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Protection of L-methionine against H_2O_2-induced oxidative damage in mitochondria

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ABSTRACT

Reactive oxygen species (ROS) is reported to be a critical pathogenic factor and mitochondria is one of the susceptible subcellular organs for oxidative damage. Methionine sulfoxide reductase A (MsrA) is a key anti-oxidant enzyme associated with cytoprotection and previous reports have revealed its importance in mitochondrial function. The anti-oxidation of MsrA is due to Met-centered redox cycle, suggesting that Met-centered redox cycle may play a critical role in mitochondrial protection. L-Methionine (L-Met), a natural amino acid with anti-oxidation activity, can mimic the effect of Met-centered redox cycle. Here, we investigated the protection of L-Met on H_2O_2-induced oxidative damage in mitochondria. Our study demonstrated that L-Met protected H_2O_2-induced injury in CHO cells. Cytoprotections of L-Met at low concentrations (1–5 mM) were abolished by dimethyl sulfoxide (DMSO), a competitive inhibitor of MsrA function, suggesting that these effects may involve the participation of MsrA. Overexpression of MsrA in CHO cells protected mitochondria from H_2O_2-induced downtrend of membrane potential and production of mitochondrial superoxide. Pre-treatment with L-Met (1 mM) produced a similar effect on the mitochondrial protection against H_2O_2. Furthermore, it was observed that topical application of L-Met can prevent 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA)-induced oxidative damage in the skin of mice. These results suggest that anti-oxidation activity of L-Met may promise a new strategy for the prevention of oxidative stress-induced damage.

1. Introduction

Mitochondrial dysfunctions, including impaired electron transfer, decreased membrane potential and ultrastructural damage, are concomitant with many diseases, such as Alzheimer's disease and Parkinson's disease. (Bueler, 2009; Chen and Yan, 2010). The excessive generation of reactive oxygen species (ROS) may lead to alterations of mitochondria, which are susceptible to oxidative damage (Fetisova et al., 2010; Gibson et al., 2008; Mandelker, 2008). Endogenous anti-oxidant systems are largely responsible for protection of mitochondria against ROS. For instance, methionine sulfoxide reductase A (MsrA), a key anti-oxidant enzyme that protect different cell types from oxidative stress, including embryonic stem cells, vascular smooth muscle cells and PC12 cells (Haenold et al., 2008; Moskovitz et al., 2001; Yermolaieva et al., 2004; Zhang et al., 2010), has been revealed its presence in the mitochondria (Brennan et al., 2010; Hansel et al., 2002). Silencing of MsrA results in loss of mitochondrial membrane potential and increased reactive oxygen species production in human lens cells (Marchetti et al., 2006). Recent study showed that the morphology of mitochondria in heart was significantly changed in MsrA gene knockout mice (Nan et al., 2010). These findings indicate that MsrA may contribute to the prevention and repairing of mitochondrial dysfunction caused by ROS attacking (Hansel et al., 2002; Vougier et al., 2003). Thus, it is desirable to identify agents to regulate the function of MsrA for prevention of mitochondria dysfunction.

MsrA-catalytic anti-oxidation depends on the Methionine (Met)-centered redox cycle (Moskovitz, 2005; Sun et al., 1999; Weissbach et al., 2002), which is due to the anti-oxidation activity of protein-bound Met residues. Considering the importance of MsrA in mitochondrial function, Met-centered redox cycle may play a critical role in the protection of mitochondria against ROS. A variety of ROS reacts with Met residues to form methionine sulfoxide (MetO), followed by MetO reductases (Msrs)-mediated reduction back to Met (Luo and Levine, 2009). This Met-centered cycle can scavenge free radicals and protect cells. Thus, Met residues in proteins are critical for the regulation of intracellular redox status as anti-oxidants. It has been demonstrated that L-Met, a natural amino acid with anti-oxidation activity, can also be oxidized to MetO and reduced by MsrA, prevents oxidation of protein-bound Met. (Wood et al., 2009) L-Met can mimic the anti-oxidation of

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Met-centered redox cycle via increasing the concentration of substrate. Here, we further asked that whether L-Met can protect CHO cells against. H_2O_2-induced cellular oxidative stress and alleviate the toxic effects of H_2O_2 in mitochondria.

2. Materials and methods

2.1. Chemicals and materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum and G418 were purchased from Gibco Invitrogen Corporation (Carlsbad, CA, USA). Trypsin, DMSO, L-Met, 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) and methylthiazolyldiphenyl-tetrazolium bromide (MTT) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Lipofectamine 2000 was obtained from Invitrogen. 5,5,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1) was obtained from Molecular Probes (Eugene, OR, USA). MitoSOX Red (3,8-phenanthridinediamine, 5-(6-triphenylphosphoniumhexyl)-5,6-dihydro-6-phenyl) was purchased from Invitrogen (Invitrogen Life Technologies, CA, USA). Plasmid containing rat MsrA cDNA (pcDNA3.1-rMsrA) was kindly provided by Dr. Bertrand Friguet (Université Paris 7-Denis Diderot, France). pcDNA3.1 vector was kindly provided by Professor Jiu-ping Ding (Huazhong University of Science and Technology, China). Primary antibodies to MsrA were purchased from Upstate-cell Signaling Solutions (NY, USA). All the other reagents were of analytical grade. Hydrogen peroxide (H_2O_2) was purchased from Merck (Darmstadt, Germany). All the chemicals were dissolved in water to make individual stock solutions, depending upon their water solubility, and stored at −20 °C.

2.2. Cell culture models of oxidative stress

Chinese hamster ovary (CHO) cell line were obtained from Chinese Type Culture Collection and the CHO cells were cultured in 1640 medium containing 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. The cells were grown in 6-well cell culture plate and maintained at 37 °C with 5% CO_2 atmosphere and subcultured every 2–3 days. For oxidative stress, cells were rinsed twice with PBS and treated with H_2O_2 (250 μM) in DMEM. Different concentrations of Met (0.125, 0.25, 0.5, 1 mM) were added to the culture medium 2 h prior to oxidative stress until the end of H_2O_2 treatment of indicated time.

2.3. Stable transfection of CHO cell line

CHO cells were transfected with plasmid pcDNA3.1(+)-rMsrA and pcDNA3.1(+) as control using the liposome transfection reagent (Lipofectamin 2000, Invitrogen, Carlsbad, CA, USA). To select stable transfectants, cells were grown in complete medium supplemented with G418 antibiotics (600 μg/mL) for 1 week and then G418 (400 μg/mL) for 2 weeks. Then, stable transfectants were tested by immunoblot analysis and were used for the analysis of mitochondrial membrane potential by flow cytometry assay after H_2O_2 treatment.

2.4. Western blotting

For CHO cells transfected with plasmid pcDNA3.1(+)-rMsrA and pcDNA3.1(+), after harvesting by digestion and centrifugation, the collection was homogenized in ice-cold extraction buffer containing 50 mM Tris–base (pH 7.4), 100 mM NaCl, 1% NP-40, 10 mM EDTA, 20 mM NaF, 1 mM PMSF, 3 mM Na_3VO_4 and protease inhibitors. The homogenates were centrifuged at 12,000g for 15 min at 4 °C. Supernatant was separated and protein concentration was determined by using the BCA protein assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA). Protein samples (30 μg) were separated by 10% SDS–polyacrylamide gel and then transferred to nitrocellulose membranes. After blocking with 5% nonfat milk for 1 h at room temperature, transferred membranes were incubated overnight at 4 °C with different primary antibodies (rabbit anti-MsrA, 1:200 dilution, Upstate). Following three washes with TBST, membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (1:4000) for 1 h at room temperature. After repeated washing, membranes were reacted with enhanced chemiluminescence reagents (Amersham Pharmacia Biotech, Inc., Piscataway, NJ, USA) for 3 min, and visualized with X-ray films (Kodak X-Omat, Rochester, NY, USA). The films were scanned and the optical density of the bands was determined using Optiquant software (Packard Instrument). Normalization of results was ensured by running parallel Western blots with β-actin.

2.5. MTT assay

After treatment with or without H_2O_2 (250 μM) for 12 h, cell viability of transfectants was measured by MTT assay. Cell cultures were incubated with MTT solution (5 mg/mL) for 4 h at 37 °C. Then, the medium was discarded and DMSO was added to solubilize the product formazan by shaking for 15 min. Absorbance was measured at 492 nm by a microplate reader (ELx800, Bio-Tek, Winooski, VT). Cell viability of control group was defined as 100%. Cell viability was expressed as a percent to the control value.

2.6. Measurement of mitochondrial membrane potential ($\Delta\psi m$)

To measure the mitochondrial $\Delta\psi m$, the lipophilic cationic probe JC-1 was used. JC-1 exhibits potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green (530 nm) to red (590 nm). Mitochondria depolarization is specifically indicated by a decrease in the red/green fluorescence intensity ratio. After treatment with or without H_2O_2 (250 μM) for 3 h, cells (1×10^6/3 mL in six-well plates) were rinsed with PBS and harvested by centrifugation at 800*g* for 10 min, whereas adherent cells were trypsinized and subsequently collected by centrifugation. The cells were washed with PBS and resuspended in 500 μl PBS. The cells were loaded for 20 min at 37 °C with JC-1 (10 μg/mL), and then measured through a FL-1 filter (530 nm) and a FL-2 filter (585 nm), respectively, on BD-LSR flow cytometer using Cell Quest software. Positioning of quadrants on JC-1 green/JC-1 red dot plots was performed using Cell Quest software. The upper right quadrant (Q2) represented live cells with normal mitochondrial membrane potential (JC-1 green +/JC-1 red +) and the lower right quadrant (Q4) represented cells with mitochondrial injury (JC-1 green −/JC-1 red +).

2.7. Measurement of mitochondrial superoxide level in live CHO cells

MitoSOX™ red reagent, which is oxidized by superoxide and exhibits red fluorescence in the mitochondria, (Li et al., 2011) was used to localize the mitochondrial superoxide level in live CHO cells. A 5 mM MitoSOX™ reagent stock solution was prepared by adding 13 μl of DMSO to one vial of MitoSOX™ mitochondrial superoxide indicator (50 μg). Next, 1.0 mL of 5 μM MitoSOX™ reagent working solution was applied to CHO cells which were adhered to cover slips and incubated for 10 min at 37 °C. Finally, the cells were gently washed with warm buffer and viewed under a Fluorescent microscope (Olympus, Japan).

2.8. In vivo evaluation of the efficacy of L-Met against damage induced by TPA

In vivo experiments were performed in hairless male KunMing (KM) mice (25–27 g) at 3 months of age described by Sharma and Sultana (2004) All animal care and experimental procedures complied with local and international guidelines on ethical use of animals and were approved by The University Animal Welfare Committee, Tongji Medical College, Huazhong University of Science & Technology. L-Met (4 mM) was dissolved into normal saline. To study the effect of L-Met on TPA-mediated cutaneous oxidative stress, 28 mice were randomly allocated to 4 groups of seven mice each. Groups: (1) control mice treated by 0.2 mL normal saline and 0.2 mL acetone (vehicle); (2) mice treated by 0.2 mL L-Met (4 mM) + 0.2 mL acetone (vehicle); (3) mice treated by 0.2 mL normal saline and 0.2 mL TPA (20 nmol/0.2 mL acetone); (4) mice treated by 0.2 mL L-Met (4 mM) + 0.2 mL TPA (20 nmol/0.2 mL acetone). Mice were treated with normal saline or L-Met by topical application and 2 h later received the topical application of TPA. Mice were sacrificed after 12 h of TPA stimulus, and the skin tissues were excised and washed with ice-cold normal saline.

2.9. Biochemical analysis

Protein-binding thiol oxidation analysis was quantified spectrophotometrically using the DTNB method described by Morgan et al. (2005) with some improvements. The skin tissues of mice were homogenized in ice-cold extraction buffer (10 μl/mg) containing 50 mM Tris–base (pH 7.4), 100 mM NaCl, 1% NP-40, 10 mM EDTA, 20 mM NaF, 1 mM PMSF, 3 mM Na_3VO_4 and protease inhibitors. After being sonicated 30 s for four times, the homogenates were centrifuged at 12,000*g* for 15 min at 4 °C. For protein-binding thiol oxidation analysis, supernatant was separated by centrifugal ultrafiltration at 4000*g* for 30 min at 4 °C. After centrifugal ultrafiltration, the endogenous small molecules with thiols were removed. Adaptive volume of 100 mM phosphate buffer, pH 7.4, was added into the protein sample and the protein concentration was determined using the BCA protein assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA). Two milligram of proteins in each sample was added either 500 μl freshly prepared 500 μM DTNB in 100 mM phosphate buffer, pH 7.4, or 500 μl buffer alone. Following incubation in dark for 30 min at 37 °C, absorbance at 412 nm was measured with a microplate reader. The OD value of the control group was defined as 100%. The absorbance of samples without DTNB was subtracted to account for background absorbance at 412 nm. The extent of lipid peroxidation was evaluated by measuring thiobarbituric acid reactive substances at 535 nm, as described by Mihara and Uchiyama (1978). Sample supernatants were incubated with an equal volume of 1% TBA in a boiling water bath for 30 min. The results were expressed as malondialdehyde (MDA) equivalents per mg of protein. The MDA content of control group was defined as 100%.