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S-Allylcysteine prevents the rat from 3-nitropropionic acid-induced hyperactivity, early markers of oxidative stress and mitochondrial dysfunction

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Abstract

We investigated the effects of *S*-allylcysteine (SAC) on early behavioral alterations, striatal changes in superoxide dismutase (SOD) activity, lipid peroxidation (LP) and mitochondrial dysfunction induced by the systemic infusion of 3-nitropropionic acid (3-NPA) to rats. SAC (300 mg/kg, i.p.), given to animals 30 min before 3-NPA (30 mg/kg, i.p.), prevented the hyperkinetic pattern evoked by the toxin. In addition, 3-NPA alone produced decreased activities of manganese- (Mn-SOD) and copper/zinc-dependent superoxide dismutase (Cu,Zn-SOD), increased LP (evaluated as the formation of lipid fluorescent products) and produced mitochondrial dysfunction in the striatum (measured as decreased 3-(3,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction). In contrast, pretreatment of 3-NPA-injected rats with SAC resulted in a significant prevention of all these markers. Our findings suggest that the protective actions of SAC are related with its antioxidant properties, which in turn may be accounting for the preservation of SOD activity and primary mitochondrial tasks.

Keywords: 3-Nitropropionic acid; *S*-Allylcysteine; Garlic-derived compounds; Superoxide dismutase; Hyperactivity; Mitochondrial reducing activity; Antioxidant defense

1. Introduction

3-Nitropropionic acid (3-NPA) is a naturally occurring fungal toxin that interrupts mitochondrial electron transport through the inhibition of succinate dehydrogenase, thereby producing cellular energy deficits and neurodegenerative events in mammals (Ludolph et al., 1991; Wüllner et al., 1994; Alexi et al., 1998). Although the precise mechanism by which the experimental and human exposure to 3-NPA results in neuronal degeneration is not yet perfectly understood, experimental evidence demonstrate that its systemic administration to animals produces selective striatal lesions involving secondary excitotoxic events through the activation of glutamate receptors (Beal et al., 1993; Fu et al., 1995a; Zeevalk et al., 1995; Greene et al., 1998), which in turn might account for acute necrotic and delayed apoptotic cell death pathways (Pang and Geddes, 1997), involving calpain as a central effector (Bizat et al., 2003). In addition, oxidative stress has been reported to play an important role in 3-NPA-induced neurotoxicity (Fu et al., 1995b; Schulz et al., 1996; Fontaine et al., 2000), as the toxin produces depleted levels of reduced glutathione (GSH), altered profiles of antioxidant enzyme activities and increased levels of reactive

Abbreviations: ANOVA, analysis of variance; DDC, diethyldithiocarbamate; GSH, glutathione; LFP, lipid fluorescent products; LP, lipid peroxidation; MTT, 3-(3,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NBT, nitroblue tetrazolium; 3-NPA, 3-nitropropionic acid; RFI, relative fluorescence intensity; SAC, *S*-allylcysteine; SOD, superoxide dismutase

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oxygen/nitrogen species (ROS/RNS) in the lesioned striatum (Binienda et al., 1998; Nam et al., 2005). These observations have been reinforced by the successful use of several endogenous and exogenous antioxidants against the 3-NPA-induced neuronal damage in rats (Fontaine et al., 2000; Nam et al., 2005). These findings lead us to suggest that oxidative stress, which develops at the early stages of 3-NPA toxicity in combination with excitotoxicity and mitochondrial dysfunction, will finally lead to neuronal damage (Alexi et al., 1998).

In particular, superoxide dismutase (SOD) is an endogenous antioxidant enzyme known to be responsible of protecting cells by detoxifying superoxide anions ($O_2^{\bullet -}$), which in turn are known to mediate oxidative damage. Superoxide anions are produced as result of electron leakage from the electron transport chain and other oxidative insults (Iqbal and Whitney, 1991). In the brain, SOD represents a major antioxidant defense system through its two isoforms, copper,zinc- (Cu,Zn-SOD) and Mn-dependent-SOD (Mn-SOD), where the first one is typically located in the cytosolic domain and the second is found in mitochondria. The study of the activity of both isoforms is relevant because their alterations represent an accurate mean to evidence risk of oxidative damage. Indeed, one of the most common expressions of oxidative stress is lipid peroxidation (LP), a deleterious process modifying both the structure and function of membrane lipids through the action of reactive oxygen species (ROS) and reactive nitrogen species (RNS).

Aged garlic extract compounds possess antioxidant and neuroprotective properties already tested under several experimental conditions. In particular, *S*-allylcysteine (SAC), the most abundant organosulfur molecule in garlic extracts, has been shown to exert antioxidant effects through its ability to scavenge ROS (Kim et al., 2001), thus reducing oxidative stress. The broad spectrum of positive actions of SAC in the central nervous system includes the reduction of edema formation in ischemic rat brain through the inhibition of LP (Numagami and Ohnishi, 2001), the amelioration of learning deficits in senescence-accelerated mice (Nishiyama et al., 2001), induction of neurotrophic actions in cultured rat hippocampal neurons (Moriguchi et al., 1997), reduction of Aβ-peptide-induced hippocampal neurotoxicity (Ito et al., 2003; Pérez-Severiano et al., 2004a) and neuroprotection against excitotoxic and oxidative damage induced by quinolinic acid in rat striatum (Pérez-Severiano et al., 2004b). Moreover, we recently demonstrated the ability of SAC to prevent 3-NPA-induced LP and mitochondrial dysfunction in rat brain synaptosomes (Pérez-De La Cruz et al., 2006). Thus, considering all the evidence together, we decided to investigate whether this antioxidant may also evoke positive responses in a toxic model produced by 3-NPA under in vivo conditions. For this purpose, in this work we evaluated the efficacy of SAC to prevent early changes in SOD activity, peroxidative damage to lipids and mitochondrial dysfunction produced in the striatum by the systemic infusion of the mitochondrial toxin 3-NP to rats, and whether these changes were related to early hyperkinetic behavior.

2. Materials and methods

2.1. Animals and reagents

All procedures with animals were carried out according to the "National Institutes of Health Guide for the Care and Use of Laboratory Animals". SAC was obtained and purified in our facilities, as previously described (Pérez-Severiano et al., 2004b). All other reagents were reagent-grade and obtained from known commercial sources. A total number of 62 male Wistar bred-in-house rats (280–320 g) were used throughout the study. For all experimental purposes, animals were housed five per cage under standard conditions. Animals were divided into four groups (six rats per group) and treated as follows: groups I (control) and III (3-NPA) received saline i.p., while groups II (SAC) and IV (SAC + 3-NPA) received a single administration of SAC (300 mg/kg, i.p.). Groups I and II also received saline 30 min after the first injection, whereas groups III and IV received a single administration of 3-NPA 30 min after the first injection. The doses and schedules of administration of SAC and 3-NPA employed in this work are both well documented in other experimental studies (Binienda and Kim, 1997; Binienda et al., 1998; Pérez-Severiano et al., 2004b). Immediately after receiving the second injection, each animal was separated and individually observed for a period of 120 min. Hyperkinetic behavioral patterns were qualitatively examined according to previous reports (Borlongan et al., 1997a,b,c), and included the following parameters: general hyperactivity, aberrant gait, paddling, recumbence and rigid extension of limbs. At the end of behavioral evaluation, animals were killed by decapitation, their brains were dissected out and their striata (caudate nuclei from both sides) properly collected and preserved at −75 °C until further analysis.

2.2. Superoxide dismutase activity assay

Total SOD activity was assessed by a competitive inhibition assay previously reported by us (Pérez-Severiano et al., 2004a,b), 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 final concentrations. Tissue samples were homogenized in 9 ml/g tissue 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 (2.8 U/l, final concentration) were 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 60 min, and dialyzed for 3 h with three changes of 400 volumes of 5 mM PBS (pH 7.8)–0.1 mM EDTA. Cu/Zn activity was obtained by subtracting the activity of the DDC-treated samples from that of total SOD activity. Results were expressed as units of SOD activity per mg protein. For these experimental procedures, protein concentrations were measured according to Lowry et al. (1951).

2.3. Measurement of lipid fluorescent products (LFP) formation

LP was estimated in striatal tissue by a fluorometric assay previously described by us (Santamaría et al., 2003). Briefly, striatal tissue samples were homogenized in 3 ml of saline solution (0.9% NaCl). One milliliter aliquots were then added to 4 ml of a mixture of chloroform–methanol (2:1, v/v). After stirring, samples were placed on ice for 30 min to allow phase separation. The fluorescent signal in the chloroform layer from each sample was recorded in a Perkin-Elmer LS55 luminescence spectrometer, using excitation and emission wavelengths of 370 and 430 nm, respectively. Results were expressed as units of relative fluorescence intensity (RFI) per mg of protein.

2.4. MTT reduction assay

Mitochondrial viability was estimated by the 3-(3,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay, making discrete

modifications to original reports (Mosmann, 1983; Berridge and Tan, 1993). The method is currently employed as an index of the functional status of the respiratory chain as the formation of formazan salts occurs through the action of mitochondrial dehydrogenases in viable cells or cell fractions (Massieu et al., 2004). Striatal tissue samples were homogenized in sucrose (0.32 M) and prepared for synaptosomes isolation as previously described by us (Pérez-De La Cruz et al., 2006), in order to assess MTT reduction in a mitochondrial-enriched fraction. Synaptosomal fractions were resuspended in HEPES buffer containing 100 mM NaCl, 1 mM NaH_2PO_4, 5 mM $NaHCO_3$, 1 mM $CaCl_2$, 6 mM glucose and 10 mM HEPES, pH 7.4. Four hundred microliter aliquots containing the synaptosomal fractions were added with 8 μl of the MTT reagent (10 mg/ml) and incubated at 37 °C for 90 min. Quantification of formazan was assessed by estimation of optical density in a ThermoSpectronic Genesis 8 spectrometer at 570 nm. Results were expressed as the percentage of MTT reduction with respect to the control value.

2.5. Statistical analysis

Except for behavioral data, all results were expressed as the mean ± 1S.E.M. and analyzed by one-way ANOVA followed by Tukey's test. Values of $P < 0.05$ were considered of statistical significance.

3. Results

Table 1 presents qualitative data from the behavioral parameters observed in 3-NPA- and SAC-treated rats. None of these features were observed in control animals, neither in the SAC group. In contrast, 3-NPA alone evoked all these behavioral alterations in moderate or intense magnitudes, thus evidencing general hyperactivity, while the pretreatment with SAC remarkably reduced – or even prevented – some of these parameters produced by 3-NPA.

The effects of SAC and 3-NPA on the activities of both isoforms of SOD are presented in Fig. 1. Basal activities of Cu,Zn-SOD and Mn-SOD were 39.3 ± 3.9 and 4.5 ± 0.5 units per mg protein, respectively (Fig. 1A and B). 3-NPA decreased the activities of both isoforms (25 and 41% below the control for Cu,Zn-SOD and Mn-SOD, respectively), while pretreatment with SAC to 3-NPA-infused rats resulted in the complete preservation of the enzyme activities up to basal levels (6% above and 2% below the control for Cu,Zn-SOD and Mn-SOD, respectively). SAC alone produced no changes in the activities of these enzymes when compared with control animals.

The levels of LP (expressed as the formation of LFP) in striatal tissue from control-, SAC- and 3-NPA-treated rats, are shown in Fig. 2. Administration of 3-NPA to rats increased the striatal LFP formation by 35% above the control, while pretreatment of rats with SAC decreased the 3-NPA-induced LP even slightly below the basal levels (13% below control). SAC alone produced no significant changes in LP when compared with control (4% above) (data not shown, $n = 2$).

Table 1
Behavioral parameters from rats treated with 3-nitropropionic acid (3-NPA) and/or *S*-allylcysteine (SAC)

	Control	SAC	3-NPA	SAC + 3-NPA
Hyperactivity	n.o.	n.o.	+++	+
Wobbly gait	n.o.	n.o.	++	n.o.
Stereotyped paddling	n.o.	n.o.	+++	n.o.
Ventral or lateral recumbence	n.o.	n.o.	++	n.o.
Rigid extension of hindlimbs	n.o.	n.o.	+++	+

Rats ($n = 6$ animals per group) were administered with SAC (300 mg/kg, i.p.) or saline 30 min before a single systemic infusion of 3-NPA (30 mg/kg, i.p.) or saline, and observed for a period of 120 min before they were killed for other experimental purposes. Qualitative frequency of occurrence of a given event is represented as follows: (n.o.) not observed; (+) scarce; (++) moderate; (+++) intense.

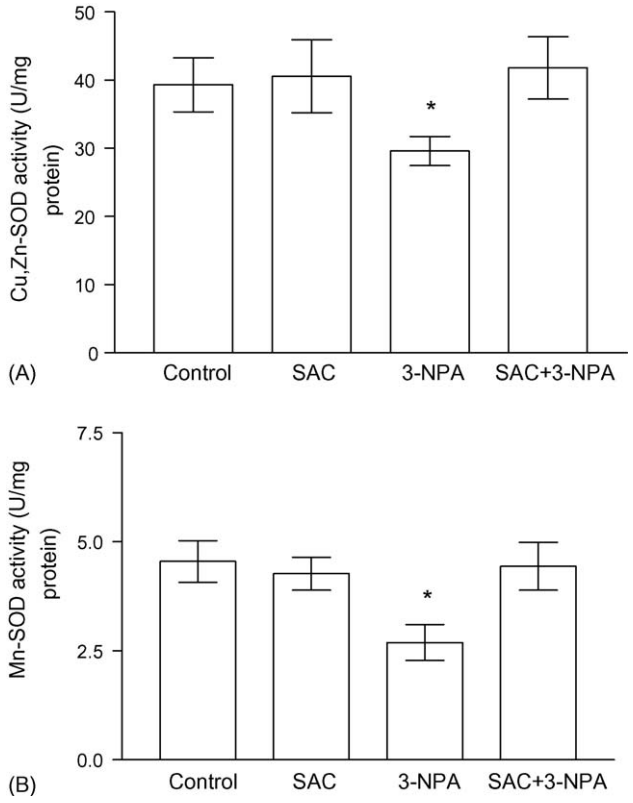

Fig. 1. (A and B) Effects of 3-nitropropionic acid (3-NPA) and *S*-allylcysteine (SAC) on the activities of copper/zinc- and manganese-dependent superoxide dismutases (Cu,Zn-SOD and Mn-SOD) in the rat striatum. Rats ($n = 6$ animals per group) were administered with SAC (300 mg/kg, i.p.) or saline 30 min before a single systemic infusion of 3-NPA (30 mg/kg, i.p.) or saline. Both isoforms of SOD were measured 120 min after 3-NPA injection. Mean values ± S.E.M. are represented. ($^*P < 0.05$), differences against control; one-way ANOVA followed by Tukey's test.

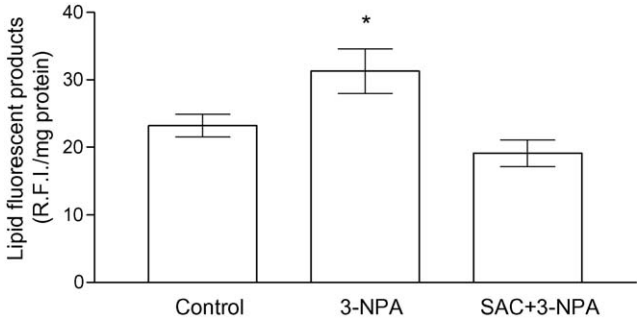

Fig. 2. Effects of 3-nitropropionic acid (3-NPA) and *S*-allylcysteine (SAC) on lipid peroxidation (lipid fluorescent products formation) in the rat striatum. Rats ($n = 4$ animals per group) were administered with SAC (300 mg/kg, i.p.) or saline 30 min before a single systemic infusion of 3-NPA (30 mg/kg, i.p.) or saline. The fluorescent signal was measured 120 min after 3-NPA injection in striatal homogenates. Mean values ± S.E.M. are represented. ($^*P < 0.05$), differences against control; one-way ANOVA followed by Tukey's test.

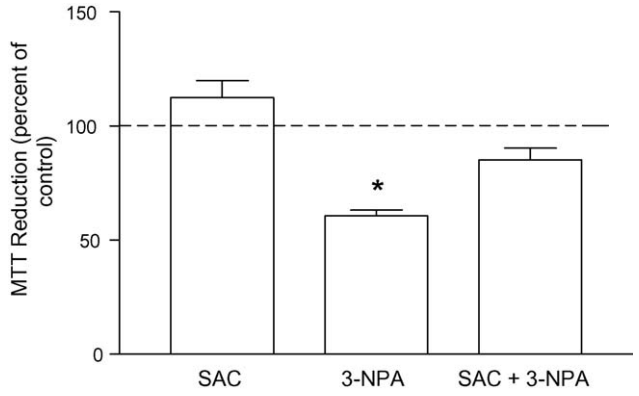

Fig. 3. Effects of 3-nitropropionic acid (3-NPA) and *S*-allylcysteine (SAC) on mitochondrial dysfunction (as decreased MTT reduction) in the rat striatum. Rats ($n = 6$ animals per group) were administered with SAC (300 mg/kg, i.p.) or saline 30 min before a single systemic infusion of 3-NPA (30 mg/kg, i.p.) or saline. The mitochondrial reductant capability was measured 120 min after 3-NPA injection in isolated synaptosomal fractions. Mean values ± S.E.M. are represented. ($^{*}P < 0.05$), differences against control (100%); one-way ANOVA followed by Tukey's test.

Fig. 3 shows the effect of SAC on the 3-NPA-induced mitochondrial dysfunction in the rat striatum assessed by the MTT reduction assay. Striatal synaptosomes of 3-NPA-treated rats exhibited a significant decrease in MTT reduction with respect to control values (39% below). In contrast, SAC partially prevented the decreased capability of MTT reduction induced by 3-NPA by 41% (14% below the control), while SAC alone given to rats produced no effect when compared with the control (12% above).

4. Discussion

In agreement with a previous report (Borlongan et al., 1997a), the systemic administration of 3-NPA to rats produced an early hyperkinetic pattern in rats characterized by altered march and movements. The toxin also produced increased levels of LP and decreased capability of mitochondria to reduce MTT in the rat caudate nucleus, suggesting early oxidative stress as well as a metabolic compromise produced by mitochondrial dysfunction. Of particular interest, and in contrast with previous observations by other groups (Binienda et al., 1998), the administration of 3-NPA alone resulted in a significant reduction in the activities of both forms of SOD in the caudate nucleus just 120 min after the toxin infusion. Considering that Binienda and co-workers described no effects of the toxin on SOD activity in the caudate nucleus of rats treated at the same dose tested in this study (a single infusion of 30 mg/kg) and after the same time of exposure (120 min), we assume that differences among the findings of both groups might be explained by the route of administration: while they injected 3-NPA subcutaneously, we injected it intraperitoneally. We therefore suggest that the i.p. administration of the toxin, instead of its s.c. infusion, may result in its higher and faster availability into the brain, thus producing biochemical alterations sooner. Moreover, the specific decrease in Mn-SOD is suggestive of early alterations in the mitochondrial metabolism in the striatum of lesioned rats.

Regarding the effects of SAC, results of this investigation clearly show that this compound was able to prevent the early alterations in SOD activity and the reductant capability of mitochondria produced by 3-NPA in the rat caudate nucleus. SAC also reduced the qualitative markers of early hyperkinetic behavior produced by 3-NPA, thus suggesting that primary excitotoxic and oxidative events evoked by this toxin during the first stages of toxicity are tightly related. At this point, we assume that the preventive actions exerted by SAC in this model can be attributed to its ability to preserve the cell redox status through its antioxidant properties, which was evidenced in this study by its preventive action on LP, and has been assessed under several experimental conditions (Moriguchi et al., 1997; Kim et al., 2001; Numagami and Ohnishi, 2001; Nishiyama et al., 2001; Ito et al., 2003; Pérez-Severiano et al., 2004a,b). In addition, our results confirm the protective properties of SAC specifically against 3-NPA toxicity, since we have recently reported the ability of this antioxidant to prevent the 3-NPA-induced LP and mitochondrial dysfunction in rat brain synaptosomes (Pérez-De La Cruz et al., 2006). In support of these findings, it has been demonstrated that several other antioxidants are effective to protect the brain against 3-NPA-induced neurotoxicity under different experimental conditions and in different biological preparations. Among them, we can mention some recent reports describing protective effects of taurine and Vitamins C and E (Rodríguez-Martínez et al., 2004), ginseng components (Kim et al., 2005; Lian et al., 2005), the azulenyl nitrone antioxidant (Yang et al., 2005), melatonin (Túnez et al., 2004) and the Nrf2-driven antioxidant stimuli (Shih et al., 2005). An immediate question emerges when we consider all evidence together: how antioxidants may protect the brain against early neurotoxic stimuli induced by 3-NPA? Based on results of other groups and our own data, we suggest that the preservation of SOD through the primary prevention of oxidative stress, mitochondrial dysfunction, energy failure and excitotoxic events results in an optimal preservation of neuronal function, which in turn may account for further cell survival. However, this suggestion still requires experimental support through additional studies using SAC against 3-NPA under other in vivo conditions, such as different times of exposure to the toxin. Whether SAC might also be preventing the reported inhibitory action of 3-NPA on glutamate uptake by synaptic vesicles (Tavares et al., 2001), is an issue remaining to be explored in further investigations. Furthermore, the potential toxicity of SAC has to be studied in a longer time scale since the high dose of SAC employed in our short-term schedule do not allow the characterization of possible adverse effects of this garlic compound. Meanwhile, we can conclude that SAC represents an interesting experimental tool with promising antioxidant and neuroprotective properties when tested against excitotoxic and oxidative events in the brain, as judged by findings of this and other works.

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