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Glutamate induces oxidative stress not mediated by glutamate receptors or cystine transporters: protective effect of melatonin and other antioxidants

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Abstract: Glutamate is responsible for most of the excitatory synaptic activity and oxidative stress induction in the mammalian brain. This amino acid is increased in the substantia nigra in parkinsonism due to the lack of dopamine restraint to the subthalamic nucleus. Parkinson's disease also shows an increase of iron levels in the substantia nigra and a decrease of glutathione, the antioxidant responsible for the ascorbate radical recycling. Considered together, these facts could make the antioxidant ascorbate behave as a pro-oxidant in parkinsonism. Since both glutamate and ascorbate are present in the synaptosomes and neurons of substantia nigra, we tested 1) if glutamate is able to induce oxidative stress independently of its excitatory activity, and 2) if ascorbate may have synergistic effects with glutamate when these two molecules co-exist. Brains were homogenized in order to disrupt membranes and render membrane receptors and intracellular signaling pathways non-functional. In these homogenates glutamate induced lipid peroxidation, indicating that this amino acid also may cause oxidative stress not mediated by its binding to glutamate receptors or cystine transporters. Ascorbate also induced lipid peroxidation thus behaving as a pro-oxidant. Both substances together produced an additive effect but they did not synergize. Given that melatonin is a potent physiological antioxidant with protective effects in models of neurotoxicity, we tested the role of this secretory product on the pro-oxidant effect of both compounds given separately or in combination. We also checked the protective ability of several other antioxidants. Pharmacological doses of melatonin (millimolar), estrogens, pinoline and trolox (micromolar) prevented the oxidant effect of glutamate, ascorbate, and the combination of both substances. Potential therapeutic application of these results is discussed.

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Introduction

Glutamate is an excitatory amino acid, which is one of the main neurotransmitters, being responsible of most of the excitatory synaptic activity in the mammalian brain [Fonnum, 1984]. This amino acid has been suggested as the major protagonist in precipitating oxidative stress in the brain. Two mechanisms have been described so far to explain this toxicity. The first one is mediated by its receptors. There are two types of glutamate receptors: ionotropic (membrane-spanning channels) and metabotropic (acting by means of G protein-initiated biochemical pathways) receptors. The first class has been implicated in the production of oxidative stress. Belonging to this type are the N-methyl-D-aspartate (NMDA) and the α-amino-3-hydroxy-5-methyl-4-isoxasole-proprionic acid (AMPA) and the kainic acid (KA) receptors (non-NMDA receptors), named according to the molecule acting as the most potent agonist in each case.

NMDA receptors (and also AMPA/KA receptors) induce elevation of intraneuronal Ca^{2+} when glutamate binds to them. It appears that this elevation may induce oxidative stress by several

pathways: (1) activating proteases, nucleases, and lipases; the peptidase calpain I catalyzes the enzymatic conversion of xanthine dehydrogenase to xanthine oxidase, whose catabolized reaction produces reactive oxygen species (ROS) [McCord, 1985]; (2) activating nitric oxide synthase [Dawson et al., 1992]; and (3) activating phospholipase A2 which increases arachidonic acid (AA) coming from the membrane. AA activates prostaglandin H synthase (PGHS) generating ROS in this process [Dumuis et al., 1988]. PGHS also stimulates the first step of the autooxidation of dopamine, some of its reactions generating ROS while some of its products being neurotoxic [Hattammal et al., 1995].

The second mechanism explaining the toxic effect of glutamate is mediated by its binding to the cystine transporter, since cystine deprivation causes a decrease in the intracellular antioxidant glutathione and accumulation of ROS [Bannai and Kitamura, 1980]. Glutamate concentration in the brain is very high. Extrasynaptic levels are in the micromolar range (160–190 μM), and synaptic cleft and intracellular concentration reach milimolar values (1–10 mM) [Marc et al., 1990; Tabb and Ueda, 1991; Clements et al., 1992; Dzubay and Jahr, 1999]. This amino acid is increased in the substantia nigra of parkinsonism, due to the lack of dopamine restraint to the subtalamic nucleus (STN) [Rodriguez et al., 1998]. This means that besides its excitatory action, which could be partly regulated and attenuated by changes in the receptors, the increase of glutamate concentration would be deleterious if this molecule would show an intrinsic oxidant activity. Glutamate is widely used as an oxidant in cell culture studies. However, as far as we know, no studies on a possible direct toxic effect of glutamate, independent from the actions described above, have ever been undertaken.

Ascorbic acid is an enzyme co-factor, an important antioxidant, and a neuromodulator in the brain. It is a broad spectrum antioxidant functioning as an electron donor (it renders two electrons to form dehydroascorbate which can be recycled by glutathione and other thiols). Ascorbate concentration is very high in neurons (10 mM), although this antioxidant is also present in glia (1 mM) and in the extracellular space (0.2–0.4 mM) [for review see Rice, 2000]. It is heteroexchanged with glutamate: when glutamate transporters in the forebrain (EAAT 2 and 3) take up glutamate from the synaptosome, ascorbate is released into it [Grunewald and Fillenz, 1984]. Due to its antioxidant properties, ascorbate is normally considered to be a neuroprotector [Halliwell, 1996]. However, in cell culture ascorbate is able to induce cytotoxycity [Andorn et al., 1996; Sakagami et al., 1997; Andorn et al., 1998; Brunet et al., 2000]. There are differences between the physiological situation in vivo and in cell culture, where neurons seem to loose intracellular ascorbate, or in vitro assays where cellular compartments are broken, with iron being released into the medium (iron in combination with ascorbate generates the highly reactive hydroxyl radical). Under pathological conditions such as iron increase or glutathione decrease (glutathione is the antioxidant that recycles the ascorbate radical), which occur in Parkinson's disease, the high levels of ascorbate could well turn this antioxidant into a dangerous molecule for the cell.

Our present study attempted to elucidate (1) if glutamate may behave by itself, independent of its actions through NMDA and non-NMDA receptors and the cystine transporter, as an oxidant molecule which, due to its intraneuronal and synaptosomal high concentration, could be cytotoxic under pathological circumstances; (2) if ascorbate, which also appears in the glutamatergic synaptosome and in the dopaminergic neurons, presents direct oxidant effects in an in vitro system and if it could present synergistic or additive effects with glutamate; and (3) the effect of melatonin and several other antioxidants on the prevention of the oxidant damage induced by each of the agents independently or given together.

Material and methods

Animals

Male Wistar rats (1 month old) were kept in the animal room of the University of Oviedo. They were maintained under a 14:10 light–dark cycle (lights on at 07:00 h) and food and water were provided ad libitum. Animals were sacrificed by decapitation 24 hr after food restriction. Brain tissue was collected, frozen in liquid nitrogen, and kept at -80°C until assayed.

Lipid peroxidation assays (LPOs)

After homogenization of tissue in Tris-HCl buffer (20 mM, pH 7.4, 4°C, 10%) and centrifugation at 2500g and 4°C for 5 min, supernatant was collected and used for the LPO. Aliquots of homogenates (450 μL) were incubated by triplicate for 1 hr at 37°C with the correspondent drug, depending of the experiment, with or without one of the several antioxidants used (a total of 50 μL). After incubation, samples were centrifuged at

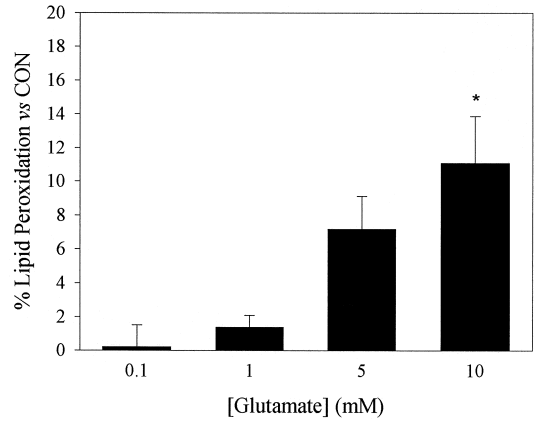

Fig. 1. Glutamate, in a dose–response manner, induces lipid peroxidation in brain tissue homogenates as measured by the N-methyl-2-phenylindole test kit (LPO586 from OxySystems). Each value represents the mean ± S.E.M. of four independent experiments. $*P < 0.01$ vs. control.

2500g for 5 min and the supernatant was taken to another tube. The lipid peroxidation was evaluated by the N-methyl-2-phenylindole test kit (LPO586 from OXIS International Inc., Portland, OR, USA). This chromogen reacts with malondialdehyde (MDA) and 4-hydroxyalkenals (4-HDA) – molecules resulting from the fragmentation of peroxides produced in the chain reaction of lipid peroxidation – yielding a chromophore with maximal absorbance at a wavelenght of 586 nm.

Statistical analysis

Data result from four independent experiments. Results are shown as the mean ± S.E. Statistical analysis was performed with analysis of variance, followed by a Student Newman–Keuls test.

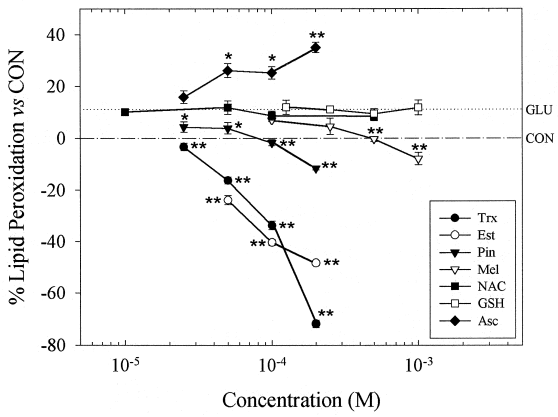

Fig. 2. Effect of several antioxidants on glutamate-induced lipid peroxidation in brain tissue homogenates. Trx, trolox; Est, estrogen; Pin, pinoline; Mel, melatonin; NAC, N-acetyl cysteine; GSH, gluthatione; Asc, ascorbate. Values represent the mean ± S.E.M. of four experiments. $*P < 0.05$; $**P < 0.01$ vs. control.

Results

To determine if glutamate induces oxidative stress independent of its excitatory properties or its binding to cystine receptors, brain tissue was homogenized. This procedure disrupts membranes, so receptors and intracellular cascades are not functional. Signs of oxidative stress induced in the homogenates should be due to the oxidant damage associated with the ability of the molecule itself to generate free radicals that initiate the lipid peroxidation chain reaction. Glutamate concentrations used were selected based on the physiological concentrations of the neurotransmiter in the extrasynaptic (160–190 μM), synaptosome, and intraneuronal compartments (1–10 mM).

Increasing concentrations of a freshly prepared glutamate solution (pH equilibrated with NaOH 1N) from 100 μM to 10 mM (in a total volume of 50 μL) were added to brain homogenates. Basal lipid peroxidation existed in the brain tissue controls in this experiment as well as in the rest of assays described in this section. The formation of MDA + 4-HDA was of 6 nm/mg of protein in the control groups. Glutamate induced a dose-dependent lipid peroxidation that was statistically significant at 10 mM (Fig. 1).

Melatonin, pinoline, estradiol, glutathione, vitamin E, and vitamin C (ascorbate) are natural antioxidants present in brain tissue while N-acetylcysteine is a good antioxidant in neuronal cultures. To determine whether some of these antioxidants prevented the oxidative stress induced by glutamate, they were added in increasing concentrations to the homogenates at the same time that glutamate was applied.

Micromolar concentrations of estradiol, pinoline, and trolox (25–200 μM), and milimolar concentrations of melatonin (0.5 and 1 mM) prevented the action of glutamate and in some cases even decreased the lipid peroxidation occurring in the control samples during the assay. Neither glutathione (125 μM–1 mM) or N-acetylcysteine (10–500 μM) prevented the effect of glutamate on lipid peroxidation. Finally, ascorbate (50–200 μM) notably increased the oxidant effects of glutamate (Fig. 2).

Once it was known that ascorbate increased the toxic effect of glutamate and given the existence of both molecules in the glutamatergic synapses, we wondered if ascorbate alone would induce oxidative stress and if so, if together these substances would present synergistic effects.

Fig. 3 shows that ascorbate alone also induces lipid peroxidation. This was dose-dependent with significant oxidant effects at all doses tested (from

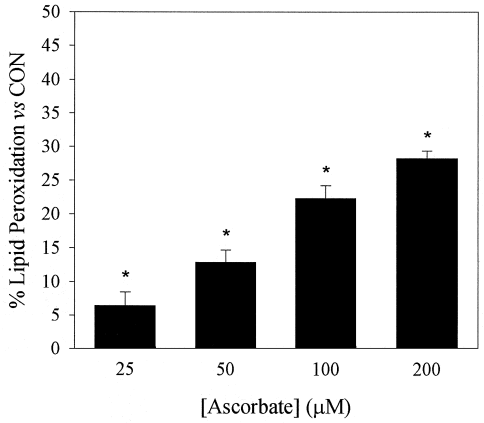

Fig. 3. Ascorbate, in a dose–response manner, induces lipid peroxidation in brain tissue homogenates as measured by the N-methyl-2-phenylindole test kit (LPO586 from OxySystems). Data were obtained from the mean ± S.E.M. of four independent experiments. $^*P < 0.01$ vs. control.

25 to 200 μM). When added together, glutamate (10 mM) and ascorbate (200 μM) showed additive but not synergistic effects (Fig. 4).

To determine if antioxidants would prevent the toxic action of ascorbate we tested whether estradiol, trolox, pinoline, or melatonin has similar effects as those shown on glutamate (Fig. 5). Gluthatione and N-acetylcysteine were not effective (data not shown). When examined each of the four antioxidants in the presence of both glutamate and ascorbate, they were protective against the toxicity of both oxidants together (Fig. 6).

Discussion

The results of the present work indicate that glutamate may exert an oxidant effect independently of its excitotoxic action or its binding to cystine

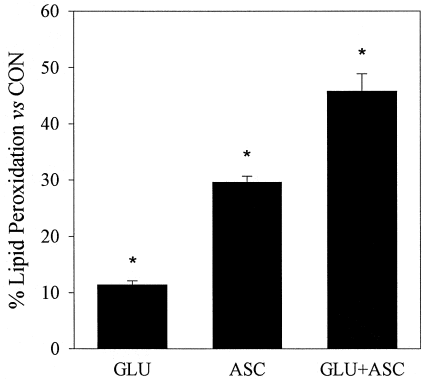

Fig. 4. Glutamate (10 mM) and ascorbate (200 μM) cause an additive effect in the induction of lipid peroxidation when added together in brain tissue homogenates. Each value represents the mean ± S.E.M. of four independent experiments. $^*P < 0.01$ vs. all groups.

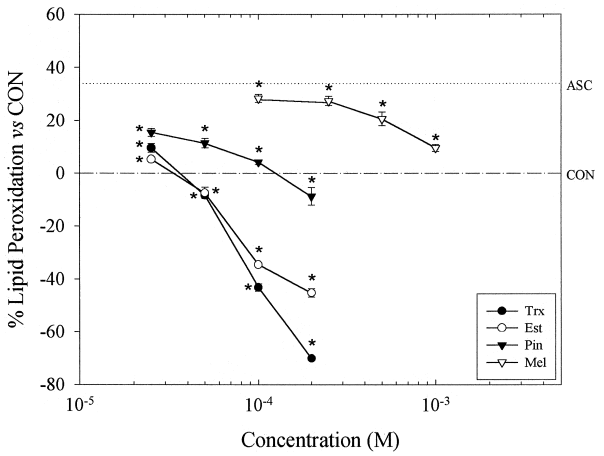

Fig. 5. Effect of several antioxidants on ascorbate-induced lipid peroxidation in brain tissue homogenates. Trx, trolox; Est, estrogens; Pin, pinoline; Mel, melatonin. Values were obtained from the mean ± S.E.M. of four independent experiments. $^*P < 0.01$ vs. control.

transporters. Glutamate synapses are present in many areas of the brain, including the substantia nigra, where dopaminergic neurons degenerate in Parkinson's disease. Glutamate toxicity has been proposed as one of the possible etiologies in the loss of neurons in this disorder, since antagonists of the glutamate receptors partially prevent parkinsonism induced by MPTP [Turski et al., 1991; Sonsalla et al., 1998]. Dopaminergic neurons produce a high level of free radicals, especially during the metabolism of dopamine catalyzed by monoamine oxidase [Cohen and Spina, 1989] and in the course of the autoxidation of monoamine to quinones [Graham, 1978]. Additionally, these neurons possess neuromelanin, a compound formed as an end product of the autoxidation of

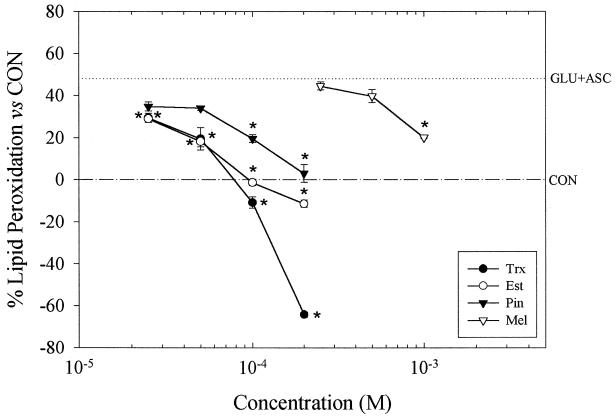

Fig. 6. Effect of several antioxidants on ascorbate/glutamate-induced lipid peroxidation in brain tissue homogenates. Trx, trolox; Est, estrogens; Pin, pinoline; Mel, melatonin. Values represent the mean ± S.E.M. of four experiments. $^*P < 0.01$ vs. control.

dopamine. Neuromelanin increases in the brain during aging and binds iron, forming melanin–iron complexes. Iron bound to melanin is reactive and may catalyze the Fenton reaction, thereby inducing the formation of the hydroxyl radical ($OH^{\bullet}$) [Olanow and Arendash, 1994]. This oxidative stress together with alterations in energy metabolism (impaired glucose uptake) may render dopaminergic cells more vulnerable to excitotoxicity and apoptosis [Mattson et al., 1999].

It is not only the higher vulnerability of neurons to excitotoxicity, however, that makes glutamate a likely contributor to the pathogenesis of Parkinson's disease. It has been demonstrated that there is greater stimulation of neurons of substantia nigra pars compacta (SNc) by the glutamatergic terminals of the STN. When dopamine is absent, inhibition of STN does not occurs, eliciting its augmented activity which in turn induces prolonged liberation of glutamate from the glutamatergic synaptosomes of the target areas of this nucleus, including the SNc [Rodriguez et al., 1998]. Glutamate present in the synapses, in addition to stimulating glutamate receptors, is captured by glutamate transporters located in glial cells (GLT1 or EAAT2) and in the postsynaptic neuron (EAAC1or EAAT3) [Rothstein et al., 1994; Fairman and Amara, 1999; Hediger and Welbourne, 1999]. Thus, the postsynaptic neuron, in this case the dopaminergic neuron, takes up an increased amount of glutamate, thereby increasing the intracellular concentration of the neurotransmitter. In summary, 1) low dopamine production by SNc permits glutamatergic neurons to liberate glutamate in the SNc for a prolonged period, 2) glutamate concentration increases in both the synaptic cleft and the postsynaptic neuron. Although the intracellular storage mechanisms and function of glutamate in the postsynaptic neuron is unknown [Fairman and Amara, 1999], it is likely that a rise in glutamate concentration will also increase, at least temporarily, free cytoplasmic glutamate, even though the glutamate may eventually be stored in vesicles. The model system used in this study relied on membrane disruption, so glutamate would have access to every subcellular structure. However, bearing in mind the likelihood of an increase of free glutamate in the dopaminergic neurons of the SNc in Parkinson's disease, it is reasonable to surmise that one of the toxic mechanisms of glutamate in this disorder, although possibly not the primary one, would be its ability to induce lipid peroxidation.

Ascorbate is generally considered a natural antioxidant [for review see Halliwell, 1996]. However, experiments in cellular models and the results presented in this work indicate that under some circumstances ascorbate may behave as a pro-oxidant [Andorn et al., 1996; Sakagami et al., 1997; Andorn et al., 1998; Brunet et al., 2000]. In most in vitro systems where oxidative stress is induced by ascorbate, iron is necessary to achieve the expected ascorbate pro-oxidant effect. In the present study, iron was not added to our system. However, brain tissue contains high levels of endogenous iron, with much of it being in the substantia nigra bound to neuromelanin and therefore being reactive. After homogenization, bound iron was presumably liberated in the solution, thus increasing its effective concentration. Realizing that iron is increased in the substantia nigra in Parkinson's disease, it is not unreasonable to think that ascorbate also may be toxic in this circumstance. In this disorder glutathione, the antioxidant that recycles ascorbate radical, is also decreased.

Given the possibility for involvement of glutamate and ascorbate to increase oxidative stress in the pathogenesis of Parkinson's disease and the location of both molecules in both synaptosomes and dopaminergic cells of the substantia nigra, we investigated the effect that these two molecules would have when added simultanwously in our system. We found that this effect is additive and not synergistic, which suggests that their mechanisms for inducing lipid peroxidation are different.

Estradiol, vitamin E, and melatonin were effective in preventing glutamate, ascorbate, or glutamate/ascorbate-induced lipid peroxidation at micromolar (estradiol and vitamin E) and millimolar (melatonin) concentrations. The physiological concentration of estradiol is in the nanomolar range and, based on our results, it would not be expected to directly prevent lipid peroxidation induced by glutamate (other actions of estradiol such as protein regulation that could exert antioxidant effects can not be excluded). Although estradiol may be given at pharmacological doses to some patients and under certain circumstances, it would be not recommendable in others. Finally, the pro-oxidant effects of some metabolites of estradiol [Markides et al., 1998; Thibodeau and Paquette, 1999] should be considered before using the hormone as a preventive drug. Vitamin E may be a good candidate for preventing glutamate and ascorbate damage in the disorders where augmented concentrations of glutamate and/or iron are anticipated. However, it has already been tested in an extensive clinical trial [the DATATOP study, Parkinson's Study Group, 1993] and no positive results were found [Parkinson's Study Group, 1996]. The poor ability of vitamin E to pass the blood–brain barrier [Pappert et al., 1996]

may be involved in the lack of results after oral administration of this agent. Melatonin, at physiological concentrations (nanomolar), also did not prevent glutamate-induced lipid peroxidation. This fact does not preclude the possibility, however, that in vivo physiological levels of melatonin could reduce glutamate toxicity by other mechanisms, i.e., regulating intracellular defenses such as antioxidant enzymes, which already have been demonstrated to be regulated by this agent [Barlow-Walden et al., 1995; Antolín et al., 1996; Mayo et al., 1998], or by reducing free radical generation in the mitochondria [Acuña-Castroviejo et al., 2001]. Under physiological conditions glutamate concentration may be as high as 10 mM, i.e., in photoreceptors. This is the lower concentration inducing significant levels of lipid peroxidation in the present work. In pathological conditions, when glutamate may be in increased concentrations, supplement addition antioxidant may have to be given to avoid lipid peroxidation.

Melatonin is a potent physiological and pharmacological antioxidant that has been used in the treatment of insomnia and jet lag. No serious side effects to melatonin usage have been described to date in humans or in animal models [Janke et al., 1999; Jan et al., 2000; de Lourdes et al., 2000]. Given its protective actions against glutamate and ascorbate toxicity and against neurotoxicity by other toxins, as described before by our group and others in cell lines [Lezoualc'h et al., 1996; Pappolla et al., 1997; Mayo et al., 1998] and also in vivo [Pappolla et al., 2000; Reiter, 2000], its use at pharmacological doses in a clinical trial in patients with incipient Parkinson's disease should be considered. This proposal does not even consider the fact that melatonin stimulates antioxidant enzymes at physiological levels. Although high (physiological) levels of melatonin are present in young individuals, elderly subjects, who are most often afflicted with neurodegenerative diseases, often show a striking decrease in melatonin concentrations. Collectively the data makes melatonin a reasonable candidate to be given at early stages of neurodegenerative diseases or even in elderly people in order to prevent the appearance of such debilitating conditions.

Acknowledgments

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