Abstract

The effects of S-allylcysteine on oxidative damage and spatial learning and memory deficits produced by an intrahippocampal injection of amyloid-β peptide 25–35 (Aβ(25–35)) in rats were investigated. The formation of reactive oxygen species, lipid peroxidation and the activities of the antioxidant enzymes superoxide dismutase and glutathione peroxidase were all measured in hippocampus 120 min after Aβ(25–35) injection (1 μl of 100 μM solution), while learning and memory skills were evaluated 2 and 35 days after the infusion of Aβ(25–35) to rats, respectively. Aβ(25–35) increased both reactive oxygen species and lipid peroxidation, whereas pretreatment with S-allylcysteine (300 mg/kg, i.p.) 30 min before peptide injection decreased both of these markers. In addition, Aβ(25–35)-induced incorrect learning responses were prevented in most of trials by S-allylcysteine. In contrast, enzyme activities were found unchanged in all groups tested. Findings of this work: (i) support the participation of reactive oxygen species in Aβ(25–35)-induced hippocampal toxicity and learning deficits; and (ii) suggest that the protective effects of S-allylcysteine were related to its ability to scavenge reactive oxygen species.

1. Introduction

Alzheimer’s disease, the most common neurodegenerative disorder in humans, is characterized by deterioration of cognitive and mental functions, including learning and memory skills. The formation of extracellular deposits of amyloid-β peptide, (Tabner et al., 2002) leading to the formation of neuritic plaques and neurofibrillary tangles in cortex and hippocampus, is a prominent pathological feature of Alzheimer’s disease. In particular, the amyloid-β protein fragment 25–35 (Aβ(25–35)) seems to be responsible of the key toxic and oxidative events leading to brain damage, such as oxidative stress-mediated changes in hippocampal long-term potentiation (Trubetskaya et al., 2003), protein oxidation in fibroblasts from Alzheimer’s disease patients (Choi et al., 2003) and in vivo oxidative damage through mechanisms involving N-methyl-D-aspartate receptors and nitric oxide synthase (Parks et al., 2001). In addition, since amyloid-β is recognized as an etiologic factor for Alzheimer’s disease, and its aggregation has also been directly associated with reactive oxygen species formation through metal-dependent mechanisms (Tabner et al., 2002), several reports have emphasized the potential therapeutic role that antioxidant agents may play for treatment of Alzheimer’s disease (Gilgun-Sherki et al., 2003; Rutten et al., 2002). Positive responses against Aβ toxicity have been reported using the Ginkgo biloba extract EGb 761 (Bastianetto and
Quirion, 2002a,b), melatonin (Shen et al., 2002; Zatta et al., 2003), acetyl-L-carnitine (Dhitavat et al., 2002), nitric oxide synthase inhibitors (Law et al., 2001) and in cells with overexpression of superoxide dismutase 1 (Celsi et al., 2004).

On the other hand, aged garlic extract compounds evoke antioxidant and protective responses under several experimental conditions. Among these constituents, S-allylcysteine, the most abundant organosulfur molecule with reported antioxidant properties (Geng et al., 1997; Kim et al., 2001; Numagami and Ohnishi, 2001), exerts its protective actions through its ability to scavenge O$_2^-$ (Kim et al., 2001) and H$_2$O$_2$ (Ide and Lau, 2001), thus preventing H$_2$O$_2$-induced endothelial cell damage and lipid peroxidation, as well as low-density lipoprotein oxidation (Ide and Lau, 2001). Additional positive actions of S-allylcysteine include inhibition of H$_2$O$_2$-induced nuclear factor kappa B (NFkB) activation (Geng et al., 1997; Ide and Lau, 2001) and regulation of nitric oxide (NO) production with associated anti-inflammatory responses (Kim et al., 2001). At a central nervous system (CNS) level, S-allylcysteine reduces edema formation in ischemic rat brain through the inhibition of lipid peroxidation (Numagami and Ohnishi, 2001), ameliorates learning deficits in senescence-accelerated mice (Nishiyama et al., 2001) and evokes neurotrophic actions in cultured rat hippocampal neurons (Moriguchi et al., 1997). Moreover, two of the most remarkable protective effects of S-allylcysteine are those produced on Aβ peptide-induced apoptosis (Peng et al., 2002) and neurotoxicity in organotypic hippocampal cultures (Ito et al., 2003). However, to our knowledge, there is no evidence available on the actions of S-allylcysteine on in vivo Aβ(25–35) toxicity. Therefore, in this work, the effects of a systemic administration of S-allylcysteine were tested on different markers of in vivo oxidative neurotoxicity evoked by an intrahippocampal injection of Aβ(25–35) to rats, in order to provide further information on the toxic mechanisms exerted by the peptide in the brain and the viability of S-allylcysteine as a potential therapeutic tool. We also explored if the toxic effect of Aβ and/or the treatment with S-allylcysteine could be associated with changes in the activity of the antioxidant enzymes, glutathione peroxidase, Mn-superoxide dismutase and Cu,Zn-superoxide dismutase. In addition, S-allylcysteine was tested against those learning/memory deficits evoked by Aβ(25–35) in rats to bring evidence of the effectiveness of this antioxidant to improve integrative physiological responses.

2. Materials and methods

2.1. Reagents

S-Alllylcysteine was synthesized by the reaction of L-cysteine with allyl bromide and purified by recrystallization from ethanol–water, according to a previous report (Maldonado et al., 2003). All other reagents were obtained from known commercial sources. Male Wistar rats (250–300 g), provided by the vivarium of the Instituto Nacional de Neurología y Neurocirugía, were used throughout the study.

2.2. Drug administration protocol

All experiments were carried out with approval of the “Local Committee of Ethics on the Use of Animals for Experimentation” from the Instituto Nacional de Neurología, and according to the “Guidelines for the Use of Animals in Neuroscience Research” from the Society of Neuroscience. Groups of 5–6 (for biochemical assays) or 12 rats (for learning/memory tests) were injected i.p. with 1.5 ml saline or S-allylcysteine (300 mg/kg). The dose of S-allylcysteine was obtained from a previous work (Numagami and Ohnishi, 2001). Animals were immediately anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and, 30 min later, infused for 2 min with bilateral intra-hippocampal injections of 1 μl of Aβ(25–35) (100 μM) dissolved in sterile saline and previously incubated at 37 °C in a shaking-water bath for 24 h. Aβ(25–35) was injected in hippocampus at the following stereotaxic coordinates: 4.2 mm posterior to bregma, ±3.0 mm lateral to bregma and 2.9 mm ventral to the dura (Paxinos and Watson, 1998). Right hippocampus from each rat served for the estimation of reactive oxygen species, while left hippocampus was used for the assay of lipid peroxidation. Control animals received intra-hippocampal and i.p. injections of sterile saline (pH 7.4). Rats from all groups were sacrificed by decapitation 120 min after Aβ(25–35) infusion, and their hippocampal tissues were dissected and stored at −75 °C until the analysis were done. Those animals employed for spatial learning and memory tests were preserved alive until the behavioral procedures were carried out.

2.3. Assay of reactive oxygen species

Reactive oxygen species were estimated by a method based on the formation of 2′7′-dichlorofluorescein in nerve tissue (Santamaría et al., 2001), adapted for in vivo experiments (Pérez-Severiano et al., 2004). Results were expressed as nanomoles of 2′7′-dichlorofluorescein formed per milligram protein per minute.

2.4. Assay of lipid fluorescent products

Lipid peroxidation was measured in hippocampal tissues by a method based on the production of lipid fluorescent products, as we have previously reported (Santamaría et al., 2003a,b). Results were expressed as fluorescence units (FU) per milligram of protein.

2.5. Measurement of superoxide dismutase activity

Total superoxide dismutase activity in hippocampal tissue samples was assayed by a method previously reported...
using nitroblue tetrazolium as the indicator reagent (Santa-maria et al., 2003b). To measure Mn-superoxide dismutase, Cu,Zn-superoxide dismutase was inhibited with diethyldithiocarbamate. Results are expressed as units of activity per milligram of protein.

2.6. Estimation of glutathione peroxidase activity

Hippocampal glutathione peroxidase activity was determined by a method based on the non-enzymatic oxidation of reduced glutathione (Santamaria et al., 2003a). One enzyme activity unit was defined as a 50% decrease in GSH in 60 min after the decrease of GSH from the non-enzymatic reaction had been subtracted. Results are expressed as units of activity per milligram of protein.

2.7. Evaluation of spatial learning and memory

Spatial learning and memory test were employed to evaluate the ability of rats to accept and remember specific tasks. For this purpose, body weight from all animals was controlled for 10 days to achieve 85% of the original weight, and preserved up to the end of the behavioral experiments. An eight-arm radial maze was employed for both measurements. Learning was recorded according to the experiments reported by Olton (1977), with a modification to make it more discriminative, which consisted of leaving food pellets only in three arms. After habituation, learning was evaluated for two days as the number of successful visits of each animal to the arms containing the pellets in a determined number of attempts. Memory was evaluated in the same animals 33 days later and just for 1 day without any habituation phase. The same arms from the maze which were loaded with pellets for learning tests were used for memory. In both cases, responses were simply qualified as “correct” or “error”, and results were expressed as percent of correct responses by trial.

2.8. Statistical analysis

All data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey’s test. In the case of learning and memory data, comparisons between groups were done trial by trial. Values of $P<0.05$ were considered of statistical significance.

3. Results

3.1. Reactive oxygen species formation

The amount of 2′,7′-dichlorofluorescein in hippocampal tissue is shown in Fig. 1A. Baseline of reactive oxygen species levels were $0.85 \pm 0.07 \text{ nmol/mg protein/min}$. S-Allylcysteine alone had no effect on 2′,7′-dichlorofluorescein levels when compared to control ($-5\%$). A significant increase in reactive oxygen species formation was found in Aβ(25–35)-treated rats (42% vs. control), while pretreatment of Aβ(25–35)-treated animals with S-allylcysteine resulted in a decrease of 2′,7′-dichlorofluorescein levels (15% vs. control).

3.2. Lipid peroxidation

Fig. 1B presents the effect of S-allylcysteine on Aβ(25–35)-induced hippocampal reactive oxygen species formation (A) and lipid peroxidation (B) in rats. Animals were administered i.p. with S-allylcysteine (300 mg/kg) 30 min before the bilateral intrahippocampal injection of 1 μl of Aβ (100 μM). Control animals received sterile saline (pH 7.4) either i.p. or intrahippocampal. Two hours after the lesions were done, the amounts of dichlorofluorescein (A) and lipid fluorescent products (B) were measured in hippocampal tissue as indexes of reactive oxygen species formation and lipid peroxidation, respectively. Mean values ± S.E.M. of five to six animals per group are shown. $^a(P<0.01)$, differences from control value; $^b(P<0.05)$ and $^c(P<0.01)$, differences from Aβ(25–35) alone; one-way ANOVA followed by Tukey’s test.
3.3. Superoxide dismutase and glutathione peroxidase activities

Baseline enzyme activities were: 4.70 ± 0.62 U/mg protein for Mn-superoxide dismutase, 9.09 ± 0.58 U/mg protein for Cu,Zn-superoxide dismutase and 0.012 ± 0.001 U/mg protein for glutathione peroxidase. No significant effects were found with any of the agents tested, or their combination, when compared with control values (data not shown).

3.4. Spatial learning and memory skills

Fig. 2A shows the effect of S-allylcysteine on learning deficits induced by Aβ in rats. The percent of correct responses from the Aβ-treated group was significantly lower (P < 0.01) than that from control group in all trials. S-Allylcysteine alone had not effect on the percent of correct responses when compared with control at any trial, whereas the pretreatment of Aβ-injected rats with S-Allylcysteine resulted in a significant improvement (P < 0.05) of correct responses in most of trials (2, 4, 5, 6 and 7).

The effect of S-Allylcysteine on Aβ-induced memory deficits in rats is presented in Fig. 2B. Aβ-treated group exhibited a decreased percent of correct responses (P < 0.01) in trials 6–8, when compared with control group. In contrast, S-allylcysteine alone produced an improved number of correct responses in most of trials when compared with control group (P < 0.05 and P < 0.01), but no positive effect was produced by S-allylcysteine in Aβ-treated animals when compared with Aβ alone.

4. Discussion

This study demonstrates protective effects of S-allylcysteine on the in vivo acute Aβ(25–35)-induced reactive oxygen species formation and lipid peroxidation in the rat hippocampus, and such positive actions seem to be related to the capability of S-allylcysteine to act as free radical scavenger (Numagami and Ohnishi, 2001; Tabner et al., 2002), suggesting that reactive oxygen species formation and lipid peroxidation constitute two key factors accounting for the pattern of toxicity elicited by Aβ(25–35). To our knowledge, this is the first report showing the viability of S-allylcysteine as antioxidant against Aβ(25–35) under in vivo conditions. Our findings are also in agreement with previous observations on the in vitro antioxidant and protective effects of S-allylcysteine against Aβ toxicity (Ito et al., 2003; Peng et al., 2002). In addition, several evidences support the antioxidant ability of S-allylcysteine; for example, this compound is able to inhibit lipid peroxidation (Ide and Lau, 2001) and to enhance the endogenous antioxidant defenses (Peng et al., 2002). Other characteristics of S-allylcysteine, such as its high water solubility and stability (up to 2 years), and low toxicity (Maldonado et al., 2003), suggest a broad spectrum of potential therapeutic actions of this aged garlic extract compound in the CNS and support its use at many experimental levels.

The protective actions of S-allylcysteine on Aβ toxicity were extended to behavioral markers of spatial learning, where in many trials of the test, Aβ-treated animals previously administered with S-allylcysteine showed important improvements in the percent of correct responses when compared with rats infused with Aβ alone. Moreover, S-allylcysteine also improved the basal memory responses from control animals, suggesting that supplementation with
antioxidants is relevant even for improvement of performance in regular tasks, which is consistent with a previous report demonstrating an ameliorative effect of S-allylcysteine on learning deficits in senescence-accelerated mice (Nishiyama et al., 2001). Another remarkable finding of S-allylcysteine was that a single dose administered just 30 min before the toxic insult with Aβ to rats was sufficient to evoke these preventive effects, pointing out its rapid absorption and partially high effectiveness. Therefore, chronic or subchronic suppletions with the antioxidant have to be tested in further studies against the deleterious effects of Aβ, either given as pretreatment or postlesion, in order to know whether different schemes of administration of this agent may improve also spatial memory and other tasks. Furthermore, in light of these findings, it is likely that the neuronal protection that S-allylcysteine might be exerting through its antioxidant properties could be directly responsible of the preservation of spatial functions, although this proposal deserves more detailed investigation.

On the other hand, despite the fact that neither both superoxide dismutase isoforms nor glutathione peroxidase, resulted affected by Aβ(25–35) infusion or stimulated by S-allylcysteine, the data presented here are not sufficient to discard a possible participation of these antioxidant enzymes as primary targets of the peptide toxicity or possible contributors of antioxidant defense, since it has to be considered that our measurements were only done during an acute phase of response of the enzymes (within the first 120 min). Therefore, further studies evaluating the contribution of these enzymes at different times after the toxic insult induced by Aβ(25–35) need to be performed.

In summary, in consideration to the positive findings of this study and all the properties described for S-allylcysteine in different experimental models of systemic and central diseases, this aged garlic extract compound is a promising candidate to be clinically and experimentally tested in neurological degenerative events with oxidative components.

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