL1, multidrug resistance like-1; SOD2, mitochondrial Mn superoxide dismutase; CAT, catalase; GSTA1, glutathione S-transferase alpha 1; GSTA2, glutathione S-transferase alpha 2; SESN3, Sestrin 3; HSPD1, heat shock protein family D (Hsp60) member 1; HSPE1, heat shock protein family E (Hsp10) member 1; DNAJA3, DnaJ heat shock protein family (Hsp40) member A3; LONP1, Lon Peptidase 1; YME1, YME1 Like 1 ATPase; CLPP, caseinolytic mitochondrial matrix peptidase proteolytic subunit; CANX, calnexin; HSP90B1, heat shock protein 90 beta family member 1.

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http://dx.doi.org/10.1016/j.bbrc.2017.03.063
0006-291X/

mitochondrial inner membrane transporter that exports peptides from mitochondrial matrix to intermembrane space. The exported peptides then diffuse into cytosol. A group of exported peptides has been suggested to be trigger molecules that transduce the subsequent signaling pathway [13]. Human ABCB10 (ATP-binding cassette subfamily B member 10) is a putative ortholog of *C. elegans* HAF-1 and yeast MDL1 (multidrug resistance like-1), a transporter that exports peptides from mitochondria [14,15]. MDL1 and ABCB10 are involved in controlling oxidative stress status of cells [16,17]. Therefore, it is possible that ABCB10 has roles similar to that of HAF-1 in the UPR^{mt} signaling pathway. However, this possibility has not been reported.

In this study, ABCB10 was depleted from HepG2 cells by using small interfering RNA (siRNA), and the resulting mRNA expression profile was examined. ABCB10 depletion increased the expression of ROS-detoxifying enzymes (SOD2, GSTA1, and GSTA2), and SESN3, a protein induced by ROS to protect the cell from oxidative stress, and decreased expression of UPR^{mt}-related mitochondrial chaperones (HSPD1 and DNAJA3), and proteases (LONP1). However, the putative activity of ABCB10 to export peptides from mitochondria was not lost by ABCB10 depletion. Altogether, these data suggest that ABCB10 is involved in UPR^{mt} signaling pathway similar to that of HAF-1, although ABCB10 probably does not participate in peptide export from mitochondria.

2. Materials and methods

2.1. Reagents

All reagents were purchased from Sigma-Aldrich (Missouri, USA), Wako (Osaka, Japan), or Takara (Kyoto, Japan) unless otherwise stated.

2.2. Cell culture and transfection

HepG2 cells were cultured in growth medium (Dulbecco's modified Eagle's medium [DMEM] plus 10% fetal calf serum) at 37 °C under 5% CO_2 and 95% atmosphere. Lipofectamine RNAiMAX (Invitrogen, California, USA) and Opti-MEM I (Gibco, New York, USA) were used to transfect siRNA. The siRNAs to reduce ABCB10 mRNA (siRNA1: 5'-rArGrCrArArUrUrArCrUrGrGrUrArArArCrArArUrArUrGAG-3′, siRNA2: 5'-rGrGrArUrGrGrArArGrArArCrGrGrUrGrUrUrArGrUrUrATT-3′) and scrambled siRNA (for control) were purchased from Origene (Maryland, USA). After transfecting siRNA, the cells were cultured for 3 days, and used for following analysis.

2.3. Quantitative PCR

Total RNA was isolated from the cells using TRIzol reagent (Invitrogen, Carlsbad, California, USA), and was reverse transcribed with a PrimeScript RT reagent Kit (Takara Bio, Osaka, Japan) to produce cDNA pool. By using the cDNA as template, quantitative PCR was performed and analyzed with a LightCycler Nano (Roche, Basel, Switzerland). The oligonucleotides used for amplification of target genes are listed in Table 1. The abundance of the target gene was normalized to that of ACTB (β-actin) mRNA.

2.4. Immunoblot analysis

HepG2 cells were harvested with phosphate-buffered saline (PBS) containing 1 mM EDTA and then washed with PBS twice. The cells were lysed by adding PBS containing 1% Triton X-100 and centrifuged at 10,000 × *g* for 5 min at 4 °C. The supernatant was recovered and mixed with SDS-PAGE loading buffer. The proteins were separated by SDS-PAGE, and transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking with 5% skim milk, the membrane was used for the immunoblot analysis using ECL Western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, England). Immunoblotting was performed with *anti*-ABCB10 antibody (Proteintech, Illinois, USA) and *anti*-Hsc70 antibody (Santa Cruz Biotechnology, California, USA). Detection of carbonylated proteins by using *anti*-2,4-dinitrophenyl (DNP) antibody (SHIMA Laboratories, Tokyo, Japan) was performed as shown previously [18].

2.5. MitoSOX staining

HepG2 cells were cultured in growth medium for 3 days after transfection, and the cells were stained with MitoSOX, a superoxide indicator, and observed according to the manufacturer's protocol.

2.6. Isolation of mitochondria

HepG2 cells were harvested with PBS containing 1 mM EDTA and then washed twice with PBS. The cells were suspended in ice-cold mitochondrial isolation buffer (3 mM HEPES-KOH [pH 7.4], 0.21 M mannitol, 0.07 M sucrose, 0.2 mM EGTA), and homogenized with a Dounce homogenizer on ice. The homogenate was centrifuged at 500 × *g* for 5 min at 4 °C to precipitate intact cells and nuclei. The supernatant containing mitochondria was further centrifuged at 10,000 × *g* for 5 min at 4 °C to precipitate mitochondria, and the mitochondria were resuspended and washed three times by centrifugation in the mitochondrial isolation buffer.

2.7. Mitochondrial activity assay

Mitochondrial activity assay (Cytochrome *c* oxidase activity assay) kit (BioChain, California, USA) was used to measure the activities of isolated mitochondria. In brief, isolated mitochondria were solubilized by n-dodecyl ß-D-maltoside to obtain electron transport complex IV, and the cytochrome *c* oxidase activity of this complex was measured by optically monitoring the catalytic activity changing the reduced form of cytochrome *c* into its oxidized form.

2.8. Peptide release assay

Isolated mitochondria were suspended in the mitochondrial isolation buffer containing 1 mM ATP, and were incubated at 37 °C for 3 h. They were cooled on ice and then centrifuged at 10,000 × *g* for 5 min at 4 °C to remove mitochondria as a pellet. The recovered supernatant was subjected to NanoSep centrifugal device with 30 kDa molecular weight cutoff (MWCO) membrane (Pall Corporation, Michigan, USA) and centrifuged at 10,000 × *g* for 10 min at 4 °C. Then, the upper fraction that did not flow through 30 kDa MWCO membrane (probably containing >30 kDa proteins) was recovered as the ">30 kDa" fraction. The lower fraction (probably containing <30 kDa proteins) was recovered and then subjected to the device with 10 kDa MWCO membrane. After centrifugation, the upper fraction (probably containing 10–30 kDa proteins) was recovered as the "10–30 kDa" fraction, and the lower fraction (probably containing <10 kDa proteins) was recovered and subjected to the device with 3 kDa MWCO membrane. After centrifugation, the upper fraction (probably containing 3–10 kDa proteins) was recovered as the "3–10 kDa" fraction, and the lower fraction (probably containing <3 kDa peptides) was recovered as the "<3 kDa" fraction. The amount of proteins and peptides in each fraction was measured by using QuantPro BCA assay kit (Sigma-Aldrich).

Please cite this article in press as: M. Yano, ABCB10 depletion reduces unfolded protein response in mitochondria, Biochemical and Biophysical Research Communications (2017), http://dx.doi.org/10.1016/j.bbrc.2017.03.063