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Short Communication

Over-expression of methionine sulfoxide reductase A in the endoplasmic reticulum increases resistance to oxidative and ER stresses

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MsrA and MsrB catalyze the reduction of methionine-*S*-sulfoxide and methionine-*R*-sulfoxide, respectively, to methionine in different cellular compartments of mammalian cells. One of the three MsrBs, MsrB3, is an endoplasmic reticulum (ER)-type enzyme critical for stress resistance including oxidative and ER stresses. However, there is no evidence for the presence of an ER-type MsrA or the ER localization of MsrA. In this work, we developed an ER-targeted recombinant MsrA construct and investigated the potential effects of methionine-*S*-sulfoxide reduction in the ER on stress resistance. The ER-targeted MsrA construct contained the N-terminal ER-targeting signal peptide of human MsrB3A (MSPRRSLPRPLSLCLSLCLCLCLAAALGSAQ) and the C-terminal ER-retention signal sequence (KAEL). The over-expression of ER-targeted MsrA significantly increased cellular resistance to H_2O_2-induced oxidative stress. The ER-targeted MsrA over-expression also significantly enhanced resistance to dithiothreitol-induced ER stress; however, it had no positive effects on the resistance to ER stresses induced by tunicamycin and thapsigargin. Collectively, our data suggest that methionine-*S*-sulfoxide reduction in the ER compartment plays a protective role against oxidative and ER stresses.

Keywords methionine sulfoxide reductase; MsrA; endoplasmic reticulum; stress resistance

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Introduction

The methionine sulfoxide reduction system is an important pathway that repairs oxidatively damaged proteins, eliminates cellular reactive oxygen species (ROS), and regulates protein function. Methionine sulfoxide reductases (Msrs) are the enzymes responsible for this reduction system [1,2]. Two stereospecific Msr families, MsrA and MsrB, have evolved for the complete reduction of a mixture of methionine-(*R*,*S*)-sulfoxide residues in proteins. MsrA specifically reduces the *S*-form of methionine sulfoxide, whereas MsrB only acts on the *R*-form.

There are three *MsrB* genes in mammalian cells and each MsrB protein is targeted to different cellular compartments [3]. MsrB1 (a selenoprotein) is present in the cytosol and nucleus, MsrB2 localized in the mitochondria, and MsrB3 is targeted to the endoplasmic reticulum (ER). In contrast, a single *MsrA* gene exists in mammals [4], although multiple MsrA variants generated by alternative splicing have been reported in mammalian cells [5,6]. The most abundant MsrA form has a typical N-terminal mitochondrial targeting sequence [7], but is distributed in the mitochondria, cytosol, and nucleus [8–10]. Other known MsrA variants are targeted to either the cytosol and nucleus or the mitochondria [5,6,11]. Interestingly, there is no evidence for the localization of MsrA in the ER or the presence of an ER-targeted MsrA form.

An important function of the ER is to control protein folding. The perturbation of this ER function, such as inhibition of disulfide bond formation and glycosylation, results in the accumulation of unfolded or misfolded proteins in the ER lumen, causing ER stress [12]. In addition, the accumulation of cellular ROS generated by the ER stress can contribute to the acceleration of ER stress [13]. We previously found that the ER-type MsrB3 enzyme functions as an antioxidant to protect cells against oxidative stress [14–16]. Moreover, MsrB3 was found to protect mammalian cells and *Drosophila* against ER stress [15,16]. In this work, we generated an ER-targeted MsrA construct and investigated the effects of MsrA in the ER on resistance to oxidative and ER stresses.

Materials and Methods

Generation of an ER-targeted MsrA construct

A DNA fragment encoding residues 22–233 of mouse *MsrA* was PCR-amplified using a pET-based MsrA construct [9] and forward (5′-ACACGAATTCTGGGCGACTCAGCTT CGAA-3′) and reverse (5′-ACACTGTACATTAGAGCTCC

GCTTTTTTAATGGCCATCGGG-3′) primers. The reverse primer was designed to insert an 'AEL' sequence to the C-terminal end of MsrA. The amplified DNA fragment was cloned into the *Eco*RI/*Bsr*GI sites of pS3a-GFP [3] derived from pEGFP-N1. The pS3a-GFP construct contains the N-terminal 31-amino acid ER-targeting signal sequence of human MsrB3A. The resulting construct, designated pMsrA-ER, encoded MsrA with an N-terminal ER-targeting signal and a C-terminal ER-retention signal sequence (KAEL). The construct was verified by DNA sequencing.

Cell culture and transfection

NIH 3T3 and A549 cells were grown in Dulbecco's modified eagle medium and Roswell park memorial institute medium (Welgene, Daegu, Korea), respectively, supplemented with 10% fetal bovine serum and 100 U/ml penicillin-streptomycin antibiotics at 37°C in a 5% CO_2 incubator. Cells were transfected with pMsrA-ER, pMsrA [9], or an empty vector (pEGFP-N1) using Lipofectamine 2000 (Invitrogen, Carlsbad, USA). The pMsrA construct (derived from pEGFP-N1) encoded the full-length MsrA containing an N-terminal mitochondrial signal peptide.

Fluorescence confocal microscopy

Mouse fibroblast NIH 3T3 cells were transfected with pMsrA-ER using Lipofectamine 2000. At 24 h post-transfection, the cells were incubated with 0.3 μM ER-Tracker (Invitrogen) for 30 min at 37°C. For the immunofluorescence staining of MsrA, the cells were fixed with 4% paraformaldehyde in buffer A (phosphate buffered saline containing 0.9 mM $CaCl_2$, 0.52 mM $MgCl_2$, and 0.16 mM $MgSO_4$), washed three times with buffer A, and treated with 0.1% NP-40 in buffer A for 10 min at room temperature. MsrA proteins were stained with polyclonal anti-MsrA antibodies followed by secondary Alexa Fluor 546 goat anti-rabbit IgG antibodies (Invitrogen). The cells were imaged using a Leica TCS SP2 laser scanning confocal microscope (Leica Microsystems, Tokyo, Japan).

Western blot analysis

Cell extracts were prepared using CelLytic-M (Sigma-Aldrich, St Louis, USA) lysis buffer. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and probed with primary antibodies, followed by horseradish peroxidase-conjugated secondary antibodies. MsrA protein levels were analyzed using polyclonal anti-MsrA antibodies. C/EBP homologous protein (CHOP) was used as an ER stress marker protein and β-actin as a protein loading control.

MsrA enzyme assay

The reaction mixture (100 μl) contained 50 mM sodium phosphate (pH 7.5), 50 mM NaCl, 20 mM dithiothreitol (DTT), 200 μM dabsylated methionine-*S*-sulfoxide, and 200 μg crude protein from the cell extract. The reaction was carried out at 37°C for 30 min, and the reaction product (dabsyl-Met) was analyzed by an high-performance liquid chromatography procedure [17]. The chromatogram is shown in **Supplementary Fig. S1**.

Oxidative stress test

NIH 3T3 cells at 24 h post-transfection were treated with 0–500 μM H_2O_2 for 3 h. Cell viability was analyzed using an established colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-based assay. Briefly, 0.2% MTT was added into each well and incubated for 3 h at 37°C in the 5% CO_2 incubator. After removal of the medium, the resulting formazan was dissolved in dimethylsulfoxide. Absorbance was recorded at 540 nm using a microplate reader (Bio-Rad, Hercules, USA).

ER stress tests

NIH 3T3 cells at 24 h post-transfection were treated with 0–1 mM DTT (MP Biomedicals, Santa Ana, USA) for 6 h, 0–5 μg/ml tunicamycin (Sigma-Aldrich) for 12 h, or 0–1 μM thapsigargin (Tocris Bioscience, Bristol, UK) for 12 h. A549 cells at 48 h post-transfection were treated with 0–1 μM thapsigargin for 16 h. Cell viability was analyzed using the MTT assay.

Statistical analysis

Cell viability was analyzed using Student's *t*-test. Prism 5 software (GraphPad, San Diego, USA) was used for the statistical analysis of data. $P < 0.05$ was considered significant.

Results

Construction of functional ER-targeted MsrA

To investigate the roles of methionine-*S*-sulfoxide reduction in the ER, an ER-targeted recombinant mouse MsrA protein (MsrA-ER) was created (**Fig. 1A**). For this targeting, an N-terminal ER targeting sequence (MSPRRSLPRPLSLCL SLCLCLCLAAALGSAQ) from human MsrB3A [3] was applied and an ER retention signal sequence (KAEL) was introduced at the C-terminus. In fluorescence confocal microscopy, the recombinant MsrA-ER protein colocalized with ER-Tracker (**Fig. 1B**), demonstrating that MsrA-ER resides in the ER.

The over-expression of MsrA-ER in transfected NIH 3T3 cells was confirmed by western blot analysis through a comparison with endogenous MsrA levels in cells transfected with an empty vector (**Fig. 2A**). In addition, a native MsrA-expressing construct was also transfected as a positive control. The over-expression level of the native MsrA form was likely higher than the MsrA-ER form due to its cytosolic and mitochondrial expression.

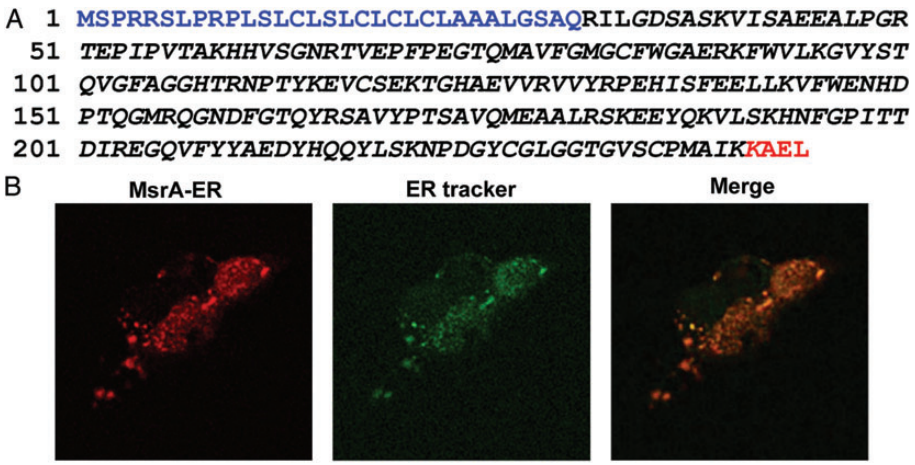

Figure 1. Construction and subcellular localization of MsrA-ER (A) Sequence of MsrA-ER. The N-terminal ER-targeting signal peptide of human MSRB3A is indicated in blue letters. An ER-retention signal sequence (KAEL) is indicated in red letters. The mouse MsrA sequence (residues 22–233) is indicated in italics. The C-terminal end residue of native MsrA is Lys. (B) ER localization of MsrA-ER. NIH 3T3 cells were transfected with pMsrA-ER. Images at 20 h post-transfection were taken under a confocal microscope ($\times$100).

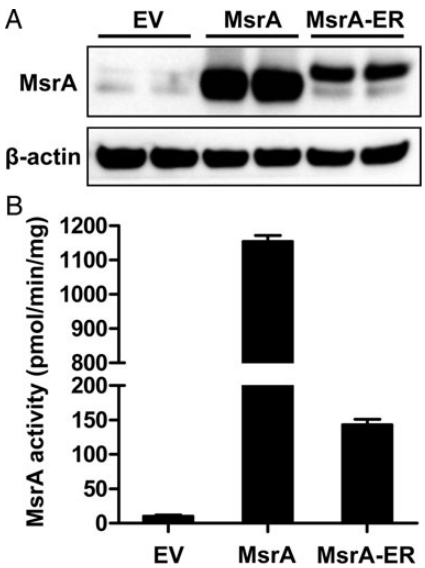

Figure 2. Over-expression of MsrA-ER NIH 3T3 cells were transfected with an empty vector (EV), pMsrA, or pMsrA-ER for 24 h. (A) Western blot analysis of MsrA protein levels. (B) MsrA activity assay.

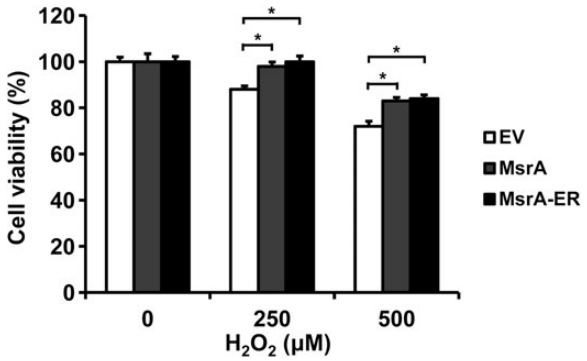

Figure 3. Effect of MsrA-ER over-expression on oxidative stress resistance NIH 3T3 cells were transfected with pMsrA-ER, pMsrA, or EV for 24 h. The transfected cells were then treated with H_2O_2 for 3 h at the indicated concentrations. Cell viability was analyzed by the MTT assay. Data are shown as mean $\pm$ SEM from three experiments (*$P < 0.05$).

The functionality of the MsrA-ER was confirmed using the MsrA enzyme assay (**Fig. 2B**). Cells over-expressing MsrA-ER showed a 14-fold increase in MsrA activity at 24 h post-transfection compared with cells transfected with an empty vector. In addition, cells over-expressing native MsrA exhibited a higher increase in MsrA activity compared with cells over-expressing MsrA-ER. The MsrA assay data were thus consistent with the western blot data.

Protective effect of ER-targeted MsrA against oxidative stress

The effect of MsrA-ER over-expression on oxidative stress resistance was tested. When treated with H_2O_2, NIH 3T3 cells over-expressing MsrA-ER showed significantly increased viability compared with cells transfected with an empty vector (**Fig. 3**). The protective effect of MsrA-ER against H_2O_2-induced cell death was similar to the native MsrA protein. These results indicated that over-expression of MsrA in the ER can protect against oxidative stress.

Effects of ER-targeted MsrA on ER stress resistance

The effect of MsrA-ER over-expression on ER stress resistance was also tested. NIH 3T3 cells over-expressing MsrA-ER exhibited a significantly increased resistance to DTT-induced ER stress, compared with cells transfected with an empty vector (**Fig. 4A**). MsrA-ER over-expression enhanced the cell viability by 24% and 33% after treatment with 0.5 and 1 mM DTT, respectively. Interestingly, the over-expression of native MsrA also significantly increased the cell viability after 0.5 and 1 mM DTT treatments.

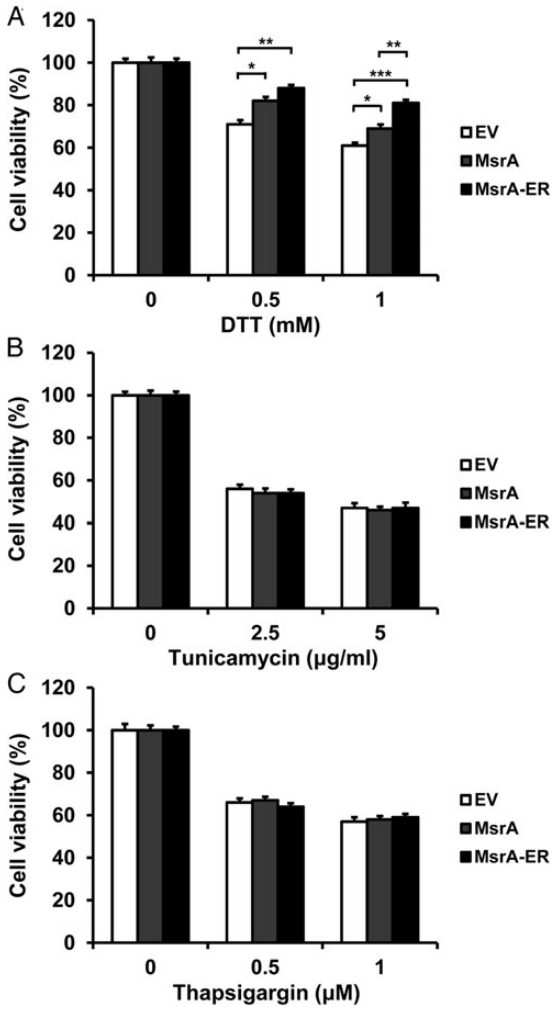

Figure 4. Effects of MsrA-ER over-expression on ER stress resistance in NIH 3T3 cells Cells were transfected with pMsrA-ER, pMsrA, or EV for 24 h. The transfected cells were then treated with DTT for 6 h (A), tunicamycin for 12 h (B), or thapsigargin for 12 h (C) at the indicated concentrations. Cell viability was analyzed by the MTT assay. Data are shown as mean ± SEM from three experiments (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

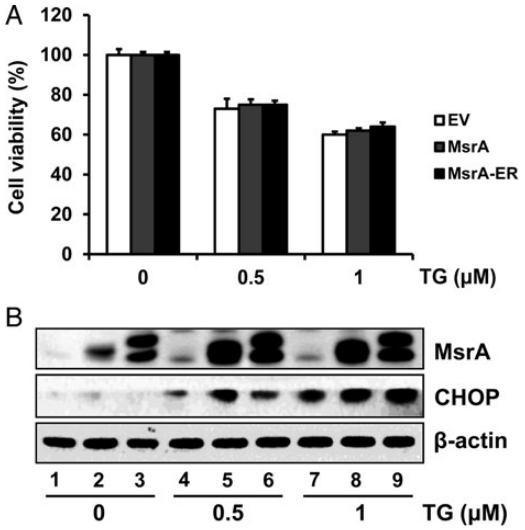

Figure 5. Effect of MsrA-ER over-expression on thapsigargin-induced ER stress resistance in A549 cells Cells at 48 h post-transfection with pMsrA-ER, pMsrA, or EV were treated with thapsigargin (TG) for 16 h at the indicated concentrations. (A) Cell viability analyzed by the MTT assay. Data are shown as mean ± SEM from three experiments. (B) Western blot analysis. CHOP was used as an ER stress marker protein. Lanes 1, 4, and 7: EV transfection; Lanes 2, 5, and 8: pMsrA transfection; Lanes 3, 6, and 9: pMsrA-ER transfection.

However, the protective effect was more profound in cells over-expressing MsrA-ER than in cells over-expressing native MsrA.

Whether the ER-targeted MsrA could protect cells from other ER stress agents, tunicamycin and thapsigargin, was also tested. MsrA-ER over-expression did not increase tunicamycin-induced ER stress resistance (**Fig. 4B**). In addition, MsrA-ER over-expression did not show a positive effect on thapsigargin-induced ER stress resistance (**Fig. 4C**). Also, native MsrA over-expression did not have a positive effect on the resistance to ER stresses induced by tunicamycin and thapsigargin.

We previously found that the over-expression of an ER-type MsrB (MsrB3) protects A549 cells against ER stress [16]. Human MsrB3A over-expression led to an increased resistance to cell death induced by DTT and thapsigargin, but had no positive effect on the resistance to tunicamycin-induced cell death. The effect of MsrA-ER over-expression against thapsigargin-induced ER stress was also tested in A549 cells after thapsigargin treatment. No positive effect of MsrA-ER over-expression against thapsigargin-induced ER stress was observed in A549 cells (**Fig. 5A**). The thapsigargin-induced ER stress was confirmed by increased levels of CHOP, an ER stress marker protein (**Fig. 5B**). Collectively, the data suggest that MsrA over-expression in the ER protects cells from DTT-induced ER stress.

Discussion

MsrA specifically reduces free and peptidyl methionine-*S*-sulfoxide to methionine. The cyclic oxidation/reduction of methionine has an important antioxidant role in cells [18,19] and MsrA functions as a crucial antioxidant enzyme in cells [20,21]. In addition, MsrA modulates specific methionine sulfoxidation involved in the function of proteins such as calcium/calmodulin-dependent protein kinase II [22] and the shaker potassium channel [23]. In mammalian cells, MsrA proteins are expressed from a single gene and localized to the cytosol, nucleus, and mitochondria. There is no evidence, however, for the ER localization of MsrA.

We recently found that methionine-*R*-sulfoxide reduction in the ER is critical for the resistance to a variety of stresses, including oxidative and ER stresses [15,16]. In this study, the ability of methionine-*S*-sulfoxide reduction in the ER to protect

against oxidative and ER stresses was examined. An ER-targeted recombinant MsrA form (MsrA-ER) was developed with N-terminal ER-targeting and C-terminal ER-retention signal sequences. The over-expression of MsrA-ER in mammalian cells resulted in significantly increased viability against H_2O_2-induced oxidative stress. Thus, our data suggest that MsrA in the ER is critical for oxidative stress reduction, similar to its function in the cytosol and mitochondria.

DTT is a strong reducing agent that blocks protein disulfide bond formation in the ER (inducing the unfolded protein response) and perturbs cellular redox homeostasis. Thapsigargin inhibits calcium influx into the ER, affecting the calcium homeostasis in the ER crucial for ER functions including proper protein folding. ER-type MsrB3 over-expression was previously found to protect cells against ER stresses induced by DTT and thapsigargin [16]. In this study, MsrA over-expression in the ER enhanced resistance to DTT-induced ER stress, but had no positive effect on thapsigargin-induced ER stress resistance. Therefore, our data suggest that methionine-*S*-sulfoxide reduction in the ER plays a protective role against DTT-induced ER stress and is associated with redox homeostasis rather than calcium homeostasis.

Supplementary data

Supplementary data is available at *ABBS* online.

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