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S-Allylcysteine, a garlic compound, protects against oxidative stress in 1-methyl-4-phenylpyridinium-induced parkinsonism in mice☆

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Abstract

S-Allylcysteine (SAC), the most abundant organosulfur compound in aged garlic extract, has multifunctional activity via different mechanisms and neuroprotective effects that are exerted probably via its antioxidant or free radical scavenger action. The 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mouse has been the most widely used model for assessing neuroprotective agents for Parkinson's disease. 1-Methyl-4-phenylpyridinium (MPP^+) is the stable metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, and it causes nigrostriatal dopaminergic neurotoxicity. Previous studies suggest that oxidative stress, via free radical production, is involved in MPP^+-induced neurotoxicity. Here, we report on the neuroprotective effect of SAC against oxidative stress induced by MPP^+ in the striatum of C57BL/6J mice. Mice were pretreated with SAC (125 mg/kg ip) daily for 17 days, followed by administration of MPP^+ (0.72 mg/kg icv), and were sacrificed 24 h later to evaluate lipid peroxidation, different antioxidant enzyme activities, spontaneous locomotor activity and dopamine (DA) content. MPP^+ administration resulted in a significant decrease in DA levels in the striatum. Mice receiving SAC (125 mg/kg ip) had significantly attenuated MPP^+-induced loss of striatal DA levels (32%). The neuroprotective effect of SAC against MPP^+ neurotoxicity was associated with blocked (100% of protection) of lipid peroxidation and reduction of superoxide radical production — indicated by an up-regulation of Cu-Zn-superoxide dismutase activity — both of which are indices of oxidative stress. Behavioral analyses showed that SAC improved MPP^+-induced impairment of locomotion (35%). These findings suggest that in mice, SAC attenuates MPP^+-induced neurotoxicity in the striatum and that an antioxidant effect against oxidative stress may be partly responsible for its observed neuroprotective effects.

Keywords: Parkinson's disease; 1-methyl-4-phenylpyridinium; S-Allylcysteine; Dopamine; Neuroprotection; Antioxidant defense

1. Introduction

Numerous sulfur-containing compounds derived from garlic (*Allium sativum*) have been reported to have various biological actions. S-Allylcysteine (SAC), the most abundant organosulfur compound in aged garlic extracts [1], has multiple beneficial effects, such as anticancer action [2], cholesterol-lowering action [3] and antioxidant and radical scavenging effects [4,5].

SAC is known to scavenge superoxide radicals [6] and to neutralize hydrogen peroxide (H_2O_2) [7], thereby preventing H_2O_2-induced cell damage and lipid peroxidation (LP) [7], as well as low-density lipoprotein oxidation [8]. SAC protective actions in the central nervous system include inhibition of neuronal damage induced by ischemia in rat brain, probably involving mechanisms linked to the inhibition of LP [9], ameliorating learning deficits in senescence-accelerated mice [10]. SAC protects against amyloid-β peptide-induced oxidative damage [11], provides neuroprotection against 3-nitropropionic acid-induced neurotoxicity and oxidative stress [12], apoptosis [13], and has neurotrophic actions on hippocampal neurons [14].

Parkinson's disease (PD) is a neurological disorder characterized by degeneration and death of the dopaminergic neurons of the nigrostriatal pathway in the brain [15]. Death of these neurons produces a decrease in striatal dopamine (DA) content. The cause of this neuronal loss is unclear, but there is increasing evidence suggesting that oxidative stress, via free radical production, plays an important role in the process [16].

Free radicals are produced constitutively under normal physiological conditions. Organisms have developed various defense mechanisms to protect themselves against injury from free radicals.

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Such defense mechanisms include antioxidant enzymes, free radical scavengers and metal chelating agents [17]. The antioxidant enzymes are catalase, glutathione peroxidase (GPx) and superoxide dismutase (SOD). Normally, there is a balance between the generation of free radicals and antioxidant defense system activity *in vivo*. When this balance is altered to favor production of free radicals due to depletion of antioxidant system components or increased generation of free radicals, oxidative stress occurs [18]. Oxidative stress leads to damage of polyunsaturated lipids by LP, a chain reaction that results in numerous degradation products as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) [19]. Postmortem studies on patients with PD have reported alterations in the substantia nigra: the level of iron is elevated [20], there is loss of glutathione [21] and there is increased of LP [22].

The therapeutic efficacy of new antiparkinsonian drugs is usually assessed using 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). MPTP is a dopaminergic toxin that causes nigral cell loss and clinical symptoms similar to those of PD. Its active metabolite, 1-methyl-4-phenylpyridinium ion (MPP^+), is taken up in dopaminergic terminals by the plasma membrane DA transporter and is accumulated in mitochondria [23]. Both MPTP- and MPP^+-induced toxicity are linked, in part, to oxidative stress, via the production of reactive oxygen species (ROS) [24,25]. Incubation of MPP^+ with mitochondrial enzymes induces free radical production [26], and the increase in free radicals can further inhibit the function of complex I [27]. Several antioxidants (radical scavengers) have been reported to protect against MPP^+- and MPTP-induced neurotoxicity [28–30]. Based on the above, it appears reasonable to propose that exogenous antioxidants may be effective in diminishing the cumulative damaging effects of oxidative stress in PD and MPP^+ neurotoxicity.

This study investigated the neuroprotective effect of SAC against neurotoxicity induced by MPP^+ in the striatum using different research tools related to evaluate oxidative stress. We analyzed ROS production, LP thiobarbituric acid-reactive substances (TBA-RS) and "MDA+4-HNE" and different antioxidant enzyme activities, such as Mn-SOD, Cu,Zn-SOD, GPx and glutathione reductase. Spontaneous locomotor activity and DA analyses were also performed.

2. Materials and methods

2.1. Chemicals

SAC was synthesized by the reaction of L-cysteine with allyl bromide and purified by recrystallization from ethanol–water [31]. The final product was compared with its identification with SAC standard by ^1H nuclear magnetic resonance spectroscopy, and its *in vitro* antioxidant activity was confirmed by measuring its scavenging activity on $O_2^{-\bullet}$ and H_2O_2, according to a previous report [31]. MPP^+, sodium octyl sulfate, sodium metabisulfite, glutathione reductase, NADPH, homovanillic acid (HVA), DA and 1-methyl-2-phenylindole were obtained from Sigma-Aldrich (St. Louis, MO, USA). Perchloric acid, TBA, EDTA (Merck, Darmstadt, Germany), polyclonal anti-Cu,Zn-SOD and polyclonal anti-Mn-SOD antibodies (Stressgen Biotechnologies, Victoria, British Columbia, Canada); a chemiluminescence detection system (Amersham, Piscataway, NJ, USA); and 2,7-dichlorofluorescein diacetate (DCFH-DA) (Cayman Chemical Company, Ann Arbor, MI, USA) were used for our experiments. All other reagents were of reagent grade and obtained from known commercial sources. Solutions were prepared using deionized water obtained from a Milli R/Q purifier system (Millipore). An Adsorbosphere (Alltech Associates, Deerfield, IL, USA) catecholamine analytical column was used.

2.2. Animals

Experiments were conducted on male C57BL/6J mice (Harlan, Mexico) at 11–13 weeks of age. Animals were maintained in standard conditions (12:12 h light/dark cycle, 21°C ±2°C) and in 40% relative humidity, and they were allowed access to food and water *ad libitum*. All experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (USA), approved by the local ethics committee and conformed to regulations specified by the animal care and use committee of our institution and the standards of the National Institutes of Health of Mexico (NOM-062-ZOO-1999). Animals were treated humanely to minimize pain or discomfort.

2.3. SAC pretreatment and MPP^+ administration

There were four treatment groups: Group I, intraperitoneal saline solution plus intracerebroventricular saline solution; Group II, intraperitoneal SAC plus intracerebroventricular saline solution; Group III, intraperitoneal saline solution plus intracerebroventricular MPP^+; and Group IV, intraperitoneal SAC plus intracerebroventricular MPP^+. Animals of Groups I and III received normal saline solution (intraperitoneal), and groups II and IV received SAC (125 mg/kg ip) daily for 17 days. This dose of SAC has been shown previously to produce protection in other models [32]. After pretreatment, animals of Groups III and IV were anesthetized and given 3 μl of a solution containing 18 μg of MPP^+ (0.72 mg/kg), injected into the right lateral ventricle (intracerebroventricular) as described previously [33]. We have reported previously that this acute dose of MPP^+ produces damage to striatal dopaminergic neurons [34]. Mice from Groups I and II, injected with saline solution (intracerebroventricular), served as controls. Mice were sacrificed 24 h after saline or MPP^+ administration, and their striata were obtained to measure DA content, spontaneous locomotor activity, LP and antioxidant enzyme activities. Additional groups of animals treated as described above were sacrificed 30 min (to measure ROS content) or 24 h (to analyze the MDA+4-HNE level) after treatment.

2.4. Analysis of striatal DA content

SAC neuroprotective effect against MPP^+-induced loss of striatal DA content was analyzed after treatment. Following sacrifice of the mice, their brains were immediately removed and the corpora striata were dissected out. The tissue concentrations of DA, 4-dihydroxyphenylacetic acid (DOPAC) and HVA were determined according to a method previously described [33]. Briefly, an aliquot (500 μl) of perchloric acid–sodium metabisulfite solution (0.1% w/v) was added to the weighed tissue and sonicated with a Lab-Line Ultratip Labsonic System (Lab-Line Instruments, Melrose Park, IL, USA). Samples were then centrifuged at 4000*g* for 10 min. Striatal content of DA was determined using an LC 200 HPLC system (Perkin-Elmer, Shelton, CT, USA) with a BASi LC-4C electrochemical detector (BASi, West Lafayette, IN, USA). The detector potential was adjusted to 0.8 V vs. Ag/AgCl reference electrode. The mobile phase consisted of aqueous phosphate buffer (pH 3.1), which contained 0.2 mM sodium octyl sulfate, 0.1 mM EDTA and 15% (v/v) methanol. An Adsorbosphere catecholamine analytical column of 100×4.8 mm with 3-μm particle diameter was used (Alltech Associates). Calibration curves were constructed for DA, DOPAC and HVA, and their concentrations were obtained by interpolation of the respective standard curves. The results are expressed as micrograms of compound per gram of tissue.

2.5. Spontaneous locomotor activity

Because alterations in striatal DA signaling are predicted to alter striatal function and therefore affect motor output, locomotor activity was investigated as previously reported [35]. Four groups of mice (n=8–10) were assigned as described previously. Spontaneous locomotor activity was measured 24 h after saline or MPP^+ administration in animals previously treated with saline or SAC.

Spontaneous locomotor activity was measured by an Opto-Varimex-Minor activity meter (Columbus Instruments, Columbus, OH, USA). The system uses sensors with high-intensity, modulated infrared light beams to detect animal motion. Locomotor activity associated with ambulatory locomotion was defined as the total distance traveled in 1 h. The activity meter consists of an array of 15 infrared emitter–detector pairs, spaced at 2.54-cm intervals, measuring activity along a single axis of motion. This device differentiates nonambulatory movements (scratching, gnawing) from ambulation (activity traveled) on the basis of consecutive interruption of the infrared beams in the horizontal plane (15 on each axis).

All experiments were conducted by one operator in the light phase of the circadian cycle, between 9 a.m. and 3 p.m. A habituation period of 1 h to the conditions of the experimental room preceded the experimental procedures. During the activity measurements, animals had no access to food or water.

After acclimatization to the experimental room, mice were individually tested in a dimly lit, sound-controlled room. The mice were removed from their home cages and placed into the cage activity system. Thereupon, the data collection system was immediately activated and the spontaneous locomotor activity of the mice was recorded for 1 h.

2.6. ROS analysis

Oxygen free radicals, more generally known as ROS, can interact with cellular lipids, proteins and DNA producing cellular dysfunction and sometimes cell death. ROS also cause LP. Four groups of mice (n=5–7) were assigned as described previously to analyze ROS.

ROS production was measured in striatal homogenate by a modified spectrophotometric method [36]. ROS formed in the homogenate were followed by measuring the conversion of DCFH-DA, a dichlorofluorescein (DCF). Homogenate was mixed with phosphate-buffered saline to a final dilution of 1%, followed by addition of DCFH-DA to a final concentration of 5 μM, and incubated for 30 min. DCF formation was detected at 500 nm in a Beckman spectrophotometer. Data are expressed as optical density at 500 nm per milligram of protein.

2.7. LP analysis

We used two methods to analyze LP: (1) TBA-RS and (2) 4-HNE+MDA, which are highly toxic products of LP. Four groups of mice (n=5–7) were assigned as described previously.

2.7.1. TBA-RS analysis

LP was measured by TBA-RS assay as an accurate method to characterize oxidative damage to membrane lipids. LP, an index of oxidative stress, was evaluated as reported previously [34]. Four groups of mice (n=8–10) were assigned as described previously. Briefly, striatum tissue was homogenized in 2 ml of ice-cold 0.05 M phosphate buffer (pH 7.0) containing 0.015 M NaCl and 0.145 M KCl. One-milliliter aliquots of this homogenate were added to 2 ml of the TBA reagent (0.5 g of TBA plus 15 g of trichloroacetic acid plus 2.5 ml of concentrated HCl in 100 ml of water), and the solution was heated for 30 min in a boiling water bath. After cooling and centrifugation (2000*g*, 10 min), the absorbance was read on a DU-6 Beckman spectrophotometer at 532 nm. Final amounts of TBA-RS, mostly MDA, were calculated by interpolation of values in a standard curve and corrected by the content of protein per sample [37]. All samples were analyzed in duplicate, and results are expressed as nanomoles of TBA-RS per milligram of protein.

2.7.2. Determination of MDA+4-HNE

MDA and/or 4-HNE reacts to 1-methyl-2-phenylindole under acidic conditions to yield a stable chromophore with intense maximal absorbance at 586 nm [38]. Two hundred microliters of the striatum homogenate was added to 650 μl of a solution of 1-methyl-2-phenylindole in acetonitrile/methanol (3:1). The final concentration of the reagent was 10 mM. The reaction was then started by adding 150 μl of concentrated methanesulfonic acid containing 34 μM Fe(III). The reaction mixture was incubated at 45°C for 30 min and measured upon 586 nm of absorbance. A standard curve was

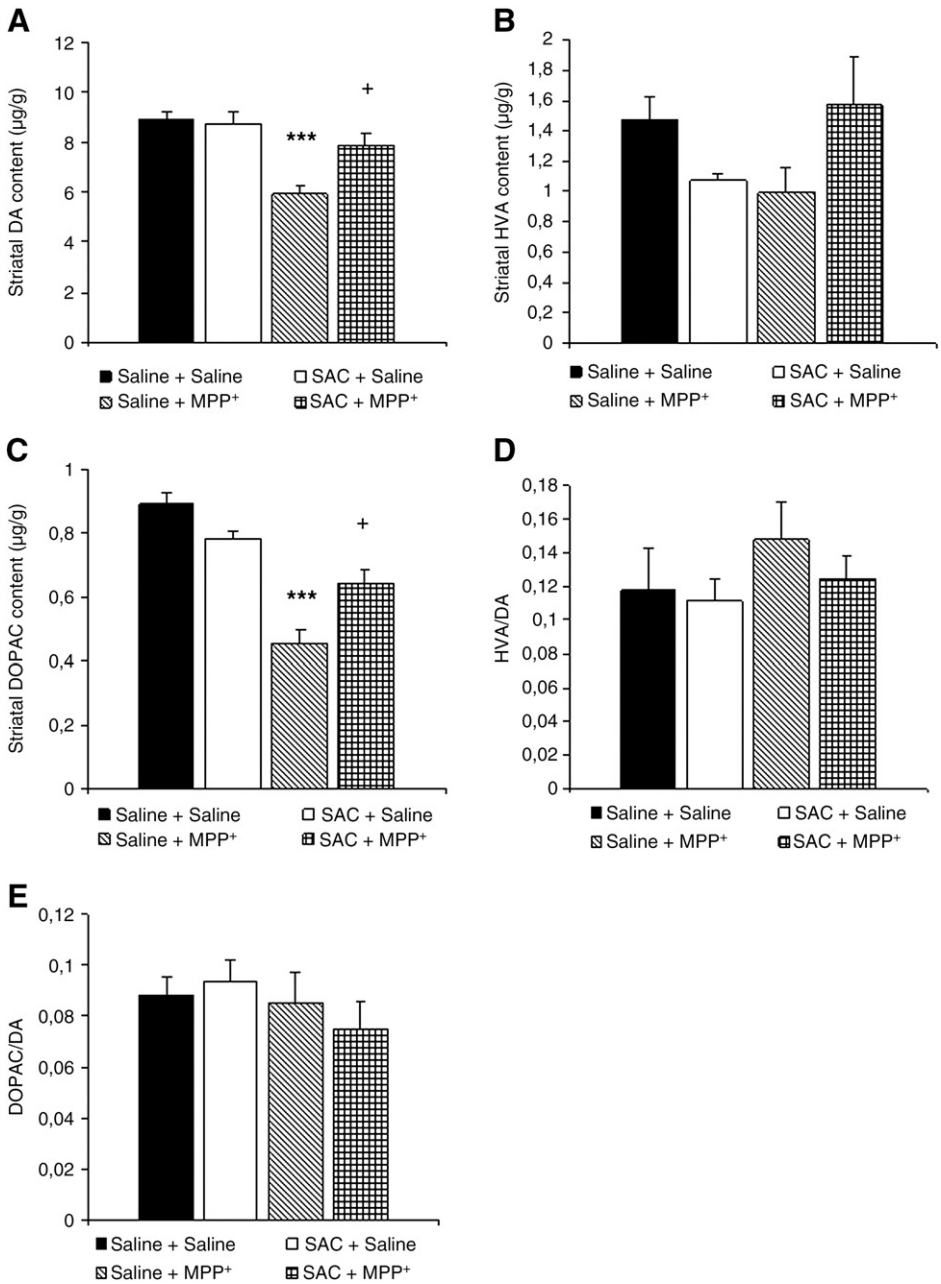

Fig. 1. SAC significantly protects animals from MPP^+-induced reductions in DA levels. Shown are the striatal DA (A), HVA (B), DOPAC (C) content, HVA/DA ratio (D) and DOPAC/DA (E) of animals treated with SAC in MPP^+ neurotoxicity. Eight to ten mice were used per group. Values are the mean±S.E.M. Differences were analyzed using one-way ANOVA followed by post hoc Duncan's test. ***Statistically different from the control group (Saline+Saline), P<.001, Duncan's test. $^+$Statistically different from the MPP^+-treated (Saline+MPP^+) group, P<.05, Duncan's test. SAC = S-allylcysteine; MPP+ = 1-methyl-4-phenylpyridinium ion; DA = dopamine; DOPAC = 4-dihydroxyphenylacetic acid; HVA = homovanillic acid.

performed with 1,1,3,3-tetramethoxypropane (0–23.7 nmol/ml), a source of MDA. Data are expressed as nanomoles of MDA+4-HNE per milligram of protein.

2.8. Antioxidant enzymes

Eight to ten mice per treatment group were used to analyze different antioxidant enzyme activities, such as Mn-SOD, Cu,Zn-SOD, GPx and glutathione reductase.

2.8.1. SOD activity

The production of superoxide radicals was determined by measuring the SOD activity in the striatum. Total SOD activity was assessed by a competitive inhibition assay previously reported by our group [39], using a xanthine/xanthine oxidase system to reduce nitroblue tetrazolium (NBT), which served as the indicator reagent. Briefly, the mixture reaction contained 0.122 mM EDTA, 30.6 μM NBT, 0.122 mM xanthine, 0.006% bovine serum albumin and 49 mM sodium carbonate, all of which are final concentrations. Tissue samples were homogenized in 9 ml/g of tissue and 50 mM phosphate buffer (pH 7.0)/Triton X-100 (1%). Five hundred microliters of striatal homogenates were added to 2.45 ml of the mixture described above, and then 50 μl of xanthine oxidase (final concentration=2.8 U/L) was added and incubated at 27°C for 30 min. Reactions were stopped with 1 ml of 0.8 mM cupric chloride, and optical density was recorded at 560 nm. The amount of striatal protein that inhibited 50% of maximal NBT reduction was defined as one unit of SOD activity. Mn-SOD was differentiated from Cu,Zn-SOD by inhibiting the latter with diethyldithiocarbamate (DDC). For these purposes, samples were incubated with 50 mM DDC at 30°C for 1 h and dialyzed for 3 h with three changes of 400 volumes of 5 mM phosphate-buffered saline (pH 7.8) with 0.1 mM EDTA. Cu,Zn-SOD activity was obtained by subtracting the activity of the DDC-treated samples from the total SOD activity. For these experimental procedures, protein concentrations were measured according to Lowry *et al.* [37]. All samples were analyzed in duplicate, and results are expressed as units of SOD activity per milligram of protein.

2.8.2. GPx activity

Striatal GPx activity was assayed by a method previously described [40]. The reaction mixture consisted of 50 mM potassium phosphate (pH 7.0), 1 mM EDTA, 1 mM sodium azide, 0.2 mM NADPH, 1 U/ml of glutathione reductase and 1 mM glutathione. One hundred microliters of a 1:3 dilution of striatal homogenate was added to 0.8 ml of reaction mixture and allowed to incubate for 5 min at room temperature before initiation of the reaction by the addition of 0.1 ml of 1.5 mM H_2O_2 solution. Absorbance at 340 nm was recorded with a Beckman DU640 spectrophotometer (Beckman Coulter, Fullerton, CA, USA) for 3 min. The activity was calculated from the slope of these lines as micromoles of NADPH oxidized per minute, noting that the millimolar absorption coefficient for NADPH is 6.22. Blank reactions were subtracted from each assay. The results are expressed as units per milligram of protein.

2.8.3. Glutathione reductase activity

Striatal glutathione reductase activity was assayed by using oxidized glutathione as substrate and measuring the disappearance of NADPH at 340 nm with a Beckman DU640 spectrophotometer, as previously reported [41]. The rate of oxidation of NADPH by glutathione disulfide at 30°C was used as a standard measure of the glutathione reductase activity. The reaction mixture of 1 ml contained the following: 1 mM glutathione disulfide, 0.1 mM NADPH, 0.5 mM EDTA, 0.10 M sodium phosphate buffer (pH 7.6) and a suitable amount of the glutathione reductase sample to give a change in absorbance of 0.05 to 0.30/min. The oxidation of 1 pmol of NADPH per minute under these conditions was used as a unit of glutathione reductase activity. The specific activity is expressed as units per milligram of protein.

2.8.4. Analysis of SOD protein by Western blot

In total, 8–10 mice were studied per treatment group. Striatal tissue was homogenized with protease inhibitors in 50 mM phosphate buffer (pH 7.4). Tissue homogenates were centrifuged at 1000*g* and 4°C for 10 min. Twenty-five micrograms of protein were fractionated by reducing 12.5% sodium dodecyl sulfate polyacrylamide gel for electrophoresis and electroblotted to a nitrocellulose membrane. Immunodetection was performed using primary antibodies specific for Mn-SOD or Cu,Zn-SOD (Stressgen Biotechnologies), as previously reported [40]. Hybrids were visualized by chemiluminescence using an enhanced chemiluminescence detection system (Amersham), followed by densitometric analysis using SigmaScan software (Aspire Software International, Ashburn, VA, USA).

2.9. Statistical analysis

The data are expressed as the mean±S.E.M. DA content, HVA, DOPAC, locomotor activity, ROS, LP, SOD, GPx and glutathione reductase activities and Western blot

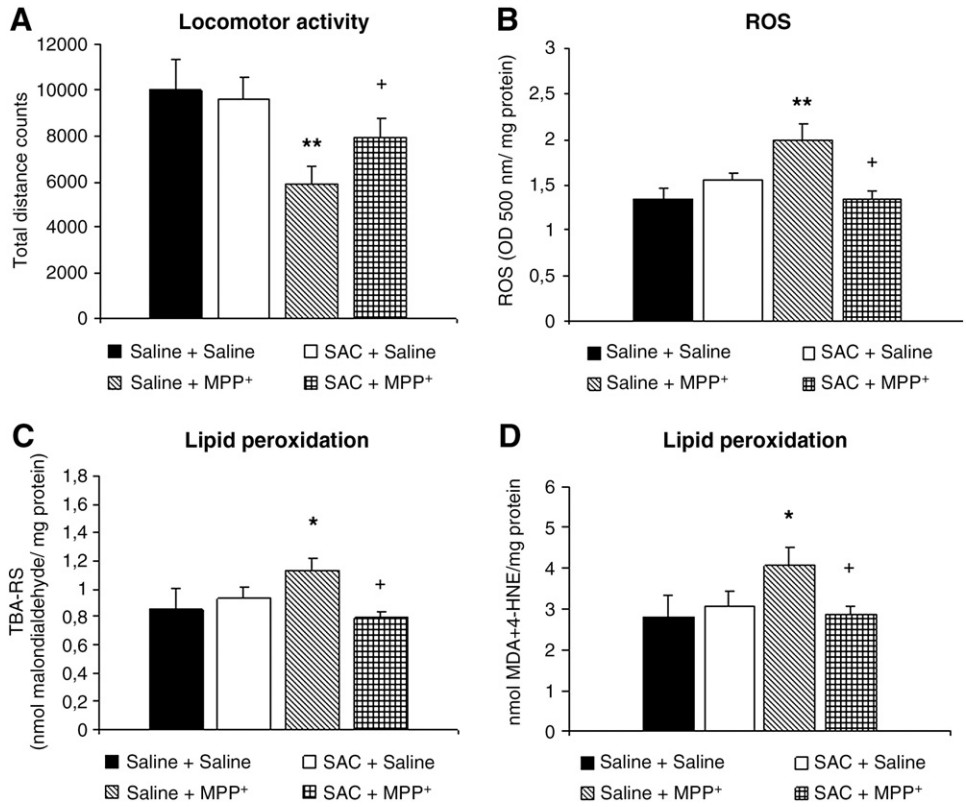

Fig. 2. SAC dramatically improves motor deficits against MPP^+-induced neurotoxicity (A), and its neuroprotective effect is linked to reduced ROS (B) and LP [analyzed by TBA-RS (C) and MDA+4-HNE (D) methods] in the striatum. Eight to ten mice were used per group. Values are the mean±S.E.M. Differences were analyzed using one-way ANOVA followed by post hoc Duncan's test. *Statistically different from the control group (Saline+Saline), $P<.05$, Duncan's test. **Statistically different from the control group (Saline+Saline), $P<.01$, Duncan's test. $^+$Statistically different from the MPP^+-treated (Saline+MPP^+) group, $P<.05$, Duncan's test. SAC = S-allylcysteine; ROS = reactive oxygen species; MPP+ = 1-methyl-4-phenylpyridinium ion; TBA-RS = thiobarbituric acid-reactive substances; MDA = malondyaldehyde; 4-HNE = 4-hydroxynonenal.

for SOD were analyzed using one-way analysis of variance (ANOVA), followed by post hoc Duncan's test. Values of $P<.05$, $P<.01$ and $P<.001$ were considered to be statistically significant.

3. Results

3.1. SAC protects striatal DA levels against MPP^+ neurotoxicity

The neuroprotective effect of SAC in preventing MPP^+-induced decreases in striatal DA levels was demonstrated by analyzing DA levels using HPLC. SAC administration to control mice did not produce a significant alteration in striatal DA content when compared with control animals ("Saline+Saline") (Fig. 1A). Mice in the Saline $+MPP^+$ group presented markedly reduced (34%) DA levels as a result of the neurotoxic action of the compound (Fig. 1A). Administration of SAC to MPP^+-treated mice significantly (32%) prevented the DA-depleting effect of the neurotoxin.

SAC administration to control mice did not produce changes in striatal HVA content when compared with control animals (Saline +Saline) (Fig. 1B). No significant changes of HVA in the striata of the Saline+MPP^+ and SAC+MPP^+ groups were produced as compared with the Saline+Saline group.

SAC administration to control mice did not produce changes in striatal DOPAC content when compared with control animals (Saline +Saline) (Fig. 1C). Mice in the Saline+MPP^+ group presented markedly reduced (50%) DOPAC levels as a result of the neurotoxic action of the compound (Fig. 1C). Administration of SAC to MPP^+-treated mice partially (41%) prevented the DOPAC-depleting effect of the neurotoxin.

No significant changes in HVA/DA and DOPAC/DA ratios were shown in any of the groups treated (Fig. 1D and E).

3.2. MPP^+-induced hypolocomotion is improved by SAC administration

The ultimate therapeutic goal of neuroprotection is to ameliorate functional impairment. Accordingly, we next determined whether the protection of nigral DA neurons was also accompanied by an absence of locomotor deficit, one important hallmark of PD.

The Saline+MPP^+ group displayed a significant reduction in locomotor activity (41%) when compared with non-MPP^+-treated control animals (Fig. 2A). In contrast, mice of the SAC+MPP^+ group significantly recovered locomotor activity (35%) as compared with MPP^+-damaged animals (Fig. 2A).

3.3. SAC protects against enhancement of ROS production induced by MPP^+ administration

The amount of DCF in striatal tissue is shown in Fig. 2B. We have shown that SAC alone had no effect on ROS levels when compared with the control group (Saline+Saline). A significant increase (60%) in ROS formation was found in the Saline+MPP^+ group. In contrast, ROS level was blocked (100% of protection) in the SAC+MPP^+ group.

3.4. Inhibition of LP is associated with the neuroprotective effect of SAC

The TBA-RS assay was performed as an index of LP to examine whether the neuroprotective effect of SAC during MPP^+ neurotoxicity

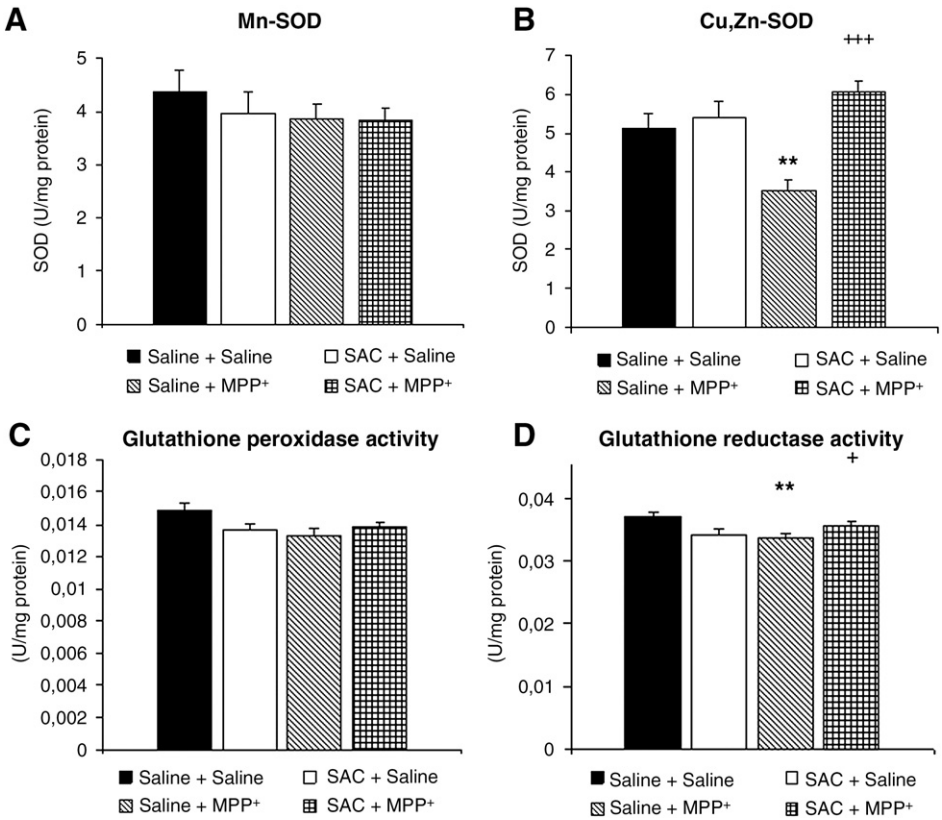

Fig. 3. The neuroprotective effect of SAC against MPP^+-induced neurotoxicity is possibly associated with a reduction of superoxide radical production indicated by an up-regulation of Cu,Zn-SOD activity. SAC did not change Mn-SOD (A) or GPx (C) in the striatum in parkinsonism induced by MPP^+. SAC up-regulates Cu,Zn-SOD in the striatum in parkinsonism induced by MPP^+ (B); the glutathione reductase activity (D) was reduced in the Saline+MPP^+ group but enhanced in the SAC+MPP^+ group. Eight to ten mice were used per group. Values are the mean±S.E.M. Differences were analyzed using one-way ANOVA followed by post hoc Duncan's test. **Statistically different from the control group (Saline+Saline), $P<.01$, Duncan's test. $^{+}$Statistically different from the MPP^+-treated (Saline+MPP^+) group, $P<.05$, Duncan's test. $^{+++}$Statistically different from the MPP^+-treated (Saline+MPP^+) group, $P<.001$, Duncan's test. SAC = S-allylcysteine; MPP+ = 1-methyl-4-phenylpyridinium ion; Mn-SOD = Mn-superoxide dismutase; Cu,Zn-SOD = Cu,Zn-superoxide dismutase.

was related to the prevention of oxidative stress. We demonstrated that SAC administration to mice produces no significant increases in LP (Fig. 2C) when compared with the control group (Saline+Saline). As shown in Fig. 2C, LP was significantly increased (34%) in the Saline +MPP^+ group. In contrast, LP was blocked (100% of protection) in the SAC+MPP^+ group.

Fig. 2D presents products of LP, such as MDA+4-HNE. SAC alone did not affect LP when compared with the control group (Saline +Saline). MPP^+ administration enhanced the striatal LP (46% vs. control group), and such effect was blocked (100% of protection) in the SAC+MPP^+ group.

3.5. Changes in different antioxidant enzymes in the neuroprotective effect of SAC

To explore the potential antioxidant effect of SAC against MPP^+ neurotoxicity, we investigated whether SAC treatment was associated with changes in the activity of antioxidant enzymes, such as Mn-SOD, Cu,Zn-SOD, GPx and glutathione reductase.

No changes were found in Mn-SOD activity across all treatment groups (Fig. 3A). Cu,Zn-SOD activity (Fig. 3B) was reduced in the Saline+MPP^+ group (32%) as compared with Saline+Saline group; the SAC+MPP^+ group showed an enhancement of 72% in Cu,Zn-SOD as compared with the Saline+MPP^+ group.

The protein content of Mn-SOD and Cu,Zn-SOD in the striatum, measured by Western blot, is shown in Fig. 4. The content of Mn-SOD and Cu,Zn-SOD remained unchanged in the four groups of mice studied except for Cu,Zn-SOD in the "SAC+Saline" group, which was enhanced (20%) as compared with that in the Saline +Saline group.

No changes were found in GPx activity in any of the groups treated (Fig. 3C). SAC administration alone did not modify the striatal basal activity of glutathione reductase (Fig. 3D). In contrast, glutathione reductase activity was reduced in the Saline+MPP^+ (10%) group and was enhanced significantly (6%) in the SAC+MPP^+ group as compared with the control group (Saline+Saline).

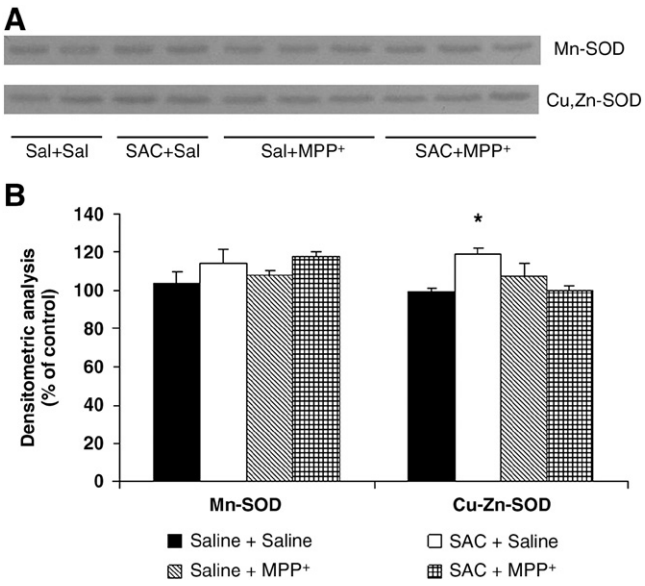

Fig. 4. (A) Representative Western blots of Mn-SOD and Cu,Zn-SOD protein levels in the neuroprotective effect of SAC in MPP^+ neurotoxicity in the striatum. (B) Densitometric analysis of Western blots of Mn-SOD and Cu,Zn-SOD in striatal tissue. Eight to ten mice were used per group. Values are the mean±S.E.M. Differences were analyzed using one-way ANOVA followed by post hoc Duncan's test. *Statistically different from the control group (Saline+Saline), $P<.05$, Duncan's test.

4. Discussion

SAC, the most abundant organosulfur compound in aged garlic extract, has multifunctional actions via different mechanisms, including neuroprotective effects, exerted probably via its antioxidant or free radical scavenger action. This study describes for the first time some protective effects of SAC on different markers of MPP^+-induced neurotoxicity and oxidative damage in the mouse corpus striatum. This protective action might be directly related to the antioxidant actions that SAC exerts in the brain, such as a decrease in striatal LP, preservation of Cu,Zn-SOD activity and improvement of MPP^+-induced impairment of locomotion and DA levels.

It has been reported that SAC administration protects partially DA levels in synaptosomal fractions against MPTP neurotoxicity [32]. This study evaluated the real neuroprotective effect of SAC using MPP^+, the stable metabolite of MPTP, which produces the neurotoxicity. We found that SAC protection is not due to a lower delivery of MPTP to the brain as could have been the case in a previous study [32]. To avoid this experimental problem, we injected MPP^+ directly in the brain and still observed neuroprotection by SAC.

We also evaluated in this study the SAC protective effect in the whole striatal tissue, which includes the glia, neurons and synaptic cleft. Our data demonstrate that SAC can provide effective protection against the damage to midbrain DA neurons arising from the neurotoxic effects of MPP^+ *in vivo*. This is clearly shown by the fact that the administration of SAC to MPP^+-injected animals (SAC +MPP^+ group) resulted in significantly attenuated MPP^+-induced loss of striatal DA levels (32%). This similar pattern has been shown by our group in the neuroprotection induced by a *Ginkgo biloba* extract (EGb 761), a well-known antioxidant and free radical scavenger, in MPP^+ neurotoxicity [39].

LP, an index of oxidative stress, may damage the cell membrane and thereby alter biophysical characteristics of the cell membrane, such as membrane fluidity and conformation of protein sulfhydryl groups. LP may also damage receptors and ion channels in the cell membrane [42], which may result in calcium influx and cause cell death.

With regard to the oxidative stress hypothesis, we previously found enhanced LP, a process dependent on free radical overproduction, to be a consequence of MPP^+ administration to mice [34]. In this study, the presence of elevated LP, indicated by TBA-RS and MDA+4-HNE content, was detected after MPP^+ administration. SAC blocked LP in the MPP^+-treated group. This suggests that the neuroprotective effect of SAC is due, at least in part, to its antioxidant properties [6,7]. Several studies in the central nervous system have reported that the protective actions of SAC are related to its antioxidant properties by reduction of edema formation in ischemic rat brain through the inhibition of LP [9], neuroprotection against excitotoxic and oxidative damage induced by quinolinic acid in rat brain [11] and that SAC prevents amyloid-B peptide-induced oxidative stress in rat hippocampus [11]. Our findings also support the antioxidant effect of SAC in MPP^+ neurotoxicity, because we found that pretreatment of SAC blocked ROS production in this model of PD.

We focused on thiol-containing compounds found in garlic (*A. sativum*), such as SAC, because various thiol compounds are known to prevent LP [43,44]. In particular, LP inhibitors were reported to have beneficial effects against MPTP/MPP^+ dopaminergic neurotoxicity [39].

In support of the free radical scavenger hypothesis of SAC in MPP^+ neurotoxicity, Cu,Zn-SOD activity was reduced in response to MPP^+ neurotoxicity and was enhanced in the SAC+MPP^+ group to prevent the early alterations produced by MPP^+ neurotoxicity. From our results, it is also clear that another contributing factor to the

protective actions exerted by SAC on MPP^+-induced neurotoxicity is the preservation of Cu,Zn-SOD activity, the enzyme responsible for efficient superoxide radical removal. We suggest that changes in Cu, Zn-SOD activity are due to a structural modification of this enzyme, but not to a reduction in the amount of the enzyme, as shown by the lack of changes in the Western blot analysis. This kind of modifications in proteins may be due to the toxic action of ROS [45]. The protective action of SAC observed on Cu,Zn-SOD could be a consequence of effective removal of superoxide radical production preventing oxidative stress in the SAC+MPP^+ group.

This suggests indirectly that an early mechanism of SAC protection in MPP^+ neurotoxicity is acting as a superoxide radical scavenger and enhancing Cu,Zn-SOD activity. Our results show that Cu,Zn-SOD and Mn-SOD activities are differently regulated by SAC in MPP^+ neurotoxicity. Further supporting evidence includes the observation that the pretreatment of mice with DDC, a SOD inhibitor, enhances MPTP-induced neurotoxicity [46], whereas transgenic mice that overexpress human Cu,Zn-SOD, showing increased SOD activity, are reported to be more resistant to MPTP than wild-type mice [47].

SAC did not stimulate GPx and glutathione reductase activities in the absence of neurotoxicity. Enhancement of glutathione reductase activity in the SAC+MPP^+ group may have provided some protection against neurotoxicity.

We propose that the antioxidant effect of SAC in MPP^+ neurotoxicity is due primarily to the scavenging of superoxide free radicals. SAC has been shown to scavenge superoxide anion [6,31,48] and neutralize hydrogen peroxide (H_2O_2) [7,31], preventing LP [7].

As alterations in striatal DA signaling are predicted to alter striatal function and therefore affect motor output, the locomotor deficits observed in MPP^+ neurotoxicity were restored by SAC, probably due to partial protection of striatal DA levels.

On the other hand, many adverse reactions to garlic can be attributed to an excess of oil-soluble organosulfur constituents, such as diallyl sulfide, diallyl disulfide and allicin. Oil-soluble sulfur compounds are known irritants and allergens; topically applied diallyl sulfide is the most allergenic [49]. Imada [50] reported the following toxicity effects of garlic: (1) allicin is one of the major irritants in raw garlic; (2) oil-soluble sulfur compounds are more toxic than water-soluble compounds; and (3) when garlic is extracted in a certain period, its toxicity is greatly reduced.

Aged garlic extract is an odorless product created through prolonged extraction of fresh garlic. This extract is aged for up to 20 months. During this aging process, the odorous, harsh and irritating compounds (many volatile and unstable chemical compounds, such as allicin) are converted to more stable compounds and safe sulfur compounds, such as SAC [49]. Aged garlic extract has been widely studied in various pharmacological and biological areas, including cancer, cardiovascular, immunological, metabolism and other categories. SAC is a nontoxic, water-soluble organosulfur compound. It is considered as one of the important biologically active constituents of garlic. SAC has come under extensive study in the light of its anticancer effects both *in vitro* and *in vivo* [51].

A number of toxicological and clinical studies of aged garlic extract, a product standardized for SAC, have been performed with no adverse effects (for a review, see Ref. [52]). The safety has been well established by the following studies: (1) acute and subacute toxicity; (2) chronic toxicity test; (3) mutagenicity tests; (4) general toxicity tests; (5) teratogenicity tests (Segments I–III); (6) toxicity test conducted by the U.S. Food and Drug Administration; and (7) clinical studies conducted on 1000 subjects. The U.S. National Cancer Institute tested the toxicity of SAC vs. other typical garlic compounds and found that it has 30-fold less toxicity than allicin and diallyl disulfide [50]. This shows that SAC is a safe compound because of its reduced toxicity as confirmed by toxicological studies and the long history of human consumption [49]. Extraction procedures used to obtain the favorable compounds of herbal material to use for health benefits are an important key. For example, commercially available *G. biloba* extract is designed to eliminate ginkgolic acid, which may cause allergic reactions.

In our study, the administration schedule of SAC is a short-term treatment to analyze its protective effect in MPP^+ neurotoxicity. However, no toxic signs have been observed after 6 months of daily aged garlic administration (2000 mg/kg), a product standardized for SAC [53]. Further studies are necessary to explore a long-term treatment in the MPP^+ model to be able to compare it in neurodegenerative diseases.

One of the important considerations in developing drug therapies for patients with PD is to prevent the potential side effects during or after long-term administration. In addition, there is worldwide interest in finding new and safe antioxidants from natural resources to prevent oxidative deterioration of living cells. The use of synthetic antioxidants has decreased due to their suspected activity as carcinogenesis promoters, together with a general consumer rejection of synthetic food additives.

In addition, SAC is widely used, has a very impressive clinical safety record and rapidly crosses the blood–brain barrier when peripherally administered [9], making it a seemingly excellent candidate for further investigation of its usefulness in the treatment of PD.

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