Impairment of excitatory amino acid transporter activity by oxidative stress conditions in retinal cells: effect of antioxidants

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ABSTRACT **In the present study we analyzed how oxidative stress conditions induced by ascorbate/Fe^{2+} affect the excitatory amino acid (EAA) transport systems in cultured chick retina cells. The uptake of D-[^{3}H]aspartate, which is transported by the same carrier as glutamate, was determined in control cells and in cells subjected to ascorbate/Fe^{2+}. The uptake of this EAA was Na^+ dependent and was inhibited by about 40% under oxidative stress conditions. To clarify the molecular mechanisms involved in the inhibition of D-[^{3}H]aspartate uptake by ascorbate/Fe^{2+}, we investigated the effect of vitamin E (Vit E), melatonin, reduced glutathione (GSH), and dithiothreitol (DTT) on the uptake of D-[^{3}H]aspartate and on the extent of lipid peroxidation in control and in peroxidized cells. Preincubation with Vit E (100 μM) abolished lipid peroxidation, but had no significant effect on the inhibition of D-[^{3}H]aspartate uptake evoked by ascorbate/Fe^{2+}. Melatonin was more effective in reducing the formation of TBARS and conjugated dienes than in preventing the D-[^{3}H]aspartate uptake inhibition evoked by the oxidant pair. Conversely, GSH (4 mM) and DTT (4 mM) completely prevented the inhibition of D-[^{3}H]aspartate uptake in cells subjected to oxidative stress, but were without effect on the extent of peroxidation. Free fatty acids, such as arachidonic acid, seem not to be involved in reducing the activity of the D-[^{3}H]aspartate uptake system, whereas the reduction of the Na^+ electrochemical gradient that occurs under oxidative stress was in part involved in the reduction of D-[^{3}H]aspartate uptake by the cells. The inhibition of D-[^{3}H]aspartate uptake by ascorbate/Fe^{2+} persisted for at least 1 h, but could be partially reverted by disulfide reducing agents. It is concluded that oxidative stress causes long-lasting modifications of the glutamate/D-[^{3}H]aspartate transport system (or systems), such as oxidation of protein sulfhydryl (SH) groups, which can be recovered by some antioxidants.—Agostinho, P., Duarte, C. B., Oliveira, C. R. Impairment of excitatory amino acid transporter activity by oxidative stress conditions in retinal cells: effect of antioxidants. *FASEB J.* 11, 154–163 (1997)**

Key Words: D-[^{3}H]aspartate uptake · lipid peroxidation · antioxidants · melatonin · oxidation of proteins

AEROBIC METABOLISM GIVES RISE to reactive oxygen species (ROS),[2] which are continuously inactivated by endogenous enzymatic (superoxide dismutase, glutathione peroxidase, catalase) and nonenzymatic (e.g., vitamin E, glutathione, and ascorbate) antioxidant systems (1). The steady state of prooxidant and antioxidant factors may be disrupted under some pathological conditions. The imbalance in favor of the former factors and in disfavoring the antioxidants leads to oxidative stress conditions that can cause citotoxicity. It is known that during aging and in several pathological conditions such as ischemia/reperfusion, stroke, and neurodegenerative diseases, the production of ROS overcomes the activity of the endogenous antioxidant systems (2, 3). ROS such as hydroxyl radical ($HO^•$), superoxide anion ($O_2^{•-}$), and hydrogen peroxide (H_2O_2) may attack cell components like the protein thiol (SH) groups, unsaturated fatty acids, and nucleotides, leading to cell injury (4).

The extracellular excitatory amino acid (EAA) levels are regulated by "high-affinity" and "low-affinity" transport systems found in neuronal and glial cells (5). The dysfunction of these transport systems and the consequent abnormal elevation of extracellular EAA levels constitute one of the major components of excitotoxicity (2, 6). The toxicity of EAA and of ROS are considered interdependent mechanisms. Indeed, it was reported that *1*) oxidative stress conditions increase the release of EAA (2, 7), *2*)

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[2] Abbreviations: AA, arachidonic acid; BHT, 2,6-di-tert-butyl-4-methylphenol; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EAA, excitatory amino acids; GSH, reduced glutathione; LDH, lactate dehydrogenase; MK-801, (+)5-methyl-10,11-dihydro-5H-dibenzo(a,d)cyclohepten-5,10-imine maleate; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; MTT, 3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NMG, N-methyl-D-glucamine; ONO-RS-082, 2-(*p*-amylcinnamoyl)amino-4-chlorobenzoic acid; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PLA_2, phospholipase A_2; ROS, reactive oxygen species; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances; TCA, trichloroacetic acid; Vit E, vitamin E; α-tocopherol acetate; $[Na^+]_i$, intracellular free Na^+ concentration.

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glutamate toxicity can be prevented by free radical scavengers (8), and *3*) excessive release of EAA under ischemic conditions is blocked by free radical scavengers and reproduced by free radical-generating systems (9).

Glutamate and ROS toxicity have been shown to be involved in retina degeneration occurring in diabetic retinopathy, senile macular degeneration, Batten's disease, and certain drug toxicities (10). The excitotoxic effects of EAA have been characterized in the intact tissue (11, 12) and by using cultured retina cells (13, 14). The retina, like the brain, is also very susceptible to oxidative stress because it has a high rate of oxygen consumption, is enriched in polyunsaturated fatty acids, and is poor in antioxidant defenses (4, 15). Oxidative stress conditions were shown to stimulate the activity of NMDA receptors (16, 17) and the Ca^{2+}-independent, carrier-mediated release of glutamate and D-[^{3}H]aspartate (7, 18) from cultured retina cells. Whereas the effect of ascorbate/Fe^{2+} on the NMDA receptors is thought to occur at a site different from the redox site (17), the molecular mechanisms responsible for the modulation of the EAA transporter activity under oxidative stress have not been investigated. In the present work we show that oxidative stress induced by ascorbate/Fe^{2+} reduces the uptake of D-[^{3}H]aspartate by retina cells in culture. This effect is shown to be due to oxidation of protein SH groups rather than to lipid peroxidation. The data reinforce the hypothesis that ROS and EAA may cooperate in the development of neurotoxic injury.

MATERIALS AND METHODS

Materials

Basal medium of Eagle (Earle's salts; BME) was purchased from Sigma Chemical Co. (St. Louis, Mo.), fetal calf serum was obtained from BioChrom KG (Berlin, Germany), and trypsin was from GIBCO (Paisley; U.K.). D-[^{3}H]Aspartic acid was purchased from Amersham International (Aylesbury, U.K.). α-Tocopherol acetate (Vit E) was obtained from Fluka Chemie AG (Switzerland) and 2-(*p*-amylcinnamoyl)amino-4-chlorobenzoic acid (ONO-RS-082) was from Biomol (Plymouth Meeting, Pa.). (+)5-Methyl-10,11-dihydro-5H-dibenzo(a,d)cyclohepten-5,10-imine maleate (MK-801) was a kind gift from Merck Sharp and Dohme (West Point, Pa.) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) was from NOVO Nordisk (Denmark). All other chemicals used were purchased from Sigma Chemical Co.

Preparation of cell cultures

Retina cells were isolated from 8-day-old chick (White Leghorn) embryos, essentially as described previously (16–18). Briefly, the retinas were dissected free from other eye tissues and incubated with 0.1% trypsin in a Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (CMF) for 15 min at 37°C. The digested tissue was centrifuged at 140 × g_{av} for 1 min, and the pellet was washed once with BME supplemented with 5% fetal calf serum. The digested retinas were then dissociated mechanically by aspirating the tissue 14 times through a large-bore 5 ml glass pipette. The cells were cultured in BME buffered with 20 mM Hepes and 10 mM $NaHCO_3$, and supplemented with 5% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). The cells were plated at a density of 0.4 × 10^6 cells/cm^2 in six-well cluster plates, coated with poly-L-lysine (0.1 mg/ml), and cultured for 6 days at 37°C in a humidified atmosphere of 5% CO_2/95% air.

A preparation similar to the one used in this study was shown to be highly enriched in anacrine-like neurons (19). This was confirmed by immunofluorescence, using a monoclonal antibody raised against the rat brain choline acetyltransferase (clone 1E6; Chemicon International, Temecula, Calif.) (authors' unpublished data). The amacrine cells are thought to be the only cholinergic cells of the retina (20).

Induction of lipid peroxidation

The oxidant pair ascorbate/Fe^{2+} was used to induce nonenzymatic lipid peroxidation. The cells were incubated with ascorbic acid (1.5 mM) and ferrous sulfate (7.5 μM) in Na^+ medium containing (in mM): 132 NaCl, 4 KCl, 1 $CaCl_2$, 1.2 H_3PO_4, 1.4 $MgCl_2$, 6 glucose, and 10 HEPES-Tris, pH7.4, for 15 min at 37°C. The medium was supplemented with antioxidants, fatty acid-free bovine serum albumin (BSA, 1 mg/ml), ONO-RS-082 (20 μM), or MK-801 (10 μM) plus CNQX (10 μM) when the effect of these drugs on the extent of peroxidation was being investigated. Vit E was added to the culture medium for the indicated periods of time before the 15 min incubation with the oxidant pair. The cells were preincubated with the other tested compounds for 10 min except where indicated in the figure captions. Lipid peroxidation was stopped by rapidly lowering the temperature to 0–4°C.

Quantification of lipid peroxidation

The extent of peroxidation was evaluated by the thiobarbituric acid (TBA) method and by measuring the formation of conjugated dienes (21). For the TBA test, the cells were scraped and then diluted three times with 15% trichloroacetic acid (TCA), 0.375% TBA, 0.25 M HCl, and 0.015% BHT, and incubated for 15 min at 100°C. The presence of BTH prevents the formation of peroxidation products during the test. The samples were centrifuged at 95.5 × g_{av} for 10 min; the supernatants were collected and the absorvance was then measured at 530 nm in a Bausch and Lomb-Spectronic-70 spectrophotometer. The amount of thiobarbituric acid reactive substances (TBARS) formed was calculated using a molar extinction coefficient of 1.56 × 10^5 $M^{-1} \cdot cm^{-1}$ and expressed as nanomoles TBARS produced per milligram of protein.

Conjugated dienes were determined after extraction of total lipids from the retina cells. The lipids were extracted using the method described by Reed et al. (22) and evaporated under N_2 atmosphere. The dried lipid extracts were resuspended in cyclohexan and conjugated dienes were detected by measuring the absorvance at 233 nm, using a Perkin-Elmer Lamba-2 spectrophotometer. The amount of conjugated dienes was calculated using the molar extinction coefficient of 2.52 × 10^4 $M^{-1} \cdot cm^{-1}$ (21), and the values were expressed in nmol/mg protein. Protein was measured by the method of Sedmak and Grossero (23), using bovine serum albumin as the standard.

D-[^{3}H]aspartate uptake

Cultured retinal cells were incubated with 5.5 nM D-[^{3}H]aspartic acid (1 μCi/ml) in Na^+ medium for 10 min at 37°C. When the effect of antioxidants, fatty acid-free BSA, or ONO-RS-082 was evaluated, a preincubation with those agents was performed, as indicated in the figure captions, before incubation with radiolabeled amino acid. The "nonspecific transport" of the amino acid was determined by measuring the uptake of D-[^{3}H]aspartic acid at 0–4°C. At the end of the experiments, the cells were washed twice and disrupted with 0.2 M HCl and the radioactivity was measured using UNIVERSOL scintillation cocktail (ICN) and a Packard 2000 Spectometer provided with dpm correction.

Determination of cell viability

The effect of oxidative stress on the viability of cultured chick retina cells was evaluated by determining the leakage of the cytoplasmic enzyme lactate dehydrogenase (LDH) (E.C. 1.1.1.27), as previously de-

TABLE 1. *Effect of oxidative stress induced by ascorbate/Fe^{2+} on the extent of peroxidation and on the viability of retina cells*[a]

	Extent of peroxidation		Cell viability	
	TBARS (nmol/mg protein)	Conjugated dienes (nmol/mg protein)	LDH leakage (% of total)	MTT test (% of control)
Control cells	0.3 ± 0.1	228.2 ± 10.7	0.8 ± 0.2	99.9 ± 1.6
Peroxidized cells	7.5 ± 0.5***	334.5 ± 10.7***	1.2 ± 0.2	95.1 ± 0.6

[a] Cultured retina cells were incubated in Na^+ medium in the presence (peroxidized cells) or in the absence (control cells) of ascorbic acid (1.5 mM) and ferrous sulfate (7.5 μM) for 15 min at 37°C. The extent of the peroxidative process was evaluated by determining the production of TBARS and of conjugated dienes. The cell viability was determined by measuring the leakage of lactate dehydrogenase (LDH) and the reduction of MTT (see Materials and Methods). Data are means ± SE of three–six experiments. *** $P < 0.001$ as compared with control cells.

scribed (24). LDH leakage into the extracellular medium was expressed as a percentage of the total enzyme activity in the cells.

The cell injury evoked by ascorbate/Fe^{2+} was also analyzed by the MTT test (25). This assay is based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) to formazan, mainly by mitochondrial dehydrogenases. MTT (0.5 mg/ml in Na^+ medium) was added to the cells previously submitted (peroxidized cells) or not (control cells) to oxidative stress and incubated for 3 h at 37°C. The blue formazan crystals were dissolved in an equal volume of 0.04 HCl in isopropanol and quantified by measuring the absorvance at 570 mm. The viability of peroxidized cells was expressed as a percentage of the absorvance determined for control cells.

Statistical analysis

Results are presented as means ± SE of the number of experiments indicated in the figure captions. Statistical significance was determined using one-way ANOVA and differences between treatments were determined using the Dunnett's test. The two-tailed Student's *t* test was used to compare control cells with peroxidized cells under the same experimental conditions.

RESULTS

Oxidative stress induced by ascorbate/Fe^{2+}

In the present study, ascorbate (1.5 mM) and Fe^{2+} (7. 5 μM) were used to induce nonenzymatic peroxidation of retinal cells in culture by promoting the formation of reactive oxygen species (ROS) through the iron-catalyzed Haber-Weiss reaction (4). These oxygen species are highly reactive and can initiate chain reactions, such as lipid peroxidation, that can alter the structural integrity and function of the cells. To determine the extent of peroxidation, we measured the production of thiobarbituric acid reactive substances (TBARS) and of conjugated dienes, since the selection of only a single test to monitor peroxidation could give misleading results (26). **Table 1** shows that incubation of cultured retina cells with ascorbate/Fe^{2+} (peroxidized cells) increased significantly ($P<0.001$) the levels of TBARS and conjugated dienes.

We also investigated whether the cell viability was affected under the oxidative stress conditions induced by ascorbate/Fe^{2+}, by measuring the leakage of LDH and the reduction of MTT to a formazan product in control and in peroxidized cells. The viability of retina cells was not affected by the oxidative stress conditions used in this study (Table 1). Indeed, the reduction of MTT and the leakage of LDH were not significantly different ($P>0.05$) in cells exposed to ascorbate/Fe^{2+} as compared with control cells.

Oxidative stress inhibits the uptake of D-[^{3}H]aspartate

ROS are thought to contribute to excitotoxicity, the disturbance of EAA transport systems being one of the mechanisms involved (2). To investigate how oxidative stress induced by ascorbate/Fe^{2+} affects the glutamate transport systems, we determined the uptake of D-[^{3}H]aspartate, which is mediated by the glutamate transporter (27), in control and in peroxidized cells. **Figure 1** shows that the amount of D-[^{3}H]aspartate taken up by peroxidized cells (0.36±0.13 pmol/10^6 cells) was significantly ($P<0.001$) lower than that accumulated by control cells (0.62±0.08 pmol/10^6 cells). The uptake of D-[^{3}H]aspartate was significantly inhibited when the experiments were performed at 0–4°C or when the extracellular Na^+ was isosmotically replaced by the nonpermeable N-methyl-D-glucamine (NMG) (Fig. 1), indicating that it was mediated by a Na^+-D-[^{3}H]aspartate cotransport system.

In an attempt to clarify the molecular mechanisms involved in the inhibition of D-[^{3}H]aspartate uptake by ascorbate/Fe^{2+}, we analyzed the effect of different antioxidants, glutamate receptor antagonists, ONO-RS-082, and free fatty acid chelation with BSA on the uptake of D-[^{3}H]aspartate by cells submitted or not to the oxidant pair (see Fig. 2 and Fig. 4). The effect of antioxidants on the extent of peroxidation was also evaluated (**Table 2**).

Lipophilic antioxidants, inhibition of phospholipase A_2, and fatty acid-free BSA do not protect the D-[^{3}H]aspartate uptake system against the effect of oxidative stress

The data of **Fig. 2** show the effect of antioxidants on the uptake of D-[^{3}H]aspartate by retina cells submitted to oxidative stress. The results are plotted as a percentage of the radioactivity taken up by control cells, i.e., cells in-

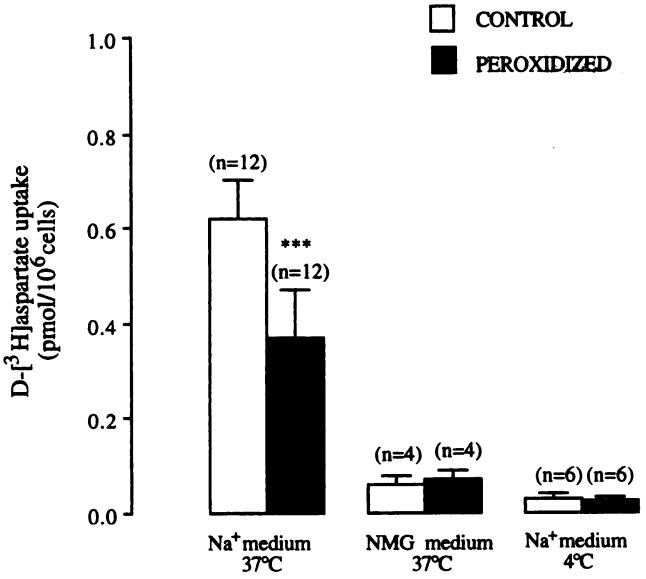

Figure 1. D-[^{3}H]Aspartate uptake by cultured retina cells cells submitted (peroxidized) or not (control) to oxidative stress induced by ascorbate/Fe^{2+}. The uptake of D-[^{3}H]aspartate was carried out for 10 min at 37°C or at 0–4°C in Na^+ medium or in NMG medium, which is identical to the Na^+ medium except that NaCl was isosmotically substituted by N-methyl-D-glucamine. The cells were exposed to ascorbate/Fe^{2+} for 15 min before the incubation with the D-[^{3}H]aspartate, and the uptake was performed in the absence of the oxidant pair as described in Materials and Methods. Data are means ± SE of number of experiments indicated. ***$P < 0.001$ as compared with control cells.

cubated with the same antioxidants but not exposed to ascorbate/Fe^{2+}.

Vitamin E (D-α-tocopherol) is the major lipid-soluble, chain-breaking antioxidant in biological systems. In the present study, we used α-tocopherol acetate because the incorporation of this type of Vit E is faster than that of α-tocopherol succinate. Moreover, α-tocopherol acetate appears to be the best form to use in media supplementation, because α-tocopherol succinate may have cytotoxic effects due to its ester groups (28). To determine the preincubation time and the concentration of Vit E acetate that inhibits the peroxidation under our experimental conditions, we tested the effect of 50, 100, or 200 μM Vit E added to the culture medium for periods of time ranging from 2 to 24 h. It was observed that 100 μM and 200 μM of Vit E protected completely against peroxidation, as measured by the production of TBARS, only after 24 h preincubation. At 50 μM Vit E did not prevent completely the production of TBARS, even after 24 h preincubation (**Fig. 3**). Therefore, we tested the effect of 100 μM Vit E on the uptake of D-[^{3}H]aspartate by cells exposed to ascorbate/Fe^{2+}.

Although 100 μM Vit E abolished the peroxidation (Table 2), the antioxidant reverted by only a small extent ($P>0.05$) the effect of ascorbate/Fe^{2+} on the uptake of D-[^{3}H]aspartate (Fig. 2). The effect of 200 μM Vit E on the D-[^{3}H]aspartate uptake was not significantly different from that obtained with 100 μM (data not shown). Dimethyl sulfoxide (1%; DMSO) used as a vehicle of the lipophilic antioxidants, Vit E and melatonin, reduced by only 8% ($P>0.05$) the formation of lipid peroxidation products in cells exposed to ascorbate/Fe^{2+} (Table 2). The antioxidant effect of DMSO, which is probably due to its SO groups (29), may account for part of the small protecting effect of Vit E on the uptake of D-[^{3}H]aspartate by cells subjected to the oxidant pair (Fig. 2).

Melatonin was reported to be a potent free radical scavenger (3, 30). In retinal homogenates it was observed that this indolamine, used at 2 mM, completely abolishes lipid peroxidation, whereas at 0.1 mM it is without effect (15). Therefore, we investigated the effect of these two concentrations of melatonin on the extent of peroxidation evoked by ascorbate/Fe^{2+} and on the D-[^{3}H]aspartate uptake in cells exposed or not to the oxidant pair. Ten minutes preincubation of retina cells with melatonin before exposure to ascorbate/Fe^{2+} affected the lipid peroxidation chain reaction (Table 2). Melatonin, at a concentration of 2 mM, abolished completely the formation of the lipid peroxidation products, whereas at 0.1 mM it reduced significantly ($P<0.001$) the formation of TBARS, by about 65%. D-[^{3}H]aspartate uptake inhibition, observed upon exposure to ascorbate/Fe^{2+} in the presence of DMSO, was significantly ($P<0.05$) reduced by the former concentration of melatonin. However, 0.1 mM melatonin did not affect significantly the uptake of D-[^{3}H]aspartate by cells subjected to oxidative stress (Fig. 2).

It is known that oxidative stress conditions cause an excessive release of free fatty acids, mainly arachidonic acid (AA), due to the activation of phospholipase A_2 (PLA_2; 31). Several studies have also reported that AA could affect the glutamate transport in neuronal or glial cells (32–34). Therefore, we analyzed the effect of a PLA_2 inhibitor (ONO-RS-082; 31) and of fatty acid-free BSA on the uptake of D-[^{3}H]aspartate by cells exposed or not to ascorbate/Fe^{2+}. **Figure 4** shows that 20 μM ONO-RS-082 or the chelation of free fatty acids with fatty acid-free BSA had no significant effect on the uptake of D-[^{3}H]aspartate by control and peroxidized cells. These two agents had no effect on the extent of peroxidation induced by ascorbate/Fe^{2+} (data not shown). The results indicate that arachidonic acid was not involved in the reduction of the activity of the D-[^{3}H]aspartate uptake system induced by oxidative stress conditions.

Glutamate receptor antagonists partially prevent the D-[^{3}H]aspartate uptake inhibition induced by oxidative stress

We have previously reported that oxidative stress conditions induced by ascorbate/Fe^{2+} increase the resting intracellular Na^+ concentration ($[Na^+]_i$) of cultured retina cells, due to the excessive activation of ionotropic glutamate receptors (35). Since D-[^{3}H]aspartate uptake system relies on the presence of a transmembrane Na^+-electrochemical gradient, we hypothesized that the reduction of D-[^{3}H]aspartate uptake in cells submitted to the oxidant pair was due to an increased $[Na^+]_i$. The resting $[Na^+]_i$ of retina cells, exposed to ascorbate/Fe^{2+} in the presence of

TABLE 2. *Effect of various antioxidants on the extent of peroxidation induced by ascorbate/Fe^{2+}* [a]

Antioxidants	Control		+Ascorbate/Fe^{2+}	
	TBARS (nmol/mg protein)	Conjugated dienes (nmol/mg protein)	TBARS (nmol/mg protein)	Conjugated dienes (nmol/mg protein)
None	0.3 ± 0.1	228.2 ± 10.7	7.5 ± 0.5	334.5 ± 10.7
DMSO (1%)	0.2 ± 0.1	227.8 ± 12.0	6.9 ± 0.8	329.5 ± 11.7
Vit E (100 μM)	0.3 ± 0.1	208.1 ± 8.6	0.4 ± 0.2***	200.2 ± 11.4***
Melatonin (0.1 mM)	0.3 ± 0.1	215.5 ± 6.9	2.6 ± 0.3**	267.0 ± 14.5**
Melatonin (2 mM)	0.2 ± 0.1	213.7 ± 7.9	0.4 ± 0.2***	200.8 ± 10.4***
GSH (4 mM)	0.4 ± 0.2	218.0 ± 3.9	6.2 ± 0.3	307.5 ± 9.9
DTT (4 mM)	0.2 ± 0.1	242.2 ± 16.1	6.4 ± 0.5	303.2 ± 15.7

[a] The cells were exposed or not to ascorbate/Fe^{2+} for 15 min in the presence or absence of the indicated concentrations of Vit E, melatonin, GSH, and DTT as indicated in Materials and Methods. A preincubation with the antioxidants for 10 min in Na^+ medium was performed when melatonin, GSH, or DTT was tested. When the effect of Vit E was determined, it was added to the culture medium 24 h before exposing the cells to ascorbate/Fe^{2+}. The effect of dimethyl sulfoxide (DMSO, used as a vehicle for Vit E and melatonin) was also tested. The quantification of TBARS and of conjugated dienes was performed as described in Materials and Methods. Data are means ± SE of three–six experiments. ** $P < 0.01$ *** $P < 0.001$, as compared with cells in the same experimental conditions in the absence of antioxidants.

ionotropic glutamate receptor antagonists, is similar to that of control cells, exposed or not to the antagonists (35). Therefore, we investigated the effect of NMDA and non-NMDA receptor antagonists MK-801 and CNQX, respectively, on the uptake of D-[^{3}H]aspartate by control and peroxidized cells. Under these conditions the $[Na^+]_i$ of peroxidized cells is similar to that of control cells, and any effect on the transport of D-[^{3}H]aspartate could not be attributed to changes in the Na^+ gradient. Figure 4 shows that MK-801 (10 μM) and CNQX (10 μM) were without effect in control cells, but reduced by about 20% the D-[^{3}H]aspartate uptake inhibition evoked by oxidative stress. However, the uptake of D-[^{3}H]aspartate by peroxidized cells in the presence of glutamate receptor antagonists was still significantly ($P<0.01$) inhibited as compared with control cells. The glutamate receptor antagonists were without effect on the extent of peroxidation (data not shown).

Disulfide reducing agents totally prevent the D-[^{3}H]aspartate uptake inhibition induced by oxidative stress

Reduced glutathione (GSH) is a hydrophilic antioxidant that protects cells from oxidative stress by protecting biomolecules from ROS damage (36). In the presence of this antioxidant, the extent of peroxidation induced by ascorbate/Fe^{2+} was not significantly ($P>0.05$) affected (Table 2). However, GSH (4 mM) completely protected the D-[^{3}H]aspartate transport system against the effect of oxidative stress, and even enhanced the uptake above the control value (Fig. 2). The effect of dithiothreitol (DTT, 4 mM) was similar to that of GSH, both in the extent of peroxidation and in the protection against the effect of oxidative stress on the D-[^{3}H]aspartate uptake system. Since DTT is a synthetic agent with selective disulfide reducing properties (37), the results suggest that the SH redox properties of GSH are involved in the protection of the D-[^{3}H]aspartate transport system against the effect of oxidative stress.

Reversibility of the effect of oxidative stress on D-[^{3}H]aspartate uptake system

To investigate whether the effects of oxidative stress on D-[^{3}H]aspartate transport system are reversible, we tested the effect of GSH, DTT, melatonin, and glutamate receptor antagonists, which are efficient protective agents (see above), on the uptake of D-[^{3}H]aspartate by cells previously exposed to ascorbate/Fe^{2+}. The cells were exposed or not to the oxidant pair in the absence of the antioxidant, and thereafter incubated in the absence of antioxidants or in the presence of GSH (4 mM), DTT (4 mM), melatonin (2 mM), or MK-801 (10 μM) plus CNQX (10 μM) for 60 min (**Fig. 5**). One hour after incubation with ascorbate/Fe^{2+}, the inhibition of D-[^{3}H]aspartate uptake persisted. However, GSH, DTT, and melatonin significantly ($P<0.05$) reversed the D-[^{3}H]aspartate uptake inhibition, whereas MK-801 (10 μM) plus CNQX(10 μM) were without effect, as compared with cells exposed to the oxidant pair for 15 min and then left in Na^+ medium for 60 min. Otherwise, neither GSH nor DTT, exposed for 25 min, were capable of reverting the inhibition of D-[^{3}H]aspartate uptake. The uptake of D-[^{3}H]aspartate by retina cells subjected to oxidative stress and further incubated for 25 or 60 min in the absence of the antioxidants (Fig. 5) was slightly higher, but not significantly different ($P>0.05$), than that determined immediately after the 15 min exposure to the oxidant pair (Fig. 2).

DISCUSSION

The molecular mechanisms involved in the dysfunction of the EAA transport system and in excitotoxicity that occurs in several neurodegenerative diseases are not completely understood (2). In the present study we show that oxidative

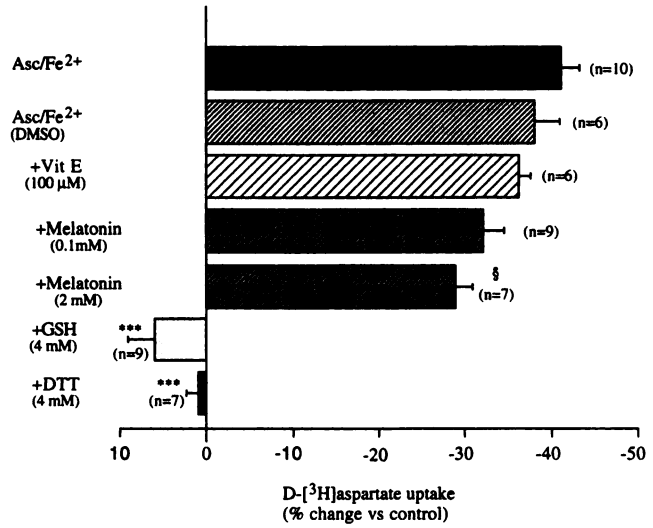

Figure 2. Effectiveness of antioxidants in protecting D-[^{3}H]aspartate uptake system (or systems) from oxidative stress (ascorbate/Fe^{2+})-induced inhibition. Cultured retina cells were incubated with ascorbate/Fe^{2+} in the presence or absence of the antioxidants for 15 min. When melatonin, GSH, or DTT were tested at the indicated concentration, a preincubation of 10 min was performed. α-Tocopherol acetate (Vit E, 100 µM) was added to culture medium 24 h before submitting the cells to oxidative stress conditions in the presence of this lipophilic antioxidant. Dimethyl sulfoxide (DMSO) was used as a vehicle for Vit E and melatonin was also tested because of its antioxidant properties. The D-[^{3}H]aspartate uptake assays were carried out in Na^+ medium for 10 min in the absence of the antioxidants. Bars show the mean percentage inhibition induced by ascorbate/Fe^{2+} in the presence or absence of the antioxidants as compared to control cells under the same experimental conditions. The effect of hydrophilic compounds (GSH and DTT) on the uptake of D-[^{3}H]aspartate by peroxidized cells was compared to that in the absence of the antioxidants (uppermost column). The effect of lipophilic antioxidants on the activity of the transporter in cells subjected to ascorbate/Fe^{2+} (Asc/Fe^{2+}) was compared with that in Na^+ medium containing DMSO (second column down). Data are means ± SE of the number of experiments indicated. ***$P < 0.001$ as compared with the percentage of inhibition in the absence of the antioxidants and of DMSO; §$P < 0.05$ as compared with the percentage of inhibition in the absence of the antioxidants and in the presence of DMSO.

stress conditions induced by ascorbate/Fe^{2+} reduce significantly ($P<0.001$) the activity of the EAA transporter, mainly by protein oxidation.

Ascorbic acid, which displays antioxidant properties, can generate ROS, and therefore lipid peroxidation, when associated with transition metals such as Fe^{2+} (4). Lipid peroxidation can cause gradual alterations on membrane functions and may ultimately lead to the loss of membrane integrity. The extent of the oxidative process induced by (1.5 mM) ascorbate/Fe^{2+} (7.5 µM) in our cell preparation was considered moderate because: *1*) a significant ($P<0.001$) rise in the formation of TBARS as well in conjugated dienes was observed in cells exposed to the oxidant pair, and *2*) the leakage of LDH, as well as the reduction of the MTT, were not affected by these oxidative stress conditions (Table 1). Therefore, the peroxidized cells used in the present study were active, and the inhibition of D-[^{3}H]aspartate uptake under the oxidative stress conditions used cannot be attributed to the loss of cell integrity or to impairment of their energy metabolism. The results are in agreement with previous reports showing that EAA transporter systems are affected by oxidative stress conditions (7, 18, 38), although the molecular mechanisms involved have not been fully elucidated.

Glutamate can be taken up by the cells through a high-affinity or a low-affinity transport systems. The former is Na^+ dependent and exists in glial cells and in glutamatergic neurons, whereas the low-affinity uptake system is Na^+ independent and exhibits K_m values above 500 µM (5). Three different glutamate transporters have been cloned (5); the *EAAC-1* is thought to be a neuronal transporter (39, 40), whereas *GLT-1* and *GLAST-1* are thought to be expressed mainly by glial cells (5, 40, 41, 42). However, immunocytochemical studies revealed that the *GLT-1* transporter is localized in photoreceptors and in bipolar cells of rat retina (43). The L-glutamate/L-aspartate transporter, *GLAST-1*, is expressed by retinal astrocytes, but not by neurons (44). However, to our knowledge the identity of the EAA transporter (or transporters) expressed by cultured retina cells used in this study has not been determined.

D-Aspartate is a nonmetabolizable analog of L-glutamate, which is transported by the same transport carrier (27). Therefore, in this study we used D-[^{3}H]aspartate as the substrate of the Na^+-dependent glutamate transporter to label cytosolic reuptake pools (45) in order to assess the effect of oxidative stress on the EAA transport system.

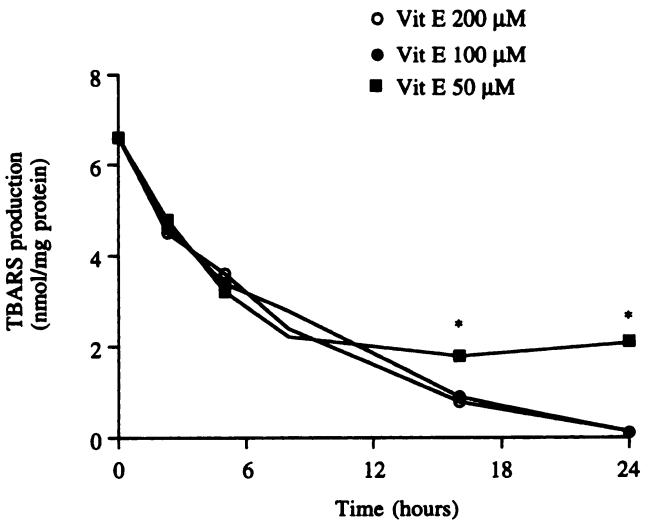

Figure 3. Time course of the effect of α-tocopherol acetate (Vit E) on the extent of peroxidation induced by ascorbate/Fe^{2+}. The cells were preincubated with the indicated concentrations of Vit E (50, 100, or 200 µM) in culture medium for the indicated time periods. After preincubation with the lipophilic antioxidant, the medium was removed and the cells were exposed or not to ascorbate/Fe^{2+} in the presence of Vit E for 15 min at 37°C. The extent of peroxidation was evaluated as described in Materials and Methods. The calculated production of TBARS (nmol/mg protein) is the difference between the total amount formed and the TBARS measured in cells not subjected to the oxidant pair. Data are means ± SE of three to six different experiments. *$P <$ 0.05 as compared with 100 or 200 µM of Vit E incubated for the same period of time.

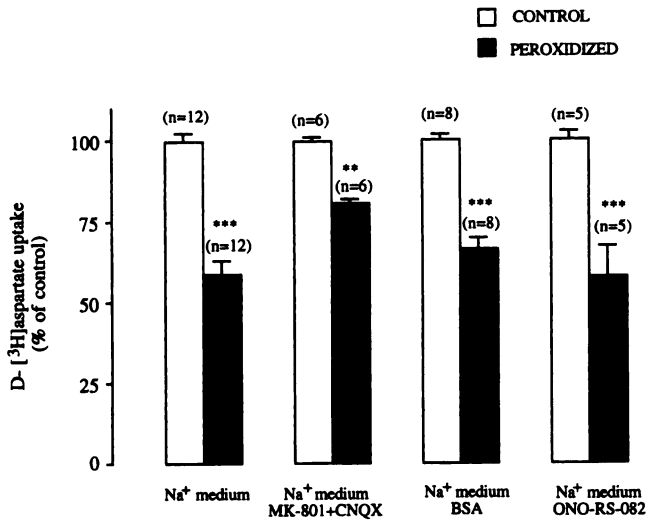

Figure 4. Effect of glutamate receptor antagonist, ONO-RS-082 and of fatty acid-free BSA on the D-[^{3}H]aspartate uptake in control and in peroxidized cells. Preincubation with MK-801 (10 μM) plus CNQX (10 μM), ONO-RS-082 (20 μM), or with fatty acid-free BSA (1 mg/ml) was performed for 25 min before the D-[^{3}H]aspartate uptake assays. The experiments were conducted similarly to those described in Fig. 2. The results are expressed as percentage of the amount of D-[^{3}H]aspartate taken up by control cells in the same experimental conditions. Data are presented as means ± SE of the number of experiments indicated. $^{**}P < 0.01$, $^{***}P < 0.001$ as compared with control cells under the same experimental conditions.

In retina cells, D-[^{3}H]aspartate uptake was almost completely abolished when Na^+ was replaced by NMG and when the uptake assays were performed at 0–4°C (Fig. 1), indicating that this uptake is Na^+ dependent and mediated by a protein carrier and not due to a nonspecific transport. The concentration of D-[^{3}H]aspartate (5.5 nM) used in the uptake assays was much lower than the K_m value for the low-affinity uptake system. Therefore, it can be concluded that under our experimental conditions the uptake of D-[^{3}H]aspartate by retinal cells was mediated by a high-affinity transport system.

To determine whether the effect of oxidative stress on the activity of the EAA transporter (or transporters) of cultured retina cells was due to modifications in the protein or in the lipidic environment, we studied the effect of several ROS scavengers on the extent of lipid peroxidation and on D-[^{3}H]aspartate uptake. Vitamin E is known to incorporate into the cell membranes to suppress fatty acid oxidation. This antioxidant contributes with hydrogen atoms from its phenolic hydroxyl group to lipid-derived peroxyl radicals, thereby preventing the propagation of lipid oxidation reactions (28, 46). Figure 3 shows that Vit E acetate prevents the formation of TBARS induced by ascorbate/Fe^{2+}, in a time- and concentration-dependent manner, the maximal inhibition being achieved at 24 h preincubation and for concentrations higher than 100 μM. These results are in agreement with the results showing that maximal accumulation of Vit E in cultured murine lymphocytic leukemia cells is obtained at 24 h and for a concentration of 100 μM (28). Although preincubation with Vit E (100 μM) inhibited the peroxidation induced by ascorbate/Fe^{2+} (Table 2), it was unable to prevent the D-[^{3}H]aspartate uptake inhibition induced by this oxidant pair. Therefore, these data suggest that the inhibition of the EAA transporter was not due to degradation of the lipids around the protein carrier.

Melatonin (N-acetyl-5-methoxytryptamine), which is a pineal neurohormone that can also be synthesized in retina, has a strong antioxidant activity. This indolamine is more efficient in scavenging both hydroxyl (HO•)− and peroxyl (ROO•)− radicals than other antioxidants such as Vit E, glutathione, and mannitol (47, 48). Melatonin donates an electron to HO• and produces indolyl cation radicals, which then presumably scavenge $O_2^{\bullet -}$, thereby further reducing the likelihood of the formation of the highly toxic HO• (49). Due to its high lipophilicity, melatonin is incorporated into the membranes all over the cell, and restricts lipid peroxidation by preventing the initiating events as well as by interrupting the chain reaction. Besides its direct dual scavenging capacity, melatonin stimulates glutathione peroxidase, an antioxidant enzyme that metabolizes H_2O_2 to H_2O (3). Melatonin was reported to inhibit the lipid peroxidation of retinal homogenates induced by $FeSO_4$ in a dose-dependent manner (15). Our data show that melatonin at a concentration of 0.1 mM induced a significant reduction ($P<0.01$) of TBARS and of conjugated dienes production evoked by ascorbate/Fe^{2+}, whereas at 2 mM it abolished their formation (Table 2). However, only the latter concentration of melatonin reduced significantly ($P<0.05$) the D-[^{3}H]aspartate uptake inhibition induced by ascorbate/Fe^{2+} (Fig. 2). Accordingly, it was reported that melatonin, besides its scavenging activity, can also protect biomolecules from free radical damage or can even be a reducing agent (47).

Reduced glutathione is considered a hydrophilic antioxidant, acting as a substrate for glutathione peroxidase in the removal of H_2O_2, as a disulfide reducing agent, or as a thiol substrate (36). The thiol group of this antioxidant is able to reduce free radical sites from biomolecules generated by the attack of ROS (50). Dithiothreitol is a thiol compound that selectively reduces disulfide bonds (37). GSH (4 mM), as well as DTT (4 mM), did not inhibit the lipid peroxidation process, but prevented completely the D-[^{3}H]aspartate uptake inhibition induced by ascorbate/Fe^{2+} (Table 2 and Fig. 2). The lack of effect of GSH on lipid peroxidation evoked by ascorbate/Fe^{2+} was probably due to the fact that this antioxidant is not lipophilic, and therefore gives little protection to lipidic damage. Accordingly, it was shown that under oxidative stress conditions, the inhibition of glutathione reduction causes an extreme loss of cell viability whereas the extent of peroxidation was not significantly increased (51). Therefore, the observed effect of GSH in preventing the inhibition of D-[^{3}H]aspartate uptake was probably due to its capacity of reducing protein thiol groups, since a similar effect was obtained with the synthetic selective disulfide reducing agent DTT. Taken together, the data suggest that oxidation of protein thiol groups, rather than lipid peroxidation, is

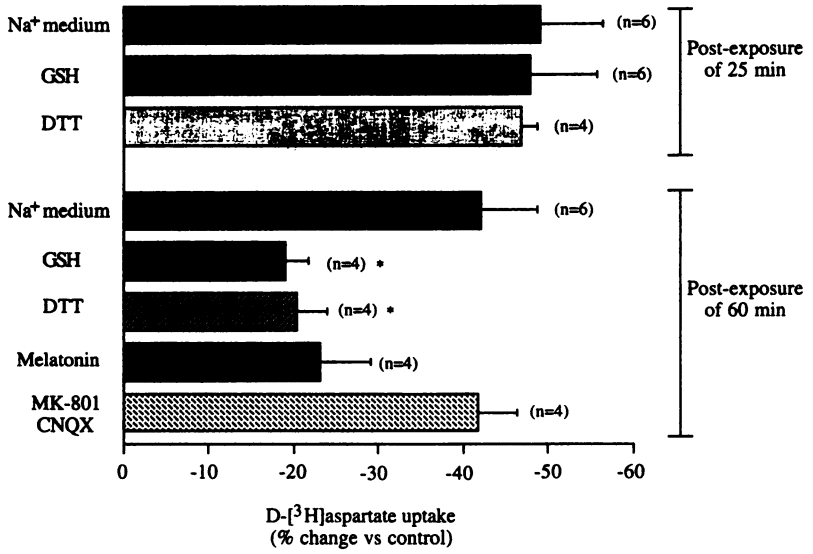

Figure 5. Effect of GSH, DTT, melatonin, and glutamate receptor antagonists on the activity of the D-[^{3}H]aspartate uptake system of cells previously subjected to ascorbate/Fe^{2+}. The cells were exposed (peroxidized) or not (control) to the oxidant pair for 15 min in the absence of the mentioned agents. Then, the cells were washed and incubated in Na^+ medium containing GSH (4 mM), DTT (4 mM), melatonin (2 mM), or MK-801(10 μM) plus CNQX (10 μM) for 60 min or 25 min. The uptake of D-[^{3}H]aspartate was performed after exposure to the indicated agents or in their absence, as described in the caption to Fig. 2. The results are expressed as percentage of the D-[^{3}H]aspartate uptake inhibition relatively to the amount of D-[^{3}H]aspartate taken up by control cells in the same experimental conditions. Data are presented as means ± SE of the number of experiments indicated. *$P < 0.05$ as compared with the percentage of inhibition in the absence of the antioxidants under the same experimental conditions.

likely to represent the mechanism leading to D-[^{3}H]aspartate uptake inhibition induced by ascorbate/Fe^{2+}. However, we cannot rule out the possible involvement of other mechanisms in the observed inhibition of D-[^{3}H]aspartate uptake by oxidative stress.

We have previously reported that oxidative stress conditions increase the resting $[Na^+]_i$ of retina cells due to the excessive activation of the ionotropic glutamate receptors (35). This Na^+ overload could also be responsible for the inhibition of D-[^{3}H]aspartate uptake due to the reduction in the Na^+-electrochemical gradient. To test this hypothesis, we determined the uptake of D-[^{3}H]aspartate by retina cells subjected to oxidative stress in the presence of the glutamate receptor antagonists MK-801 and CNQX. Although in these conditions the resting $[Na^+]_i$ of peroxidized cells is not significantly different from that of control cells (35), the present study shows that D-[^{3}H]aspartate uptake inhibition caused by ascorbate/Fe^{2+} was only partially ($P<0.01$) reduced by glutamate receptor antagonists (Fig. 4). These data suggest that the $[Na^+]_i$ rise in retina cells under oxidative stress could also contribute to inhibition of the D-[^{3}H]aspartate uptake. However, the observation that MK-801 and CNQX did not completely reverse the inhibition of D-[^{3}H]aspartate uptake caused by oxidative stress indicates that other mechanisms are also involved.

A number of neurotransmitters use protein kinase C (PKC) and/or cAMP-dependent protein kinase (PKA) to induce phosphorylation of the transporter proteins, either directly or indirectly (see ref 52 for review). Phosphorylation by PKC was shown to activate the glial, but not the neuronal, glutamate uptake system in primary cultures from the rat cerebral cortex (53), suggesting that the kinase may not be responsible for the effects reported here.

We have previously reported that ascorbate/Fe^{2+}-induced oxidative stress potentiates the NMDA-receptor activity due to the release of AA (17). Free arachidonic acid was shown to inhibit the activity of the glutamate transporter by binding either to the transporter protein or to the protein-lipid boundary (34, 54, 55). However, arachidonic acid seems not to be involved in the inhibition of D-[^{3}H]aspartate uptake under the oxidative stress conditions used here, since the preincubation of cells with the PLA_2 inhibitor ONO-RS-082 or the fatty acid chelator BSA had no significant effect on the activity of the transporter, both in control and in peroxidized cells (Fig. 4). It is possible that the AA released did not reach high enough concentrations to affect the activity of the EAA transporter. Indeed, it was reported that 50 μM of AA inhibits the glutamate uptake in cerebrocortical neurons of rat, whereas 3 μM of AA is sufficient to potentiate NMDA receptor activity of cerebellar granule cells of rat (54). ROS could promote the release of AA via activation of PLA_2, and ROS could be further generated during AA metabolism (31, 54). However, this appears not to be the case under our experimental conditions, because the extent of peroxidation evoked by ascorbate/Fe^{2+} was not affected when the cells were subjected to oxidative stress in the presence of a PLA_2 inhibitor or with fatty acid-free BSA (data not shown). Accordingly, it was shown that AA induces detectable superoxide accumulation in astrocytes for concentrations higher than 100 μM (56), which are well above those required to inhibit the glutamate transporter (34).

D-[^{3}H]Aspartate uptake inhibition induced by ascorbate/Fe^{2+} was partially maintained even 1 h after removal of the oxidant pair (compare Fig. 2 and Fig. 5). These results indicate that ROS cause long-lasting modifications of the high-affinity glutamate/D-[^{3}H]aspartate transport system. A persistent inhibition of glutamate uptake was also observed in rat cortical astrocytes exposed to xantine/xantine oxidase (35). Figure 5 shows that addition of GSH or DTT to retina cells previously subjected to oxidative stress for 1 h reduced significantly ($P<0.05$) the inhibition of D-[^{3}H]aspartate uptake evoked by ascorbate/Fe^{2+}. Melatonin also reversed the inhibition by about 18%, but this effect was not statistically significant ($P>0.05$, Dunnett's test). Therefore, the data suggest that the activity of the EAA transporter was not irreversibly affected by oxidative stress conditions.

Surprisingly, neither GSH nor DTT were able to reverse the D-[^{3}H]aspartate uptake inhibition when incubated for 25 min. This contrasts with the observations reported in rat cerebrocortical astrocytes showing that 10–20 min postexposition to DTT reverted significantly the glutamate uptake inhibition induced by either H_2O_2 or xantine/xantine oxidase (38). A likely explanation for the lack of effect of short incubations with GSH or DTT under our experimental conditions (Fig. 5) could be that the peroxidation chain reactions proceed after the removal of the oxidant pair; thus, even though these agents repair some of the transporter proteins, others are still being affected. For longer incubations, the oxidative chain reactions may be attenuated by the action of the endogenous antioxidants, GSH and DTT being more available to repair the D-[^{3}H]aspartate transport systems. Moreover, it was reported that under oxidative stress conditions, such as cerebral ischemia, glutathione is released and decreases the amount of hydroxyl radicals (57). Therefore, in an early phase of the oxidative injury of retina cells induced by ascorbate/Fe^{2+}, the added GSH may have limited the excessive production of ROS rather than being used to recover the damaged protein transporters.

The glutamate receptor antagonists MK-801 and CNQX reverse the $[Na^+]_i$ rise due to the incubation with ascorbate/Fe^{2+}, within about 30 min, to levels similar to the resting $[Na^+]_i$ of control cells. The postexposure of MK-801 and CNQX had no effect on the D-[^{3}H]aspartate uptake inhibition, indicating that changes in $[Na^+]_i$ were not the main cause of the sustained reduction of the transporter activity.

In conclusion, our data show that the D-[^{3}H]aspartate uptake by cultured retina cells, which is mediated by the high-affinity EAA transport system, was inhibited by oxidative stress conditions induced by ascorbate/Fe^{2+}. Protein oxidation, more than lipid peroxidation, seems to be the main cause leading to the impairment of the transporter activity. However, this inhibition was partially reversible, and this may be important for pharmacological therapeutic interventions. Therefore, those antioxidants able to preserve or recover the physiological redox state of the proteins can be useful in protecting against excitotoxicity. The association of lipophilic antioxidants that are most effective in preventing lipid peroxidation with agents that recover damaged proteins and the Na^+ electrochemical gradient could result in a more successful neuroprotection against the excitotoxic cell damage.

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