Astragaloside IV rescues MPP^+-induced mitochondrial dysfunction through upregulation of methionine sulfoxide reductase A

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Abstract. Methionine sulfoxide reductase (Msr) repairs oxidatively damaged proteins through acting as an antioxidant. Oxidative stress has been postulated to cause the mitochondrial dysfunction that is associated with aging and certain diseases, including Parkinson's disease (PD). The present study investigated the protective effects of astragaloside IV (AS-IV) on 1-methyl-4-phenylpyridinium (MPP^+)-induced mitochondrial dysfunction through MsrA in PC12 cells. This revealed that oxidative stress reduced the expression of MsrA following MPP^+ treatment. AS-IV was demonstrated to protect PC12 cells from MPP^+-induced oxidative damage through upregulating MsrA. MsrA expression was dependent on the Sirt1-FOXO3a signaling pathway. In addition, knockdown of MsrA reduced the protective effects of AS-IV, indicating that the antioxidant effects of AS-UV occurred through MsrA. These results suggest that AS-IV exerts antioxidant effects and regulates mitochondrial function. Thus, AS-IV may serve as an effective therapeutic agent for aging and PD.

Introduction

Oxidative stress is induced by an imbalance between the production and elimination of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (1). Although ROS and RNS are generated under normal physiological conditions, numerous cellular functions are disturbed by the reaction of ROS/RNS with cellular components, including DNA, lipids and proteins (2-6). For example, sulfur-containing amino acids (methionine and cysteine) are sensitive to ROS (7). Mitochondria are a major source of ROS (8). The free radical $\cdot O_2^-$ is produced by complex I and complex III of the electron transport chain (ETC) and released into the mitochondrial matrix (9). The mitochondrial dysfunction caused by oxidative stress and can result in cell loss leading to neurodegenerative diseases and ischemic brain injury (10). Thus, pro-oxidant antioxidant generation needs to be highly regulated (11).

Methionine sulfoxide reductase (Msr) A and B, which reduce free and protein-based methionine sulfoxides to methionine, are encoded by a single MsrA and three MsrB genes, respectively, in the mammalian genome (7,12). Mammalian MsrA is expressed in the mitochondria, cytosol and nucleus (13,14). Methionine oxidation and reduction serves an important role in cellular signaling, metabolism and oxidative stress under physiological and pathological conditions (15). MsrA repairs oxidatively-damaged proteins and functions as an antioxidant enzyme (16). MsrA has been identified to protect various cell types against oxidative stress-induced death (17,18).

Astragaloside IV (3-O-beta-D-xylopyranosyl-6-O-beta-D-glucopyrannosyl-cycloastragenol; AS-IV) is purified from the Chinese medicinal herb, Astragalus membranaceus (19). The molecular structure of AS-IV is illustrated in Fig. 1A. AS-IV has comprehensive pharmacological functions, including anti-inflammatory and antioxidative activity, and is able to reduce infarct size and improve post-ischemic brain function (20-22). However, few studies have investigated whether AS-IV prevents mitochondrial dysfunction via MsrA. The present study utilized a 1-methyl-4-phenylpyridinium (MPP^+)-induced oxidative damage cell model to investigate this. MPP^+ is toxic and acts by interfering with oxidative phosphorylation in the mitochondria through inhibiting complex I, which induces oxidative stress (23,24). MPP^+ can reproduce the clinical and pathological features of Parkinson's disease (PD) in animal models (25-27). Therefore, present study aimed to investigate the protective effects of AS-IV against MPP^+-induced oxidative damage and the potential molecular mechanisms underlying these effects.

Materials and methods

Antibodies and reagents. Anti-MsrA (cat. no. ab16803), anti-forkhead box protein O3 (FOXO3a; cat. no. ab47409) and anti-β-actin (cat. no. ab6276) antibodies were purchased from Abcam (Shanghai, China). AS-IV (cat. no. 74777) was purchased from the National Institutes for Food and Drug

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Key words: astragaloside IV, mitochondrion, methionine sulfoxide reductase A, oxidative stress

Control (Beijing, China). Ex527 (cat. no. E7034), MPP^+ (cat. no. D048) and Protease Inhibitor Cocktail (cat. no. P8340) were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). TRIzol reagent (cat. no. 15596-018), Lipofectamine® 2000 (cat. no. 11668-019), MitoTracker® Red CMXRos (cat. no. M-7512), CM-H2DCFDA (cat. no. C6827) and MTT (cat. no. M-6494) were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). QuantiTect Reverse Transcription kit (cat. no. 205314) and QuantiTect SYBR® Green PCR kit (cat. no. 204145) were purchased from Qiagen, Inc. (Valencia, CA, USA).

Cell culture, and MPP^+-induced injury and AS-IV treatment. Rat adrenal gland pheochromocytoma PC12 cells were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in high glucose Dulbecco's modified Eagle medium containing 10% fetal bovine serum (cat. no. 12484-010, Thermo Fisher Scientific, Inc.) and 2 mM L-glutamine (cat. no. 25030081, Thermo Fisher Scientific, Inc.) in a incubator at 37˚C with 5% CO_2. PC12 cells were randomly divided and treated with 1, 10 μM, 50 or 100 μM MPP^+ for 24 h. In the AS-IV treatment experiment, the MPP^+-treated PC12 cells were simultaneously treated with 50 μM AS-IV.

Cell viability assay. Cell viability was determined using the MTT assay. Cells were seeded in 96-well plates at a density of $1x10^4$ cells/well. After MTT and AS-IV treatment, 0.5 mM MTT was added into each well and the plates were incubated at 37˚C with 5% CO_2 for 4 h. Dimethylsulfoxide was added to each well in order to dissolved the formazan crystals and the absorbance at 570 nm was measured using a microplate reader. Cell viability was expressed as a percentage of the untreated control cells.

Western blot analysis. Cells were washed with PBS and resuspended in lysis buffer (cat. no. P0013G; Beyotime Institute of Biotechnology, Haimen, China) with a Protease Inhibitor Cocktail (cat. no. 04693132001; Roche Diagnostics, Basel, Switzerland; 1 tablet per 10 ml). The protein was quantified using a BCA Protein Assay (cat. no. 23225; Pierce; Thermo Fisher Scientific, Inc.). Protein samples (50 μg per lane) were separated by SDS-PAGE on a 10% gel, followed by electrotransfer onto polyvinylidenefluoride membranes. The blots were incubated with anti-MsrA (cat. no. ab16803; 1:1,000) and anti-β-actin (cat. no. ab6276; 1:1,000) primary antibodies at 4˚C for 12 h. After washing with Tris-buffered saline-Tween 20, the blots were incubated with Goat Anti-Rabbit IgG H&L conjugated to horseradish peroxidase (HRP; cat. no. ab6721; Abcam; 1:3,000) and Goat Anti-Mouse IgG H&L conjugated to HRP (cat. no. ab6789; Abcam; 1:3,000) at room temperature for 3 h. Protein expression was normalized to β-actin according to the gray scales by chemiluminescence, and quantified using a using a Gel Doc™ XR+ system version 4.0 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Small interfering (si)RNA and plasmid transfection. MsrA and FOXO3a-specific siRNAs were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The siRNAs sequences were as follows: MsrA-specific siRNA, 5'-CCAUGAAUCAUUUGC CAAAUCGCUU-3'; FOXO3a- specific siRNA, 5'-CAACCT GTCACTGCATAGT-3'; and negative control siRNA, 5'-CCA GCACUAACACCCAUCCCACAAA-3' (Sangon Biotech Co., Ltd.). A total of $1x10^6$ cells were seeded in 60 mm plates and transfected with siRNA or plasmids using Lipofectamine 2000 according to the manufacturer's protocol and incubated at 37˚C for 48 h, with fresh medium added every 6 h. Subsequently, the cells were collected for mRNA and protein extraction.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis of MsrA mRNA expression. Total cellular RNA was extracted from the cells using TRIzol reagent according to the manufacturer's protocol. cDNA was synthesized from total RNA using the QuantiTect Reverse Transcription kit according to the manufacturer's protocol. qPCR was performed using the QuantiTect SYBR Green PCR kit according to the manufacturer's protocol. The volume of PCR tube was 50 μl including 25 μl SYBR Green, 1 μl primer A, 1 μl primer B, 1 μl cDNA and 22 μl RNase-free water. The qPCR protocol was as follows: Denaturation at 95˚C for 15 sec, annealing at 60˚C for 30 sec, extension at 72˚C for 30 sec (40 cycles). This was followed by a dissociation step at 95˚C for 15 sec, 60˚C for 15 sec, and then 95˚C for 15 sec. The relative mRNA levels were normalized to β-actin and quantified using the $2^{-\Delta\Delta Cq}$ method (28). Specific qPCR primers were synthesized by Sangon Biotech Co., Ltd. The primers for qPCR were as follows: MsrA forward, 5'-TCTGGGTCTTGA AAGGAGTGTA-3' and reverse, 5'-AGGTATTGCTGGTGG TAGTCTTC-3'; and β-actin forward, 5'-CCACTGCCGCAT CCTCTTCCTC-3' and reverse, 5'-CAGCAATGCCTGGGT ACATGGTG-3'.

ROS detection. CM-H2DCFDA, an ROS indicator, was used to measure the ROS production in PC12 cells. A total of $1x10^5$ cells were inoculated into 24-well plates. A working concentration of 5 μM CM-H2DCFDA was produced and added to the plates, which were incubated at 37˚C for 30 min. The cells were washed with prewarmed PBS at 37˚C. After washing the cells, fluorescence was detected using a microplate reader (495/520 nm) and expressed as a percentage of the value of the untreated control cells.

Mitochondrial morphology staining and analysis. PC12 cells were cultured on poly-L-lysine-coated slides. A total of $1x10^5$cells were seeded into 24-well plates. PC2 cells were treated by MPP^+ and/or AS-IV in the no siRNA-interfering group and MsrA-knockdown group by siRNA. After treatment, 10 nM MitoTracker Red CMXRos was added into the culture medium and the cells were incubated for 15 min at 37˚C with 5% CO_2. After staining, the cells were washed with fresh prewarmed PBS and observed using a fluorescence microscope. The analysis was performed as described previously (29).

Statistical analysis. The statistical significance of differences between groups was analyzed using an unpaired Student's t-test or one-way analysis of the variance. GraphPad Prism software (version 5.01; GraphPad Software Inc., La Jolla, CA, USA). All data are presented as the mean ± standard error of mean

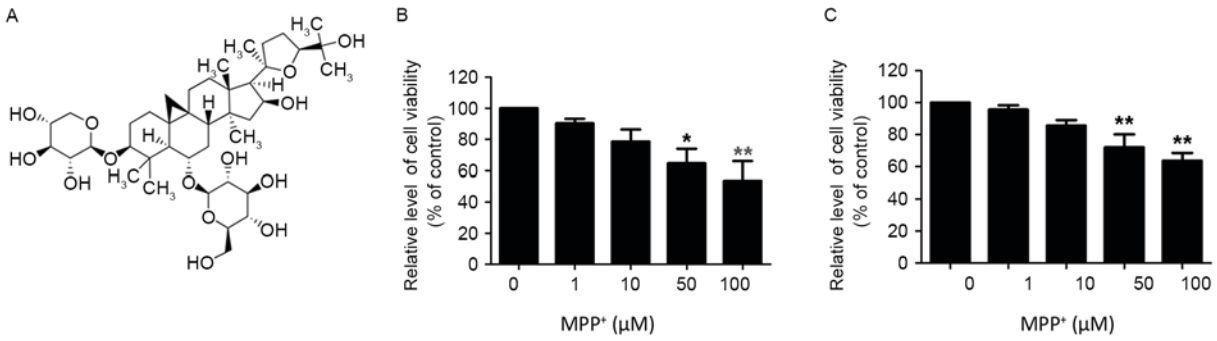

Figure 1. Protective effect of AS-IV against MPP+-induced oxidative damage. (A) Chemical structure of AS-IV. (B) PC12 cells were treated with increasing concentrations of MPP+ for 24 h. (C) AS-IV -0 μM) treatment rescued cell viability following MPP+ treatment. *P<0.05 and **P<0.01 vs. the control group (one-way analysis of the variance). AS-IV, astragaloside IV; MPP+, 1-methyl-4-phenylpyridinium.

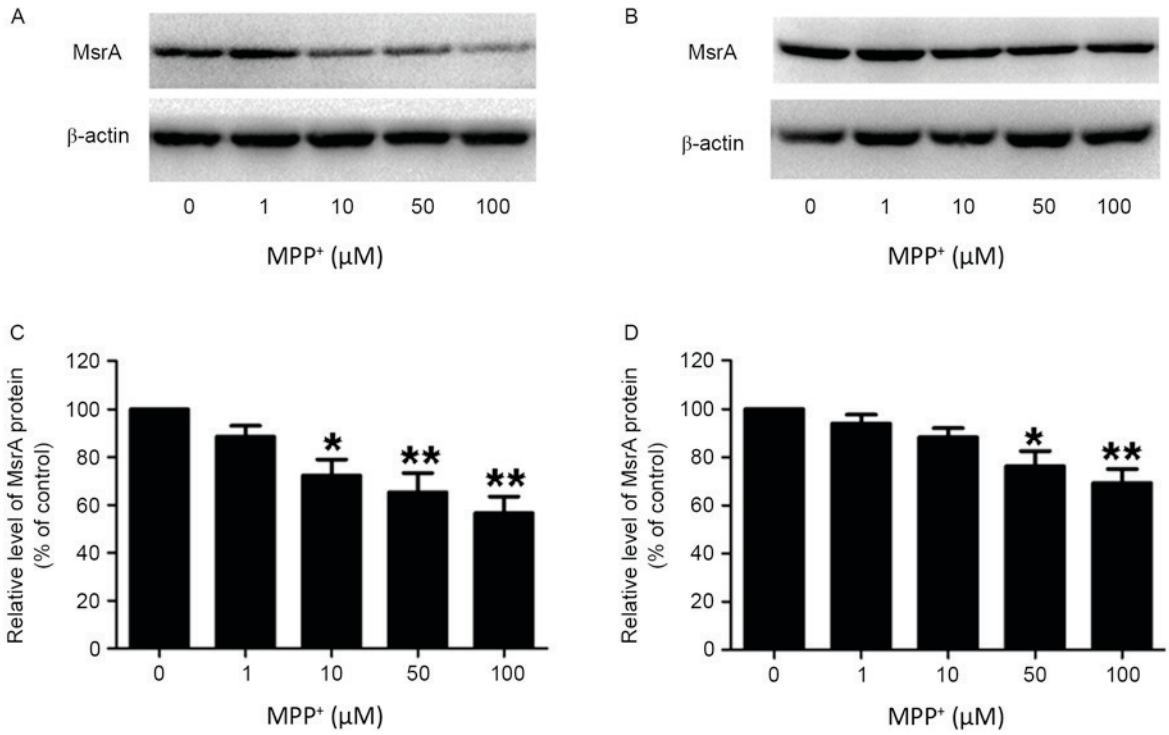

Figure 2. Protein levels of MsrA following oxidative damage by MPP+ with and without AS-IV treatment. Western blots for MsrA after (A) MPP+ treatment, and (B) AS-IV and MPP+ treatment. Densitometry analysis of western blots for MsrA after (C) increasing MPP+ treatment, and (D) AS-IV (50 μM) and increasing MPP+ treatment. Simultaneous treatment with AS-IV attenuated MPP+-induced reductions in MsrA levels. *P<0.05 and **P<0.01 vs. the control group (one-way analysis of the variance). MsrA, methionine sulfoxide reductase A; AS-IV, astragaloside IV; MPP+, 1-methyl-4-phenylpyridinium.

of three independent experiments. P<0.05 was considered to indicate a statistically significant difference.

Results

AS-IV alleviates MPP+-induced oxidative damage in PC12 cells. To investigate the effect of AS-IV against oxidative damage, PC12 were treated cells with increasing MPP+ concentrations as a model of oxidative stress. The MTT assay results revealed that cell viability was reduced with increasing MPP+ concentrations, with a significant decrease in cell viability to 53.7±12.4% with 100 μM MPP+ compared with the untreated control group (P<0.01; Fig. 1B). Treatment with 50 μM AS-IV partially rescued the viability of PC12 cells treated with MPP+ (Fig. 1C). AS-IV treatment significantly increased cell viability to 63.7±4.9% with 100 μM MPP+ treatment compared with the control group, a 1.18-fold increase (P<0.01; Fig. 1C).

AS-IV rescues MsrA protein expression after MPP+-induced oxidative damage. MsrA repairs oxidatively-damaged proteins and functions as an antioxidant enzyme (16,18). To investigate whether MsrA is associated with the protective effect of AS-IV, protein levels of MsrA after MPP+-induced oxidative damage with or without AS-IV were measured using western blotting (Fig. 2). The results demonstrated that the protein levels of MsrA gradually decreased with increasing MPP+ (Fig. 2A and C). AS-IV treatment alleviated the MPP+-induced decrease in MsrA protein levels, with MsrA returned to near normal levels in cells treated with 1 μM and 10 μM MPP+ (Fig. 2B and D).

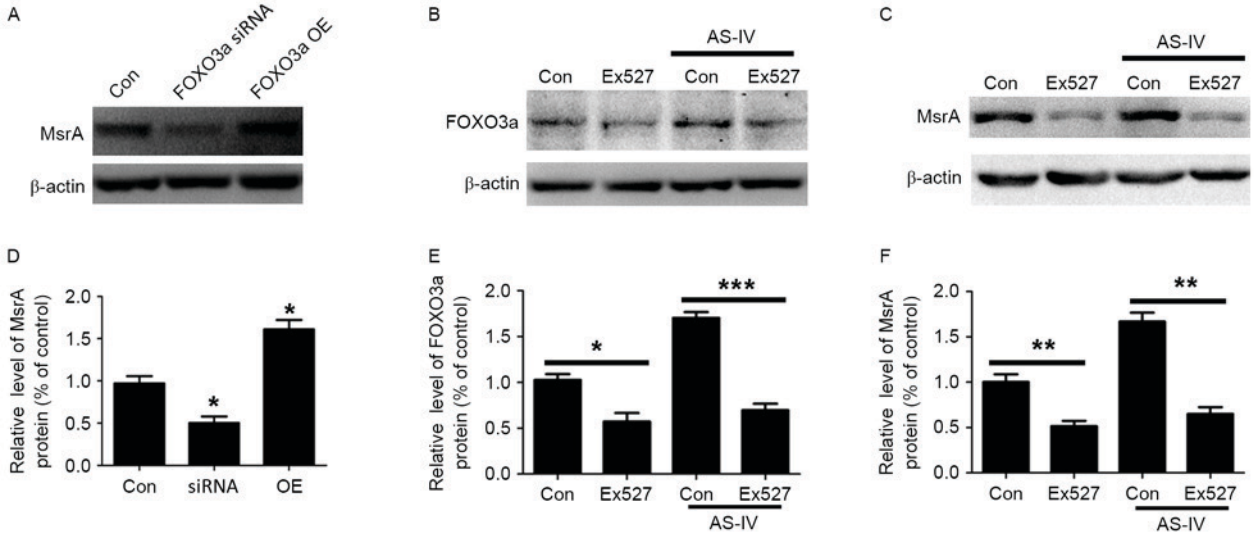

Figure 3. AS-IV increases MsrA expression through the NAD-dependent protein deacetylase sirtuin-1-FOXO3a signaling pathway. Western blot for (A) MrsA after FOXO3a-specific siRNA and OE treatment, (B) FOXO3a after 1 μM Ex527 treatment with or without AS-IV, and (C) MrsA after 1 μM Ex527 treatment with or without AS-IV. Densitometry analysis of western blots for (D) MrsA after FOXO3a-specific siRNA and OE treatment, (E) FOXO3a after 1 μM Ex527 treatment with or without AS-IV, and (F) MrsA after 1 μM Ex527 treatment with or without AS-IV. *P<0.05, **P<0.01 and ***P<0.001 compared with the Con group (t-test). AS-IV, astragaloside IV; MsrA, methionine sulfoxide reductase A; FOXO3a, forkhead box protein O3, siRNA, small interfering RNA; OE, overexpression; Con, control.

AS-IV increases MsrA protein expression through the NAD-dependent protein deacetylase sirtuin-1 (Sirt1)-FOXO3a pathway. The underlying molecular mechanism of MsrA upregulation by AS-IV was explored. A previous study indicated that MsrA expression is regulated via the forkhead box protein/FOXO signaling pathway (30). Sirt1 regulates tyrosine hydroxylase expression and the differentiation of neuroblastoma cells via the FOXO3a signaling pathway (31). Whether Sirt1-FOXO3a could regulate the expression of MsrA in PC12 cells was assessed via siRNA treatment, Ex527 treatment and western blotting (Fig. 3). MsrA protein expression was significantly reduced by FOXO3a-specific siRNA, but significantly increased by FOXO3a overexpression, in PC12 cells compared with the control group (both P<0.05; Fig. 3A and D). Subsequently, Ex527, a Sirt1 inhibitor, was demonstrated to significantly reduce FOXO3a expression compared with the control group (P<0.05; Fig. 3B and E). Similarly to FOXO3a-specific siRNA treatment, Ex527 also significantly inhibited MsrA protein expression compared with the control group (P<0.01; Fig. 3C and F).

MsrA decreases MPP^{+}-induced oxidative damage via acting as an antioxidant. The effect of MsrA on MPP^{+}-induced oxidative damage was examined. Levels of MsrA mRNA and protein were significantly downregulated by MsrA-specific siRNA and significantly upregulated by an MsrA-overexpressing plasmid compared with the control group (both P<0.05; Fig. 4A-C). Subsequently, ROS levels were detecting using CM-H2DCFD in cells treated with MPP^{+} (Fig. 4D). ROS levels were significantly increased in the MsrA knockdown group compared with the control group (P<0.05). By contrast, ROS levels were significantly reduced in cells overexpressing MsrA compared with the control group (P<0.05). These results suggest that MsrA exhibits an antioxidant function against MPP^{+}-induced oxidative damage in PC12 cells.

AS-IV inhibits MPP^{+}-induced mitochondrial fragmentation via MsrA. The phenomenon of mitochondrial fragmentation has been observed during oxidative stress, and there is an association between mitochondrial fragmentation and ROS release (29,32). Fragmented mitochondria have been demonstrated to sensitize cells to apoptosis regulator BAX insertion and facilitate the release of apoptogenic factors (32). To determine whether AS-IV inhibits MPP^{+}-induced mitochondrial fragmentation via MsrA, the density of mitochondria in PC12 cells was measured after treatment with 100 μM MPP^{+} with or without 50 μM AS-IV (Fig. 5). Imaging revealed that MPP^{+} induced mitochondrial fragmentation and reduced the density of mitochondria (Fig. 5A, B, E and F), while AS-IV increased the density of mitochondria (Fig. 5C, D, G and H). In the presence of MsrA, AS-IV reduced the MPP^{+} induced mitochondrial damage (Fig. 5B and C). By contrast, in the absence of MsrA, the protective effect of AS-IV was blocked (Fig. 5C and D). These data revealed that the protective effect of AS-IV against MPP^{+} induced mitochondrial fragmentation was dependent on MsrA (Fig. 5I).

Discussion

ROS are primarily generated by mitochondria. The ROS $\cdot O_2^-$ is produced by the ETC complexes I and III, released into the mitochondrial matrix and converted into H_2O_2 through dismutation by the antioxidant manganese superoxide dismustase (MnSOD) (33). However, excess $\cdot O_2^-$ damages Fe-S containing enzymes in the tricarboxylic acid cycle, disrupts ETC complexes and alters mitochondrial DNA (4,9,34). ROS damage cellular macromolecules, including DNA, proteins and lipids. Oxidation of free and protein-based methionine may alter protein structure and function, and cause mitochondrial dysfunction and the release of cytochrome C, ultimately leading to apoptosis (15,18,35). Increasing evidence indicates

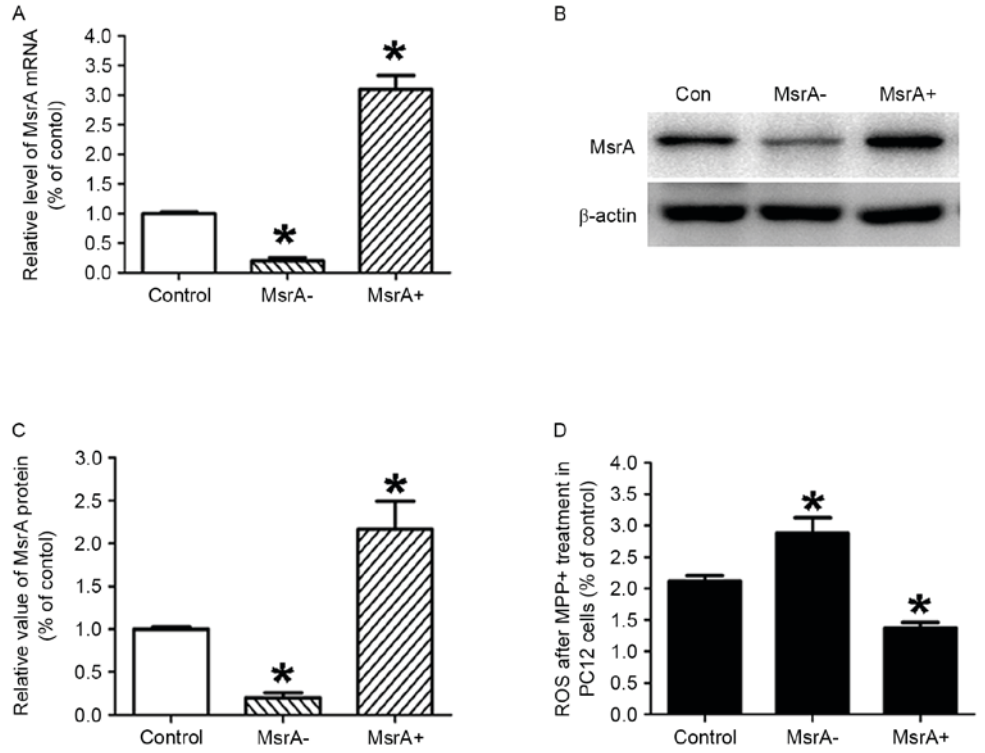

Figure 4. MsrA attenuates MPP^+-induced oxidative damage via exerting an antioxidant effect. (A) mRNA level of MsrA after MrsA- or MsrA+. (B) Western blot and (C) densitometric analysis of the protein levels of MsrA after MrsA- or MsrA+. (D) ROS levels in cells treated with 100 μM MPP^+ after MrsA- or MsrA+. $^*P<0.05$ vs. the control group (one-way analysis of the variance). MsrA, methionine sulfoxide reductase A; MrsA-, MrsA-specific small interfering RNA treatment; MrsA+, MrsA overexpression; MPP^+, 1-methyl-4-phenylpyridinium.

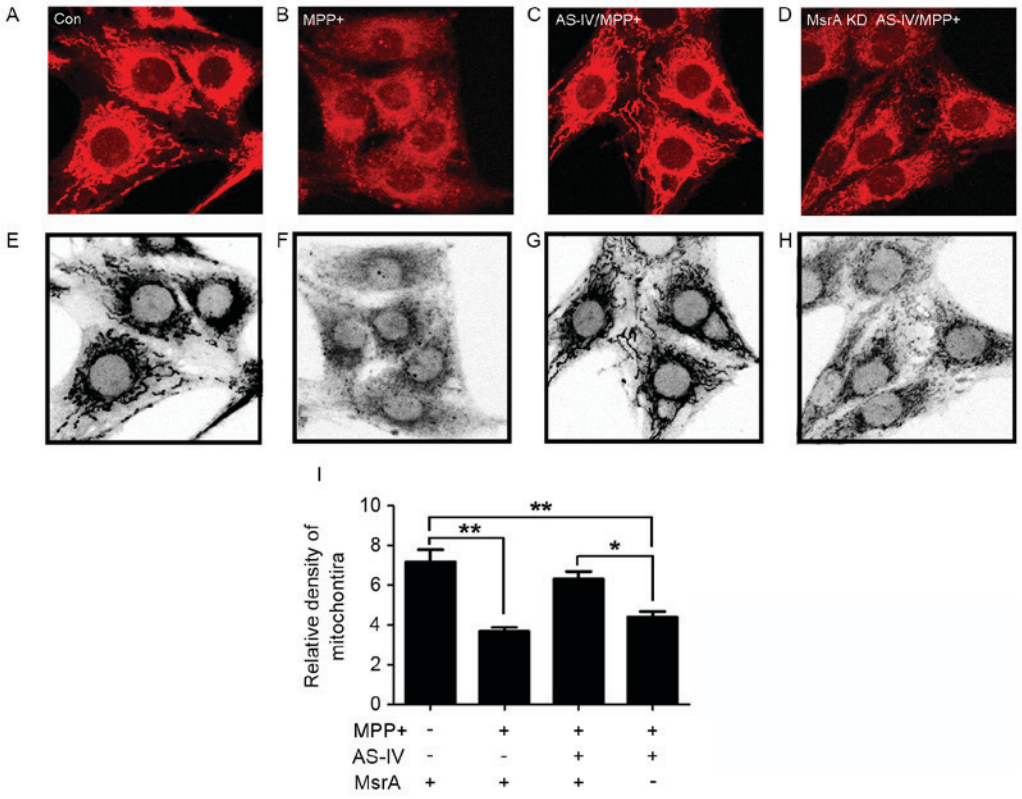

Figure 5. AS-IV reduced MPP^+-induced mitochondrial fragmentation via MsrA. Micrographs of (A) the control group, (B) the MPP^+ (100 μM) treatment group, (C) the AS-IV (50 μM) and MPP^+ treatment group, and (D) the MsrA-specific siRNA treatment group. The micrographs were converted to black and white images of (E) the control group, (F) the MPP^+ (100 μM) treatment group, (G) the AS-IV (50 μM) and MPP^+ (100 μM) treatment group, and (H) the MsrA-specific siRNA treatment group. (I) Quantification of relative mitochondrial density. $^*P<0.05$ and $^{**}P<0.01$ vs. the control group (one-way analysis of the variance). AS-IV, astragaloside IV; MPP^+, 1-methyl-4-phenylpyridinium; MsrA, methionine sulfoxide reductase A; siRNA, small interfering RNA; +, MsrA was existed and undisturbed in cells; -, MsrA in cells was knocked down by siRNA.

that oxidative stress is an etiological factor for numerous diseases, including PD, Alzheimer's disease and brain ischemia (4,9,36). Therefore, antioxidant intervention is a potential treatment option for these diseases.

MPP^+ is toxic and acts by interfering with the activity of tyrosine hydroxylase and thus oxidative phosphorylation in the mitochondria by inhibiting complex I of the ETC (37). MPP^+ causes increased electron leakage from the ETC, leading to the production of $\cdot O_2^-$ (24). MPP^+ thus exerts its toxic effects by decreasing ATP synthesis and inducing oxidative stress (37). MPP^+ can reproduce the clinical and pathological features of PD in animal models (25,27). MPP^+-induced neurodegeneration is specific to dopamine neurons and can be prevented by drug treatment with ginsenoside Rg1 or astragaloside IV (38,39). Recent studies have demonstrated that certain botanical extracts have an antioxidant effects against oxidative-stress induced damage and PD (38,40). Standardized extracts of ginsenoside Rg1 can efficiently terminate the toxic effects of MPTP in PD animal models (38). Rg1 activates the Wnt/β-catenin signaling pathway *in vivo* and *in vitro*, thus exerting a protective effect against neurodegeneration (38). AS-IV has been used as an effective treatment for ischemia-reperfusion injury, myocardial infarction and type 2 diabetes (21,39,41). The protective functions of AS-IV are attributed to an increased cellular content and activity of superoxide dismutase (SOD), and the inhibition of calcium pumps.

In a previous study, AS-IV was demonstrated to exert notable protective effects against MPP^+-induced oxidative damage in SH-SY5Y neuroblastoma cells (41). However, the antioxidant mechanism of AS-IV remains unclear. In present study, AS-IV treatment was identified to increase the expression of MsrA via the Sirt1-FOXO3a signaling pathway and protect against MPP^+-induced oxidative damage. These protective effects were dependent upon MsrA, indicating that the antioxidant effects of AS-IV are associated with MsrA. Previous studies have demonstrated that the loss of MsrA renders cells hypersensitive to damage by H_2O_2, nitrite and S-nitrosogluthatione, and results in the accumulation of higher levels of oxidized proteins (13,15,17,18,42). In addition, AS-IV has been identified to confer increased resistance to oxidative stress (18,21). These results indicate that AS-IV may regulate other potential antioxidative molecules, including SOD.

In the current study, AS-IV was observed to regulate mitochondrial function under conditions of oxidative stress via upregulating MsrA. The density of mitochondria was lower in the MsrA knockdown group compared with the control group under oxidative stress. AS-IV also prevented MPP^+-induced mitochondrial fragmentation via MsrA, while knocking down MsrA attenuated this protective effect. This effect may be attributed to the fact that MsrA protects mitofusin from oxidation and maintains normal mitochondrial function (43). Since mitochondria are the primary source of ROS and are a major target of oxidative damage, mitochondrial protein dysfunction likely increases MPP^+-induced oxidative damage. Thus, irreparable mitochondrial dysfunction may give rise to mitochondrial degradation, which ultimately leads to cell death.

In conclusion, the results of the present study indicate that AS-IV attenuates MPP^+-induced oxidative damage in PC12 cells. The protective effect of AS-IV was associated with the upregulation of MsrA. Thus, acting through MsrA, AS-IV enhances the resistance of the mitochondria to oxidative stress. Therefore, AS-IV is a potential drug for the treatment of oxidative damage-induced disease.

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