

S-methyl-L-cysteine Protects against Antimycin A-induced Mitochondrial Dysfunction in Neural Cells via Mimicking Endogenous Methionine-centered Redox Cycle*

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Summary: Mitochondrial superoxide overproduction is believed to be responsible for the neurotoxicity associated with neurodegeneration. Mitochondria-targeted antioxidants, such as MitoQ, have emerged as potentially effective antioxidant therapies. Methionine sulfoxide reductase A (MsrA) is a key mitochondrial-localized endogenous antioxidative enzyme and it can scavenge oxidizing species by catalyzing the methionine (Met)-centered redox cycle (MCRC). In this study, we observed that the natural L-Met acted as a good scavenger for antimycin A-induced mitochondrial superoxide overproduction in PC12 cells. This antioxidation was largely dependent on the Met oxidase activity of MsrA. S-methyl-L-cysteine (SMLC), a natural analogue of Met that is abundantly found in garlic and cabbage, could activate the Met oxidase activity of MsrA to scavenge free radicals. Furthermore, SMLC protected against antimycin A-induced mitochondrial membrane depolarization and alleviated 1-methyl-4-phenylpyridinium (MPP⁺)-induced neurotoxicity. Thus, our data highlighted the possibility for SMLC supplement in the detoxication of mitochondrial damage by activating the Met oxidase activity of MsrA.

Key words: methionine sulfoxide reductase A; Met oxidase; S-methyl-L-cysteine; neurotoxin; 1-methyl-4-phenylpyridinium

Reactive oxygen species (ROS), especially the free radicals, are implicated in the toxic mechanism of neurotoxins, including rotenone^[1-3], lipopolysaccharide^[4] and 1-methyl-4-phenylpyridinium (MPP⁺)^[5], etc. Mitochondria are key cell organelles that control many processes by maintaining energy production. To produce energy, the mitochondrial electron transport chain is coupled with the production of ROS, such as superoxide anion or hydrogen peroxide. Mitochondrial superoxide overproduction is considered to be the major factor in oxidative toxicity

associated with neurodegeneration. Mitochondria-targeted antioxidants, such as MitoQ and mitotempo, have emerged as potentially effective antioxidant therapies^[6-8]. However, mounting evidence indicates that the mitochondrial ROS are related with multiple essential cellular functions^[9,10] and these mitochondria-targeted antioxidants may disrupt physiological signaling^[11,12].

As a result of the constant production of ROS, mitochondria are protected by various antioxidant systems, such as glutathione reductase, thioredoxin reductase and manganese superoxide dismutase^[13]. These antioxidant systems only diminish overproduced mitochondrial ROS, but not affect ROS signaling apt for physiological signaling. The thiomethyl group that exists on the surface exposed methionine (Met) residues has been recognized as a key endogenous antioxidant defense^[14-17]. Methionine in proteins is often thought to be a generic hydrophobic residue on the surface. A variety of ROS react with Met residues to form methionine sulfoxide (MetO), which is followed by MetO reductases-mediated reduction back to Met,

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named Met-centered redox cycle (MCRC)^[15, 17]. As a key member of MetO reductases, MetO reductase A (MsrA) has been reported to be abundantly localized in the mitochondrion^[18–20]. MsrA repairs oxidative damage of proteins and increases resistance of cells against many toxins, including zinc, hydrogen peroxide, 6-hydroxydopamine and acetaminophen^[21–25]. MsrA also reduces mitochondrial ROS levels in response to insults^[24, 26] and protects mitochondrial dysfunctions in a mouse model of Alzheimer's disease (AD)^[27]. Although its action via repairing oxidized proteins is easily accepted, the mechanism by which MsrA scavenges ROS remains largely elusive. MsrA has historically been considered a reductase, not an ROS scavenger, for the reaction of Met with oxidizing species was widely viewed as a natural process. Recent studies from our lab have indicated that the antioxidant capability of MsrA may involve a Met oxidase activity that facilitates the reaction of Met with ROS, which may underlie the effect of MsrA on neuroinflammation and lifespan^[28, 29].

Exogenous substrates like L-methionine (L-Met) could activate the Met oxidase activity^[29], but dietary supplementation with L-Met is not applicable for mitochondrial dysfunction, because Met is rapidly incorporated into protein or metabolized, and high dietary Met intake confers an increased risk of acute coronary events in patients with cardiovascular diseases^[30, 31]. S-methyl-L-cysteine (SMLC) is a hydrophilic cysteine-containing compound naturally found in *Allium* plants such as garlic and onion^[32]. Previous studies reported its safety and benefits, including no adverse effect and toxicity in mammals, lowering of the serum cholesterol level, a delay in diabetes pathogenesis and prevention in PD symptoms^[33–36]. Furthermore, SMLC has been revealed as a substrate in the catalytic antioxidant system mediated by MsrA^[36]. In the present study, we found that SMLC protected against antimycin A-induced mitochondrial dysfunction in neural cells via activating Met oxidase activity of MsrA and mimicking endogenous Met-centered redox cycle.

1 MATERIALS AND METHODS

1.1 Chemicals and Materials

Dulbecco's modified Eagle's medium (DMEM), horse serum, fetal bovine serum and G418 were purchased from Gibco Invitrogen Corporation (USA). Trypsin, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich (USA). Plasmid containing rat MsrA cDNA (pcDNA3.1-rMsrA) was provided courtesy of Dr. Bertrand Friguet of the Université Paris 7-Denis Diderot. L-Met, SMLC and dithiothreitol (DTT) were purchased from

Sigma-Aldrich (USA). All the other reagents were of analytical grade. Amicon ultra-4 centrifugal filter devices were purchased from Millipore Carrigtwohill, Co. Cork (Ireland). H₂O₂ was purchased from Merck (Germany). All the chemicals were dissolved in water to make individual stock solutions, depending upon their water solubility, and stored at –20°C. Other general agents were available commercially. Distilled water was deionized in a Milli-Q SP reagent water system (Millipore, USA).

1.2 Preparation of Rat Recombined MsrA

The rat recombinant MsrA was prepared and purified from *E. coli* BL21, as described in our previous report^[37]. In brief, MsrA coding region was ligated into the restricted pET-32a (+) vector using phage T4 DNA ligase, and BL21 cells were transformed with the recombinant plasmid and grown in LB medium. The cells were harvested, and applied to a Ni-Trap nickel-chelating column. The N-terminal His-tag of recombinant protein was removed using a Thrombin kit. The activity of recombinant MsrA was monitored by detecting both total MetO reducing activity and methyl sulfoxide-initiated oxidation of DTT.

1.3 Electron Paramagnetic Resonance (EPR)

EPR was performed as described in our previous reports with some modifications^[28, 29]. DMPO was used as a free radical trapper. EPR signals were detected with a Bruker e-scan EPR spectrometer (Bruker, Germany). We produced hydroxyl radical (OH·) by the Fenton reaction in the mixture of 0.25 mmol/L H₂O₂ and 0.1 mmol/L FeSO₄ in the presence of 0.1 mmol/L DMPO. The spin traps, L-Met (0.1 or 1 mmol/L), SMLC (0.1, 1 or 10 mmol/L) and rMsrA/non-active rMsrA (1 μmol/L) were added before the Fe (II) and H₂O₂. Samples (20 μL) were loaded into a quartz tube and the EPR spectra were recorded at room temperature. The EPR microwave power was set at 4.88 mW. The modulation frequency was 9.76 GHz. The time constant was 81.92 ms. The conversion time was 81.92 ms and a sweep time of 41.94 s was used. Each sample was scanned once. A sweep width of 100 G was used for experiments with DMPO. The receiver gain was set at 3.17 × 10³. Simulation and fitting of the EPR spectra were performed using the Bruker WinEPR program.

1.4 Cell Cultures and Treatment Paradigm

Cells were cultured as described in our previous reports with slight modifications^[37, 38]. Rat PC12 cells were obtained from Chinese Type Culture Collection and have been differentiated to be neuron-like by treating with nerve growth factor. PC12 cells were cultured in DMEM containing 5% heat-inactivated fetal calf serum, 10% heat-inactivated horse serum, 100 μg/mL streptomycin and 100 U/mL penicillin in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. The culture medium was changed and the cells were passaged by trypsinization every 3 to 4 days. For

oxidative stress, cells were rinsed twice with PBS and subjected to H₂O₂ (250 μmol/L) in DMEM. Different concentrations of L-Met were added to the culture medium 12 h prior to oxidative stress until the end of H₂O₂ treatment.

The human dopamine neuronal cell line, SH-SY5Y, was obtained from Cell Centre of Chinese Academy of Medical Sciences (China) and cryopreserved after subculturing various passages. Each subculture was limited to no more than 10 passages. SH-SY5Y cells (passages 30–40) were cultured in DMEM/F12 medium containing 10% heat-inactivated FBS, 50 U/mL penicillin and 50 mg/mL streptomycin in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. Medium was changed every 2 days, and cells were passaged at 80% confluence every 3–4 days. In the experiments, cells were synchronized at the G₀ phase by serum deprivation. Different concentrations of L-Met and SMLC in DMEM/F12 medium without FBS were used to treat SH-SY5Y cells for 12 h. After preconditioning, a final concentration of MPP⁺ (2 mmol/L) was used to treat SH-SY5Y cells for 48 h. MPP⁺ treatments were performed in DMEM/F12 medium without FBS under dark condition to avoid photo-catalytic decomposition reaction.

1.5 ROS Staining

The total intracellular ROS were determined by the MitoSOX red assay. In brief, PC12 cells were seeded in a 6-well plate and loaded with MitoSOX red (Invitrogen, USA) for 1 h, which was followed by pretreatment with L-Met (0.1, 0.5, and 1 mmol/L) or SMLC (0.1, 0.5, and 1 mmol/L) for 30 min, and then treatment with antimycin A (100 ng/mL) (Sigma-Aldrich, USA). After incubation at 37°C for 30 min, the fluorescence images were immediately taken using a fluorescence microscope (System Microscopy IX70; Olympus, Japan). For the detection of cellular superoxide, we used 5 μmol/L MitoSOX (Invitrogen, USA), and took images using excitation and emission filters of 543 and 565 nm, respectively. Fluorescent signals intensities of cells were obtained using Image-Pro Plus (IPP) software.

1.6 Construction of Short Hairpin RNA (shRNA) Lentiviral Expression Vector and MsrA Knockdown

shRNA lentiviral expression vector was constructed as described in our previous reports with some modifications^[29]. A third generation of self-inactivating lentivirus vector (GeneChem, China) containing a CMV-driven GFP reporter and a U6 promoter upstream of the cloning sites (*EcoR* I and *Age* I) was used for cloning MsrA shRNA. The target sequence for control scrambled shRNA was 5'-TTCTCCGAACGTGTCACGT-3' and rat MsrA was 5'-AGCACGTCAGCTTTGAGGA-3'. PC12 cells were infected with lentivirus at a multiplicity of infection (MOI) of 20 for 10 h. Then, the medium

was replaced with fresh complete medium. After 72 h, cells were observed under the fluorescence microscope to confirm that more than 80% of cells were GFP-positive. The knockdown of MsrA was evaluated by Western blotting.

1.7 Mitochondrial Membrane Potential

To determine the mitochondrial membrane potential, PC12 cells were seeded in a 6-well plate and incubated with 2 μg/mL JC-1 at 37 °C for 30 min, which was followed by pretreatment with L-Met (1 mmol/L) or SMLC (1 mmol/L) for 30 min, and then treatment with antimycin A (100 ng/mL). After incubation at 37°C for 30 min, cells were then washed twice with PBS and immediately examined by fluorescence spectrometry using a fluorescence microplate reader (System Microscopy IX70; Olympus, Japan). A 488 nm filter was used for excitation of JC-1, and emissions at 535 and 595 nm were used to quantify the population of mitochondria with green (JC-1 monomers) and red (JC-1 aggregates) fluorescence respectively. The red/green ratio was used to reflect the mitochondrial membrane potential.

1.8 MTT Assay

Cell viability was measured by MTT assay based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenases. In live cells, mitochondrial enzymes have the capacity to transform MTT to insoluble formazan. Cell cultures were incubated with MTT solution (5 mg/mL) for 4 h at 37°C. Following this, the medium was discarded and DMSO was added to solubilize the reaction product formazan by shaking for 15 min. Absorbance at 492 nm was measured with a microplate reader (ELx800, Bio-Tek, USA). Cell viability of vehicle-treated control group was defined as 100%. Cell viability was expressed as a percentage of the value in control cultures.

1.9 MsrA Homology Modeling

MODELLER suite (version 9.11, Sali & Blundell 1993) was used to construct the model of rat MsrA, and the template was a known complex of Met sulfoxide reductase A from *Neisseria meningitidis* (PDB ID 3BQF) as described previously^[29]. Then residue CYS72 of MsrA was modified to its oxide state, just called CYO72 here, and the modified-MsrA was named CYO72-MsrA for convenience. The coarse model of CYO72-MsrA was then refined by a 12 ns standard molecular dynamics in AMBER 12 (Case 2012b) until the ensemble attained its equilibrium state to get the stable conformation of CYO72-MsrA. During the molecular docking process, the CYO72-MsrA was solvated in a truncated octahedron box with 0.1 mol/L NaCl and several other Na⁺ ions added to counter solute charges. L-Met was prepared with academic maestro 9.6 from DESMOND molecular dynamics suite and minimized energetically for further studies. According to the known crystal structure of

MsrA (PDB ID 3BQF), all of the ligands were docked manually to the active site of CYO72-MsrA to form various complexes, and the S-Me group was consistent with that of 3BQF structure. Again, all of complexes were refined in AMBER 12 with explicit solvent environment and the same ion strength mentioned above. After that, the ligands were pulled 16 angstroms away from the active site to obtain the starting structures for later Steered Molecular Dynamics simulations.

To distinguish the difference of action between ligands and CYO72-MsrA, steered molecular dynamics (SMD) method was conducted for those starting structures prepared above. First, those structures were solvated with similar conditions as described before. One minimization was then done with restraint on solutes following one non-restraint minimization to get rid of steric clashes inside the ensembles. Next, all of those systems were heated within 200 ps from 0 K to 300 K with weak restraints on solutes to prevent ligands shifting away from enzyme. After heating those systems completely, NVT simulations were arranged to all systems at 300 K. All of NVT simulations were sustained for a longer period of 10 ns with time step 2 fs and 5 000 000 steps allegor. Finally, several one-ns SMD simulations were performed for every ligand, and they pulled the ligands into the active site of CYO72-MsrA from 16 angstroms to their own equilibrated states of complexes. In the meantime backward pulling simulations were done to pull ligands away from the active site of CYO72-MsrA. The forward and backward works of pulling *vs.* a series distances could be figured for later analysis. All of MD simulations described here were completed in Amber 12 (Case 2012a) with CYO72-MsrA parameterized using the Amber 99SB-ildn force field^[39]. CYO72, which represents the oxidized form of Met, was parameterized with antechamber of Amber

suites by using the GAFF force field and the charges of all non-standard residues including CYO and L-Met were calculated from an ORCA 2.8 quantum chemistry program package from the development team at the University of Bonn. Structure was optimized at the HF/6-31 g (d) level of theory (Neese. 2010). Spin-restricted Kohn-Sham determinants were chosen to describe the closed-shell wave functions, employing the resolution of identity (RI) approximation and the tight self-consistent field (SCF) convergence criteria provided by ORCA. In addition, some calculations were conducted in parallel on GTX 690 GPU or 3.2G multi-core Xeon 5580 CPU to accelerate the processes of simulations.

1.10 Statistical Analysis

Data were expressed as means±standard deviation (SD). Comparison between two groups was evaluated by the unpaired Student's *t*-test. Multiple comparisons were tested by one-way analysis of variance (ANOVA) followed by Duncan's multiple-range test. Differences were considered significant at $P < 0.05$.

2 RESULTS

2.1 L-Met Reduces Antimycin A-induced Superoxide Generation in PC12 Cells via a MsrA-dependent Mechanism

We induced $O_2^{\cdot-}$ production in PC12 cultured cells by a mitochondrial respiratory complex III inhibitor, antimycin A, as previously described^[29]. Antimycin A increased levels of $O_2^{\cdot-}$, which were judged by the fluorescence signals emitted by the oxidized forms of Mito-SOX (fig. 1). Bath application of antimycin A (100 $\mu\text{g}/\text{mL}$) for 0.5 h induced an increase of ROS level to $320\% \pm 42\%$ of control group ($n=9$, $P < 0.01$ *vs.* blank control), which could be alleviated by co-incubation

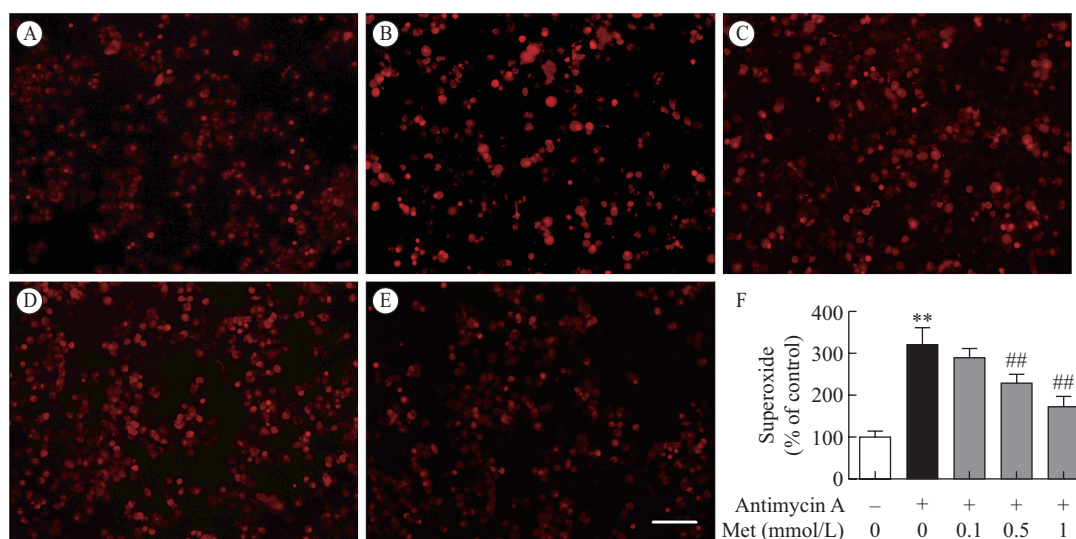


Fig. 1 L-Met reduces antimycin A-induced superoxide generation in PC12 cells.

PC12 cells were pretreated with 0 (A, B), 0.1 (C), 0.5 (D), or 1 mmol/L L-Met (E) for 30 min and then incubated with vehicle (A) or antimycin A (B-E, 100 $\mu\text{g}/\text{mL}$ for 30 min). The levels of superoxide were detected with MitoSOX red. Scale bars represent 100 μm . The fluorescence intensity of superoxide was determined (F). ** $P < 0.01$ *vs.* control group, ## $P < 0.01$ *vs.* antimycin A group

with L-Met. L-Met (0.1, 0.5 and 1 mmol/L) reduced the superoxide level to $291\% \pm 21\%$, $230\% \pm 21\%$ and $174\% \pm 22\%$ of control group, respectively ($n=6$, $P < 0.01$ vs. model, one way ANOVA, fig. 1).

To explore whether MsrA is necessary for anti-oxidant activity of L-Met, we knocked down the MsrA expression in the PC12 cells. After treatment with lentiviral-expressed specific shRNAs against MsrA, the MsrA expression levels were significantly

decreased. The effect of L-Met (1 mmol/L) on antimycin A-induced superoxide increase in the PC12 cells was abolished, suggesting that MsrA is essential for the anti-oxidant activity of L-Met in cells (fig. 2A and 2B; $201\% \pm 19\%$ of control group in L-Met with scrambled shRNA group vs. $337\% \pm 32\%$ in vehicle with scrambled shRNA group; $357\% \pm 34\%$ in L-Met with MsrA shRNA group vs. $325\% \pm 41\%$ in vehicle with MsrA shRNA group).

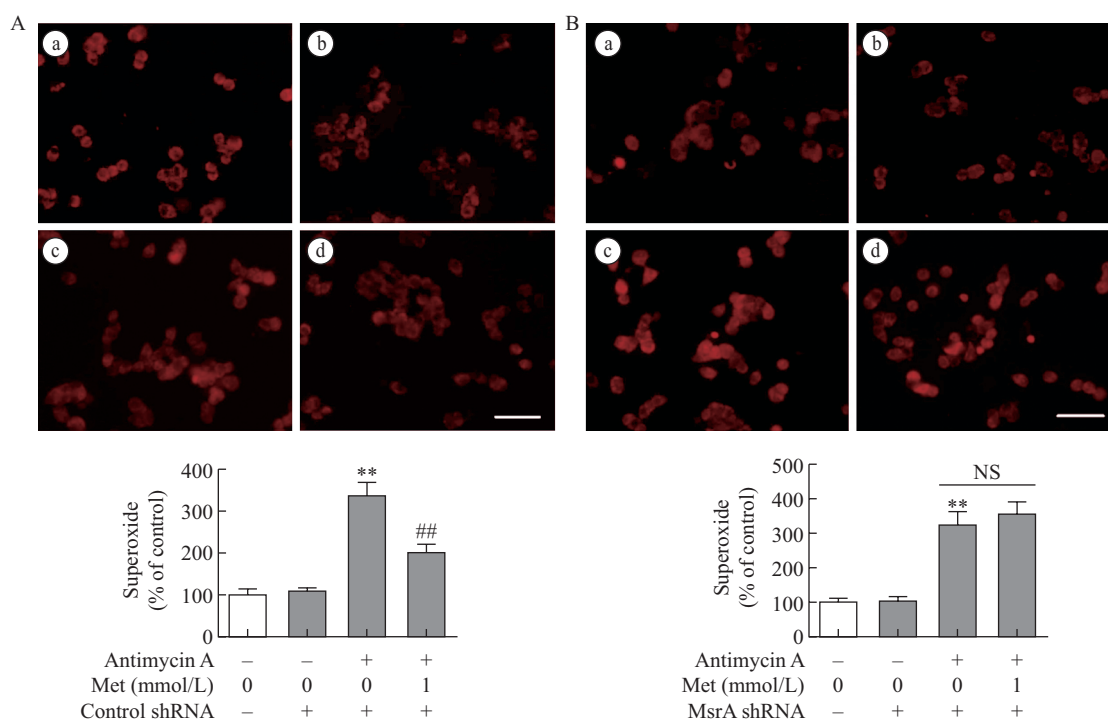


Fig. 2 L-Met reduces antimycin A-induced superoxide generation in PC12 cells via a MsrA-dependent mechanism.

A, B: PC12 cells were transfected with scramble shRNA (A) or MsrA shRNA (B). The transfected cells were pretreated with vehicle (a–c) or L-Met (d, 1 mmol/L for 30 min) and then incubated with antimycin A (c, d, 100 μ g/mL for 30 min). The release of superoxide was determined by the mitoSOX red fluorescence. And the fluorescence intensity of mitoSOX red was calculated in the statistics. Data are expressed as means \pm SD ($n=6-9$), ** $P < 0.01$ vs. control group, ## $P < 0.01$ vs. model group, or vs. untreated L-Met with scrambled shRNA group

2.2 MsrA Enhances the Radical Scavenging Property of Thiomethyl Group on Met via Formatting Sulfoxonium Intermediate

To confirm the antioxidant activity of L-Met *in vitro*, we evaluated the ROS-scavenging activity of L-Met by EPR. In EPR assay, hydroxyl radical was induced by Fenton reaction and trapped by DMPO to form a stable spin adduct DMPO-OH. L-Met reduced the signal of spin adduct to $58.8\% \pm 6.1\%$ of vehicle-treated group at 1 mmol/L ($P < 0.05$, $n=6-8$, one way ANOVA, fig. 3A–3D). Meanwhile, 0.1 mmol/L L-Met could not reduce the spin adduct signal ($94.1\% \pm 11.6\%$ of vehicle, $n=6$, $P > 0.05$).

To validate this hypothesis, we observed the effect of MsrA on the reaction between ROS and L-Met *in vitro* using a recombinant rat MsrA (rMsrA). In our previous study, we found that neither MsrA nor non-active MsrA alone has perceptible effects on EPR

signals. EPR assay (fig. 2E–2G) showed that co-treatment of L-Met with non-active MsrA or active MsrA decreased the DMPO-OH signal amplitude to $88.6\% \pm 11.0\%$ ($P < 0.05$, Student's *t* test, $n=6-8$) or $59.9\% \pm 2.6\%$ ($P < 0.05$, Student's *t* test, $n=6-8$) of vehicle group in the presence of 0.1 mmol/L L-Met, respectively. Compared to non-active MsrA group, active MsrA group significantly decreased the DMPO-OH signal ($P < 0.01$, $n=6-8$, Student's *t* test), indicating that MsrA facilitates the reaction of L-Met and ROS.

Then, to understand how MsrA catalyzes the oxidation of L-Met, homology modeling was used to visualize structural interactions between Met and MsrA. First, we observed that L-Met could bind to the catalytic pocket of MsrA (fig. 2H). The space of the pocket was formed by the side chains of Cys72, Phe73, Trp74, Tyr103 and Glu115. Previous studies had shown that the first step of MsrA-catalytic oxidation is to form

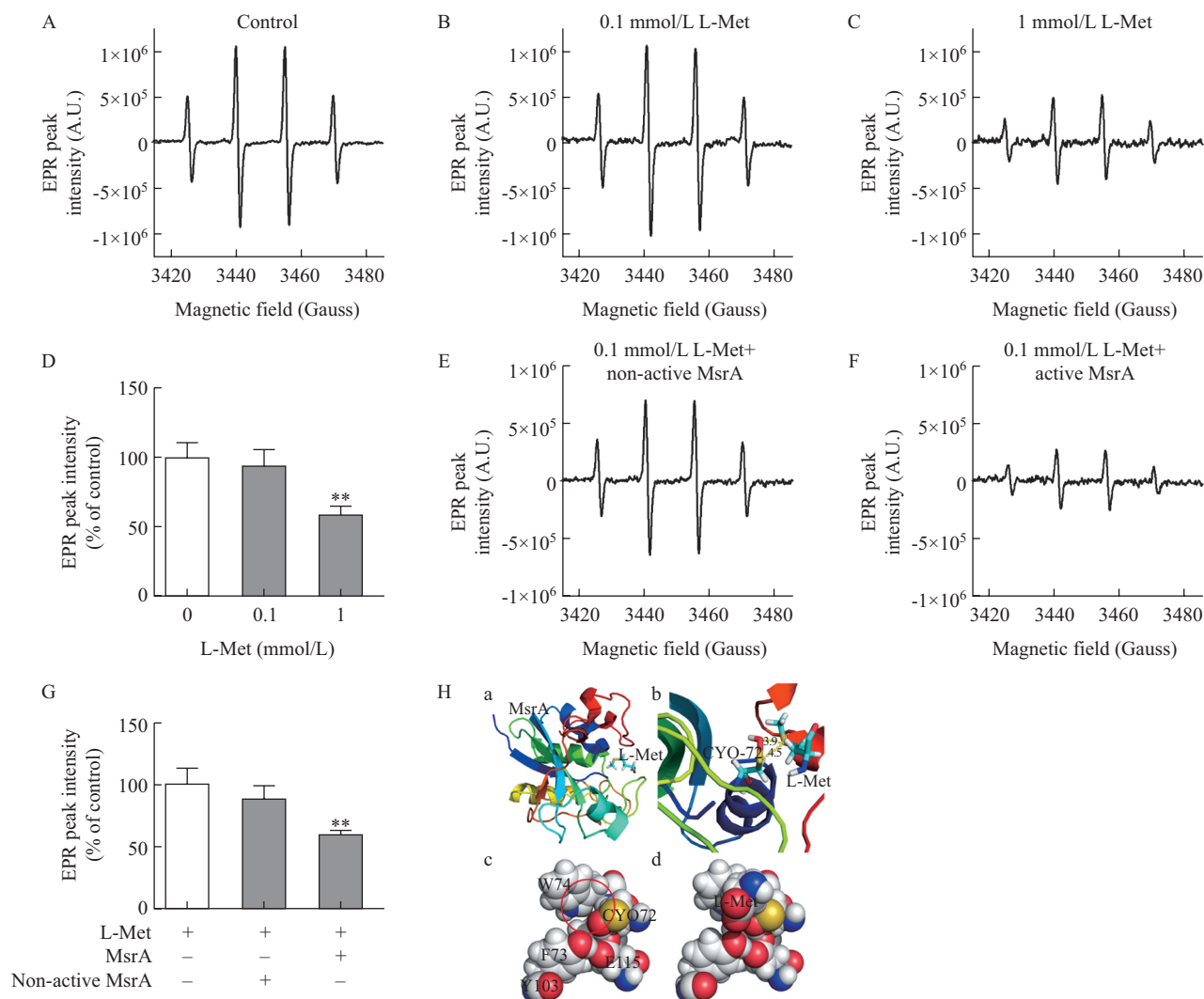


Fig. 3 MsrA enhances the radical scavenging property of thiomethyl group on Met via formatting sulfonium intermediate.

A–C: EPR spectra of DMPO-OH spin adduct during the reaction of 0.25 mmol/L H_2O_2 with 0, 0.1, or 1 mmol/L L-Met. L-Met (1 mmol/L) decreased the ROS signal. D: The levels of EPR peak intensity were obtained after treatment with L-Met ($n=6-8$), and the untreated control was set as 100%. E, F: Active or non-active MsrA enzyme was added into 0.1 mmol/L L-Met reaction tube. The ROS signal of active MsrA group was reduced significantly. G: The levels of EPR peak intensity were measured after treatment with L-Met and MsrA or non-active MsrA ($n=6-8$), and the non-enzyme control was set as 100%. H-a: Structural interactions were visualized between L-Met and MsrA. Catalytic site of MsrA consisted with CYO72, F73, W74, Y103 and E115. H-b: L-Met binding to CYO72 site of MsrA with highlighted dash line to indicate the distances. The unit is Angstrom. Hc, Hd: Molecular docking suggests MsrA has a binding site for L-Met. Data are expressed as means \pm SD ($n=6-8$), ** $P<0.01$ vs. vehicle-treated control group

a sulfenic acid residue on Cys72 by ROS^[29]. Thus, to induce the sulfoxide structure, the reaction between oxygen atom on the Cys72 (CYO72-S/O) and S atom from substrates is the limited step. To increase the reaction rate, a preferred orientation in the pocket is required for the substrate. Thus, we analyzed the orientation of different MsrA substrates in the active site of MsrA and found that L-Met adopted a preferred orientation than most of the others with the distance of O-S 3.9 Å. We found that residues Tyr103, Glu115, Tyr155, Phe73 and Trp74 contributed to the oxidation of L-Met, especially Trp74, Tyr103 and Glu115. When L-Met bound, the distances of donor and acceptor

atoms (D-H...A) between the O atom from CYO72 and the H atom from Tyr103-OH and Glu115-COOH were 3.0 Å and 2.7 Å, respectively. The two hydrogen bonds helped the transfer of O atom from CYO72 to the S atom from sulfide and facilitated the oxidation of L-Met.

2.3 SMLC, an L-Met Analogue and ROS Scavenger, Emerges as a Substrate of MsrA-catalytic Met Oxidase Activity

To confirm the antioxidant activity of SMLC, a hydrophilic cysteine-containing compound naturally found in garlic and onion (fig. 4A), we evaluated the ROS-scavenging activity of SMLC by EPR. In the

same experiment settings of L-Met, SMLC reduced the signal of spin adduct to 26.9%±6.7% of vehicle-treated group at 10 mmol/L ($P<0.01$, $n=5$, one way ANOVA, fig. 4B–4F). Meanwhile, 0.1 or 1 mmol/L SMLC could not reduce the spin adduct signal (98.9%±7.4% and 94.8%±10.7% of vehicle group, $P>0.05$).

Then we observed the effect of MsrA on the reaction between ROS and SMLC. The EPR assay showed co-treatment of 0.1 mmol/L SMLC and non-active MsrA (boiled) or active MsrA decreased the DMPO-OH signal amplitude to 64.61%±1.8% ($P<0.05$, $n=5-7$, Student's t test, fig. 4G–4J) or 17.5%±1.7% ($P<0.01$,

$n=7-8$, Student's t test) of vehicle group in the presence of 0.1 mmol/L SMLC, respectively. Compared to non-active MsrA group, active MsrA with SMLC exerted a much better anti-oxidant activity in the Fenton system ($P<0.01$, $n=5-8$, Student's t test), which indicated that MsrA significantly facilitates the reaction of SMLC and ROS.

2.4 SMLC Reduces Antimycin A-induced Mitochondrial Membrane Depolarization in PC12 Cells

As mitochondria are critical for apoptosis, it was logical to explore the effect of Met or SMLC on mitochondrial function. In this study, we used JC-1 as a

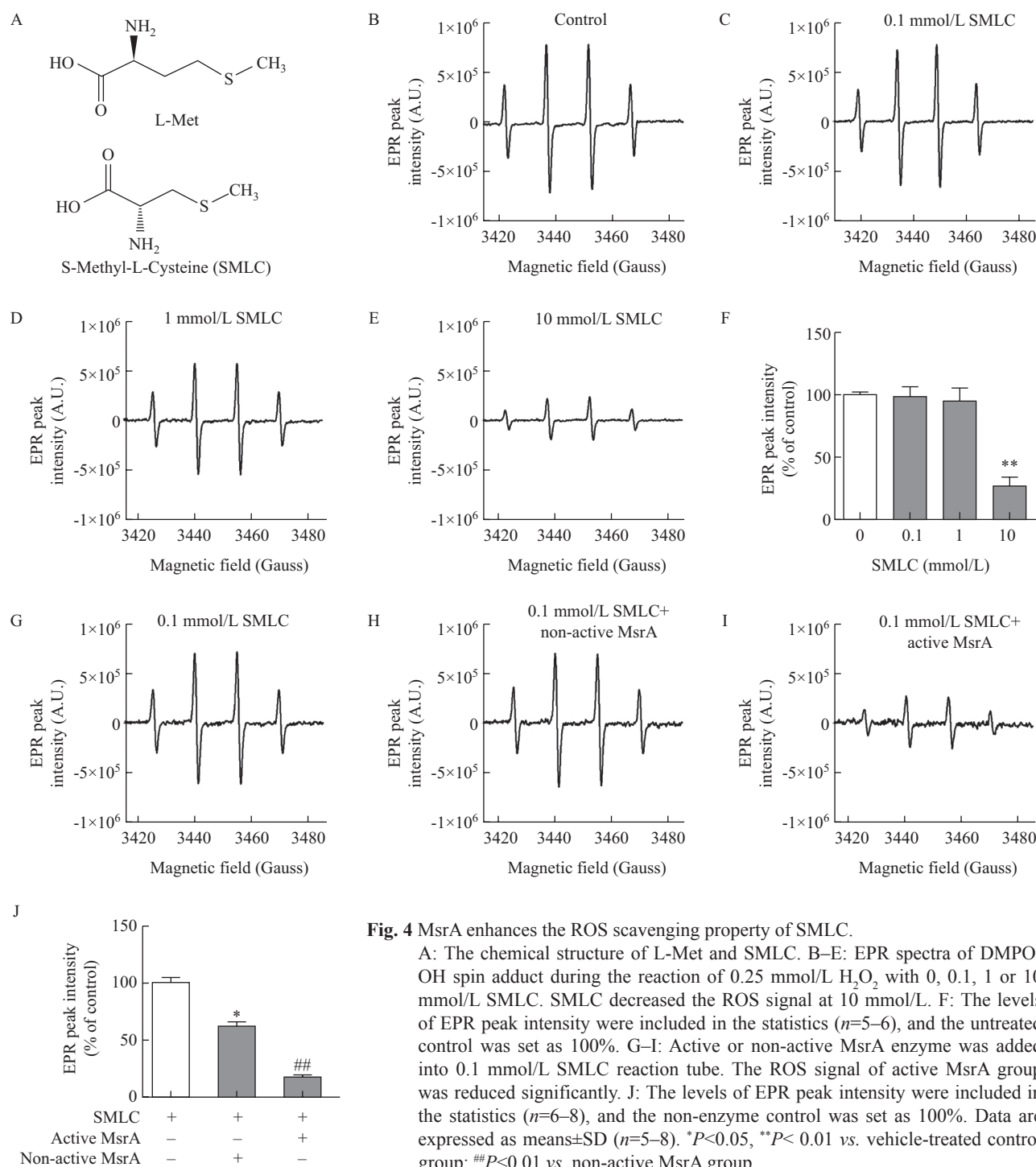


Fig. 4 MsrA enhances the ROS scavenging property of SMLC.

A: The chemical structure of L-Met and SMLC. B–E: EPR spectra of DMPO-OH spin adduct during the reaction of 0.25 mmol/L H_2O_2 with 0, 0.1, 1 or 10 mmol/L SMLC. SMLC decreased the ROS signal at 10 mmol/L. F: The levels of EPR peak intensity were included in the statistics ($n=5-6$), and the untreated control was set as 100%. G–I: Active or non-active MsrA enzyme was added into 0.1 mmol/L SMLC reaction tube. The ROS signal of active MsrA group was reduced significantly. J: The levels of EPR peak intensity were included in the statistics ($n=6-8$), and the non-enzyme control was set as 100%. Data are expressed as means±SD ($n=5-8$). * $P<0.05$, ** $P<0.01$ vs. vehicle-treated control group; ## $P<0.01$ vs. non-active MsrA group

fluorescent probe to detect the change in mitochondrial membrane potential by the change in fluorescence color. Compared with control group, treatment with 100 ng/mL antimycin A for 30 min led to mitochondrial membrane potential collapse as shown by enhanced red/green ratio. Then cells were pretreated with 1 mmol/L SMLC or 1 mmol/L L-Met at 0.5 h before adding antimycin A until the end. The red/green ratio was increased by SMLC or L-Met treatment (SMLC: 31.3%±4.1%, L-Met: 31.7%±4.8%, *n*=8, *P*<0.01, fig. 5) compared with antimycin A-treated vehicle group (22.4%±1.0% of control, *n*=5, *P*<0.01 vs. control).

2.5 SMLC Protects SH-SY5Y Cells against MPP⁺-induced Cytotoxicity

Then, we examined whether the radical-scavenging effect of L-Met and SMLC conferred neuroprotection. We used a MPP⁺-induced neurotoxicity model. SH-SY5Y cells were treated with 2 mmol/L MPP⁺ for 8, 16, 24 and 48 h. MTT assay showed that MPP⁺ reduced the cell viability in a time-dependent manner (fig. 6A). The cell viability decreased to 81.2%±6.4%, 55.9%±12.4%

and 25.2%±4.7% of control group in 8, 16, 24 and 48 h-treated groups. Then cells were pretreated with various concentrations of L-Met at 0.5 h before adding MPP⁺ until the end. The cell viability was increased by L-Met treatment (0.1 mmol/L: 31.3%±4.1%, 0.5 mmol/L: 31.7%±4.8% and 1 mmol/L: 40.2%±5.6%, *n*=8, *P*<0.01, fig. 6B) compared with MPP⁺-treated vehicle group (22.4%±1.0% of control, *n*=8, *P*<0.01 vs. control). SH-SY5Y cells were pretreated with L-Met for 0.5 h before adding MPP⁺. Then we tested whether SMLC could alleviate MPP⁺-induced neurotoxicity. Compared with MPP⁺-treated group (22.5%±5.3% of control, *P*<0.01), the cell viability was increased by SMLC treatment (0.1 mmol/L: 34.6%±4.5%, 0.5 mmol/L: 41.1%±2.7% and 1 mmol/L: 45.5%±2.5%, *n*=8, *P*<0.01, fig. 6C), when the cells were pretreated with various concentrations of SMLC for 0.5 h before adding MPP⁺ until the end. These data indicated a protective role of MsrA-catalytic Met oxidase activity initiated by L-Met and SMLC in MPP⁺-induced neurotoxicity.

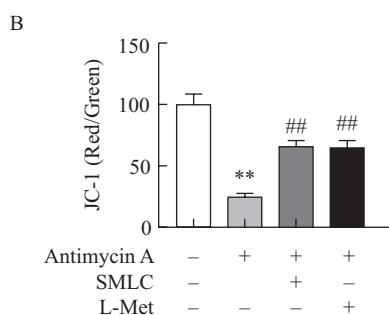
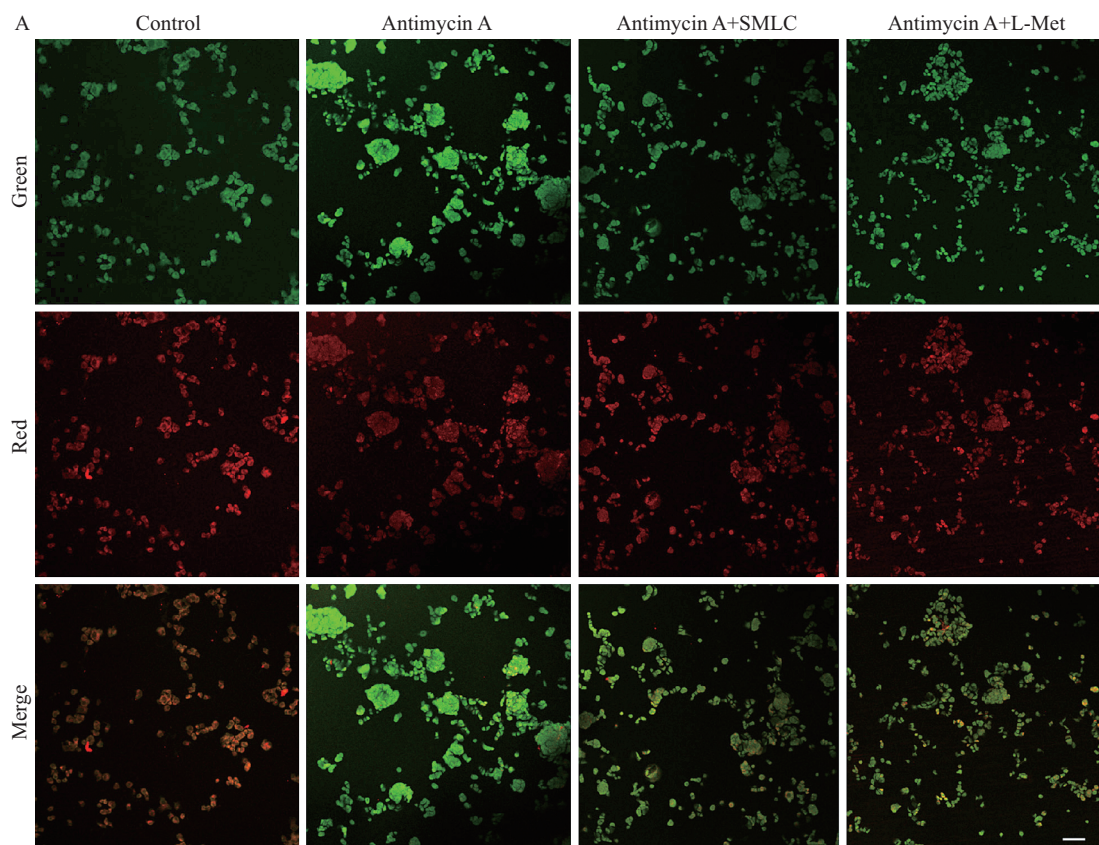


Fig. 5 SMLC reduced antimycin-induced mitochondrial membrane depolarization in PC12 cells.

A, B: PC12 cells were pretreated with 1 mmol/L SMLC or 1 mmol/L L-Met for 30 min and then incubated with antimycin A (100 µg/mL for 30 min). The mitochondrial membrane potential (JC-1 staining, red/green fluorescence ratio) was photographed and quantified by fluorescence microscopy. Data are expressed as means±SD. *n*=5, ***P*<0.01 vs. control group, ##*P*<0.01 vs. model group

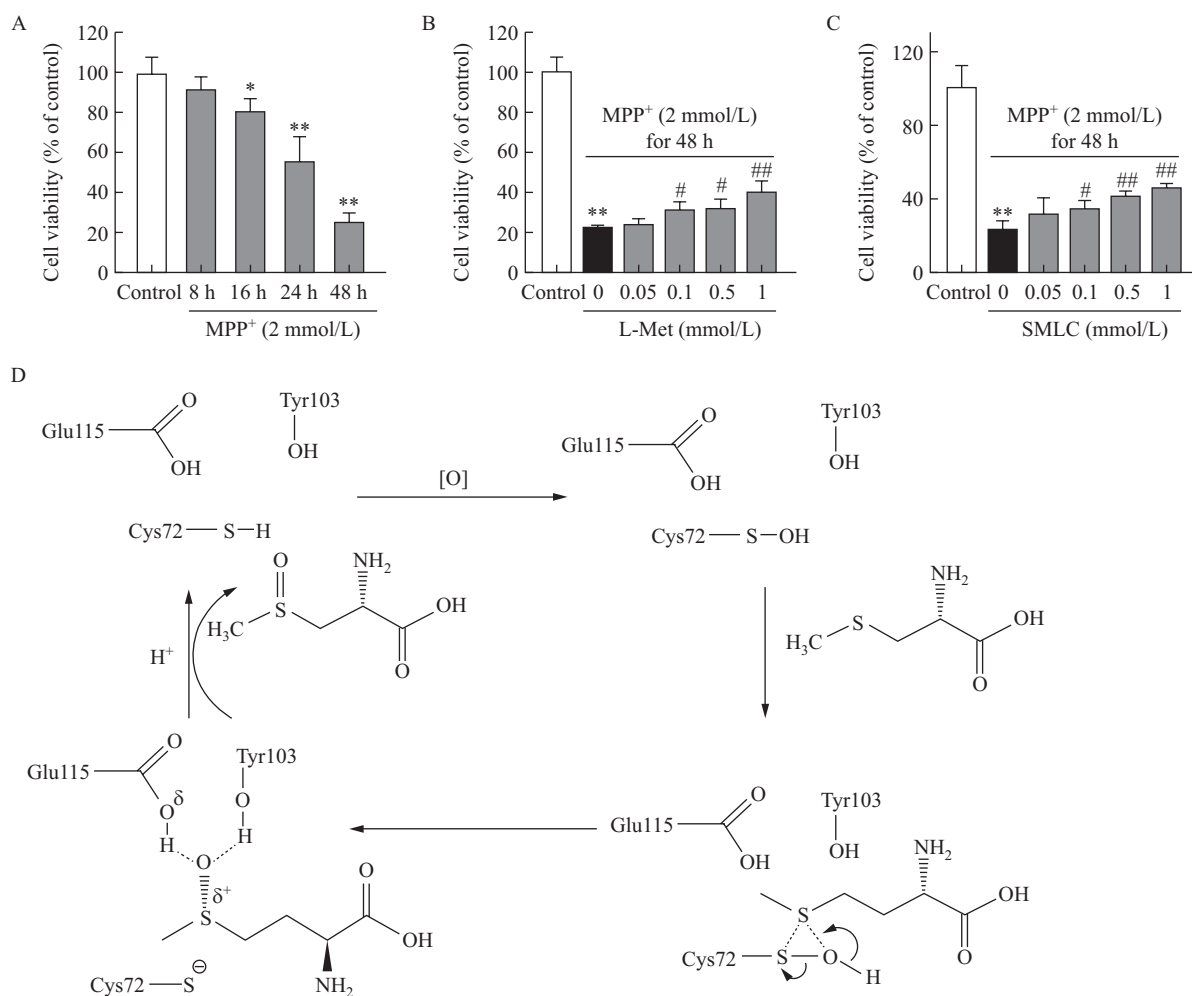


Fig. 6 Protective effect of L-Met and SMLC against MPP⁺-induced cytotoxicity in SH-SY5Y cells

A: effects of MPP⁺ treatment time on the cell viability of SH-SY5Y cells. SH-SY5Y cells were incubated with 2 mmol/L MPP⁺ for 8, 16, 24 or 48 h, and the cell viability was assayed using MTT. B: the effect of L-Met on MPP⁺-induced cytotoxicity in SH-SY5Y cells. Cells were pretreated with various concentrations of L-Met for 0.5 h, and MPP⁺ (2 mmol/L) was added for an additional 48 h. C: The cell viability rates showed the effect of 0.05 to 1 mmol/L SMLC on MPP⁺-induced cytotoxicity in SH-SY5Y cells. D: proposed mechanism of SMLC oxidation catalyzed by MsrA. Data are expressed as means±SD. *n*=8, ***P*<0.01 vs. control group; #*P*<0.05, ##*P*<0.01 vs. MPP⁺-treated group

3 DISCUSSION

Our study provided evidence that MsrA helped L-Met and its analogue SMLC scavenge radicals via forming sulfonium intermediate, which protected cells from oxidative stress and neuron toxin-induced neurotoxicity. With release of SMLC sulfoxide, the reduced form of MsrA is formed. After MsrA knockdown, the anti-oxidant activity of L-Met in PC12 cells was nearly abolished. Meanwhile, computational chemistry evidence showed that Met-like structure was a substrate of oxide-MsrA. We believe that the oxygen atom on the sulfenic acid was derived from ROS or radicals. A lone pair of electrons on the Met or SMLC sulfur attacked the sulfonicsulfur to form the positively charged sulfonium intermediate. Glu115 and Tyr103 acted as a proton donor and were stabilized

by hydrogen bonding with SMLC (fig. 6D). These data highlight the role of MsrA in the detoxication of mitochondrial ROS-generating toxins.

Epidemiologically, environmental neurotoxins, such as pesticides, MPP⁺, 6-hydroxydopamine, rotenone, 3-nitropropionic acid and paraquat, induced neurotoxicity via triggering mitochondrial oxidative stress burdens^[1-5]. Levels of ROS exceed the antioxidant capacity of the mitochondria of neurons. These neurotoxins are promising candidates for causative factors of neurodegenerative diseases, including PD. Aging-associated decline in the endogenous antioxidant defense increased the susceptibility to neurotoxins. Among the endogenous antioxidant defenses, MCRC, which depends on the anti-oxidation activity of protein-bound Met residues and their redox cycle containing Met-MetO-Met, plays a critical role in the protection

of key proteins. In the MCRC, Met residues in proteins act as a “sink” for many reactive species, and Msrs catalyze a thioredoxin-dependent reduction of MetO back to Met. This cyclic interconversion of protein Met residues between oxidized and reduced forms may be considered an efficient ROS-scavenging mechanism *in vivo*. Although most studies have indicated a role of MCRC in the aging-related diseases^[40, 41], much less is known about its role in the neuro-toxicology of ROS-generating toxins. Our previous studies revealed that Met alleviates H₂O₂-induced mitochondrial injury and 12-O-tetradecanoyl-phorbol-13-acetate (TPA)-induced skin oxidation^[42]. Selective dopaminergic neuronal cell death caused by environmental neurotoxin MPP⁺ has been widely used as a cell model of PD. In the current study, we further observed that Met also alleviates MPP⁺-induced neurotoxicity of SH-SY5Y cells. All the data raised the critical role of MCRC in the detoxication of mitochondrial ROS-generating toxins.

It is conventionally believed that the mitochondrial ROS scavenging effect of MCRC is mainly due to the natural anti-oxidation activity of Met residues. Under oxidative stress, MsrA can maintain an effective concentration of Met by reducing MetO timely via activating its reductase. Our previous study found that under oxidative stress, more than 80% of protein-bound Met residues are kept in a reduced form^[43], indicating that there is a sufficient store of Met. Although L-Met can act as an effective H₂O₂ scavenger *in vitro* in the presence of a high concentration of H₂O₂ (above 1 mmol/L), after inhibition of MsrA function by DMSO, the antioxidation of Met (1–5 mmol/L) on CHO cells is largely abolished^[42]. Recently, it has been demonstrated that MsrA also serves as a stereospecific methionine oxidase^[43], which may facilitate the reaction of Met with ROS. Here, we observed that knockdown of MsrA by shRNA nearly abolished the antioxidation of Met in the mitochondrial ROS generation. In Fenton system, EPR spin-trapping assay revealed that MsrA facilitated the Met (1 mmol/L)-mediated scavenging effect on hydroxyl radicals. Thus, Met is not merely an antioxidant, and its action may require a participation of MsrA *in vivo*.

MsrA has historically been considered a reductase to repair damaged proteins, not an ROS scavenger^[44, 45]. In spite of its impressive protective effects in neurotoxicity^[46, 47], the precise mechanisms and fundamental chemical principles for MsrA-mediated mitochondrial ROS elimination remain largely unclear. Although MsrA may exhibit antioxidation via maintaining an effective concentration of Met by reducing MetO timely, this study, together with our previous work, has demonstrated that the stereospecific Met oxidase activity may play a vital role in the anti-oxidation of MsrA, which may afford the antioxidant effect against neurotoxins, including H₂O₂, antimycin and MPP⁺.

By computation, we found that the lone pair electrons on the methylthiol-sulfur nucleophilicity of sulfide attacked the sulfenic sulfur in active site of MsrA to form the positively charged sulfonium intermediate. Then, MsrA catalyzed the transition and released the sulfoxide. When ROS bursted *in vivo*, ROS induced a sulfenic acid residue on Cys72 of MsrA, which was followed by the reaction between oxygen atom on CYO72 and S atom of DMS. The Phe73 and Trp74 in MsrA facilitated Met to bind the active center via hydrophobic bond, and Tyr103 and Glu115 increased the oxygen atom transfer from the CYO72 to the S atom of Met via the hydrogen bond. The peroxidase activity of MsrA significantly facilitated the reactivity of Met and SMLC at a relatively low concentration. The mechanisms of Met and SMLC-mediated radical-scavenging capacity were illustrated in fig. 6D.

Pharmacological enhancement of MsrA function seems to be reasonable therapeutic strategy against aging in clinical practice. Only very few approaches are available to enhance MsrA function^[29, 37, 48, 49]. Comparing to the reducing activity of MsrA for repairing oxidized proteins, activation of Met oxidase activity of MsrA required a relatively high substrate concentration. To increase the concentrations of substrate, Met supplement may not be an appropriate approach, especially in aging-related diseases, like PD and AD, for Met is rapidly incorporated into proteins and it increases cardiovascular risk^[30, 31]. In fact, dietary Met restriction, not supplementation, induces beneficial effects^[50]. For the doubtful problem for Met supplementation, other Met analogs could be considered. SMLC is a safe sulfur containing amino acid naturally found in garlic and onion^[33–35], and has been reported to have anti-lipidemic activity and anti-PD effects in *Drosophila*^[36, 51]. We found that SMLC exerted a better natural anti-oxidant activity and its anti-oxidation was also facilitated by MsrA. SMLC works as a substrate in the Met oxidase activity of MsrA. Moreover, it exerted a good neuroprotection in MPP⁺-induced injury in SH-SY5Y cells. However, more *in vivo* evidence to support this view is still required. Whether dietary supplementation with SMLC or L-Met in humans is applicable remains to be tested.

In summary, our data supported that the Met oxidase activity of MsrA might be targeted therapeutically to prevent mitochondrial ROS-generating toxins and aging-associated neurodegenerative diseases by activators like SMLC in the future.

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Conflict of Interest Statement

The authors declare that there is no conflict of interest with any financial organization or corporation or individual that can inappropriately influence this work.

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