Glutamate Uptake Inhibition by Oxygen Free Radicals in Rat Cortical Astrocytes

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Formation of reactive oxygen species and disfunction of the excitatory amino acid (EAA) system are thought to be key events in the development of neuronal injury in several acute and long-term neurodegenerative diseases. Recent evidence suggests that the two phenomena may be interdependent. The present study is aimed at exploring possible molecular mechanisms underlying oxygen radical–EAA interaction. Exposure of cortical astrocytic cultures to either xanthine + xanthine oxidase (X/XO), a free radical-generating system, or hydrogen peroxide (H_2O_2) results in a marked decrease of high-affinity glutamate transport. Within 10 min of X/XO application, uptake falls to $\approx 60\%$ of its control value. In parallel no detectable release of lactate dehydrogenase occurs. X/XO effect is abolished in the presence of a mixture of scavenger enzymes (superoxide dismutase + catalase) or by the disulfide-reducing agents glutathione and dithiothreitol (DTT), but not by lipophilic antioxidants or ascorbate. The time course of inhibition shows an almost linear decline of glutamate transport during cell exposure to free radicals, while upon their inactivation the decline stops but established inhibition persists for at least 1 hr. In this situation, application of DTT significantly restores transport function. These data suggest that free radicals inhibit glutamate uptake primarily by long-lasting oxidation of protein sulfhydryl (SH) groups. Chemical modifiers of free SH groups, such as *p*-chloromercuribenzoate and *N*-ethylmaleimide, also induce uptake inhibition. Na^+/K^+ ATPase is a known target of oxygen radicals and may be involved in glutamate uptake inhibition. Indeed, ouabain, a blocker of the pump, reduces uptake in astrocytes. However, its effect is largely additive with that of radicals. Electrophysiological recording of astrocytic resting conductance shows, in some cells, a Ba^{2+} -insensitive, inward current in response to H_2O_2 . However, in the majority of the cells, the oxidant has no effect on membrane current or voltage. In the same cells, application of glutamate in the presence of inhibitors of ionotropic EAA receptors elicits a large inward current representing electrogenic uptake. In six of seven tested cells, H_2O_2 significantly inhibited such current. These results indicate that inactivation of Na^+/K^+ ATPase can be only part of the mechanism by which oxygen radicals inhibit glutamate uptake and that a direct action on glutamate transport is likely.

In all, our data suggest that free radicals may induce extracellular accumulation of glutamate by reduction of glial uptake. In pathologies such as ischemia/reoxygenation or amyotrophic lateral sclerosis, where evidence for both oxidative stress and EAA uptake disfunction exists, this mechanism may link oxygen radical toxicity to excitotoxicity and represent an important step in the genesis of neurotoxic damage.

[Key words: oxygen free radicals, glutamate uptake, astrocytes, excitotoxicity, antioxidants, disulfide-reducing agents, protein thiol groups, redox modulation]

Oxygen-derived free radical species are formed during brain insults such as trauma and hypoxia/ischemia, particularly when followed by reoxygenation, and participate in the induction of neurotoxic injury (McCord, 1985; Braughler and Hall, 1989; Hall and Braughler, 1989; Siesjö et al., 1989). They have also been implicated in the toxicity associated with several long-term neurodegenerative disorders (Halliwell, 1992; Lees, 1993). In pathological conditions oxygen radical production is thought to overcome the endogenous protection system of scavenger enzymes and antioxidant molecules, either by “oxidative stress” (Halliwell, 1987) or “reductive stress” (Demopoulos et al., 1980). As a consequence, reactive species, including superoxide anion ($O_2^{\cdot-}$) and, in particular, hydroxyl radical ($OH^{\cdot}$), can attack cellular components like nucleic acids and nucleotides, protein SH (sulfhydryl) groups, or unsaturated fatty acid moieties of phospholipids (Slater, 1984). All these reactions are potentially toxic and may eventually lead to cell death. Membrane disruption and destabilization of Ca^{2+} homeostasis have been proposed as the major mechanisms by which free radicals induce neuronal damage (Boobis et al., 1989; Orrenius et al., 1989). However, the details of the mode of action of radicals, including the molecular targets and the sequence of events that lead to damage, remain still largely undefined. Another pathological event in common to ischemic and traumatic insult is extracellular accumulation of excitatory amino acids (EAA), which leads to excessive stimulation of glutamate receptors (Benveniste et al., 1984; Choi, 1988; Faden et al., 1989). Overfunctioning of such receptors, in particular of the NMDA type, is involved in the induction of irreversible neuronal damage (Rothman and Olney, 1987). Similar to oxidative stress, disfunction of the EAA system has been implicated in the pathogenesis of a number of long-term neurodegenerative disorders (Choi, 1992).

Several recent observations suggest that the mechanisms of oxygen free radical and EAA toxicity may be at least partially interdependent. In summary, (1) kainate-induced degeneration of cerebellar neurons in culture could be prevented by free radical scavengers (Dyckens et al., 1987); (2) glutamate administration in a neuronal cell line led to glutathione (GSH) depletion

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Figure 1. Glutamate uptake inhibition by free radical-generating systems and blockade of the effect in the presence of scavenger enzymes. ³H-glutamate uptake assay was run for 10 min in astrocytic cultures exposed to control conditions (C) or to X (500 μ M) or XO (50 mU/ml) alone, X/XO mixture without or with the scavenger cocktail SOD (90 U/ml) + CAT (3000 U/ml), or H₂O₂ (500 μ M) without or with CAT. Each column of the histogram represents the mean $\pm$ SD value of uptake obtained from 12–16 single determinations. One-way ANOVA reveals high statistical difference between treatments. In particular, as shown by the asterisks, X/XO and H₂O₂ are significantly different from any other group. All the remaining treatments do not determine uptake values different from controls (**, $P < 0.01$, one-way ANOVA followed by the Tukey method for multiple comparisons).

with peroxide formation (Murphy et al., 1989); (3) 21-amino-steroid compounds, inhibitors of lipid peroxidation, partially reduced excitotoxic neuronal death of cortical cell cultures (Monyer et al., 1990); (4) massive release of EAA in hippocampal slices subjected to “*in vitro* ischemia” was blocked by free radical scavengers and reproduced by free radical-generating systems (Pellegrini-Giampietro et al., 1990); and (5) oxidative inhibition of glutamine synthetase in parallel to free radical production was observed during early postischemic reperfusion *in vivo* (Oliver et al., 1990). On the other hand, Ca²⁺ influx through the NMDA receptors was reduced by free radicals acting at a redox modulatory site (Aizenman et al., 1990).

High-affinity glutamate reuptake systems, present in synaptic terminals and astrocytes, represent the major mechanism to control the extracellular levels of EAA and to keep them below neurotoxic values (McBean and Roberts, 1985). Defects in glutamate uptake function have been reported both in acute (Silverstein et al., 1986) and long-term (Rothstein et al., 1992) neurodegenerative pathologies associated with oxidative stress. Based on the hypothesis that an interaction between free radicals and EAA takes place during the development of neuronal damage, we became interested in exploring whether free radicals may act by inhibiting glutamate reuptake.

Materials and Methods

Astrocytic cell cultures. Primary astroglial cell cultures were obtained from the cerebral cortices of newborn rats by mechanical dissociation, plated, and grown *in vitro* for 2 weeks as previously described (Volterra et al., 1992). The cultures were then enriched (>95%) in type 1 astrocytes following the procedure by McCarthy and de Vellis (1980), replated, and kept for 4–5 d until reaching monolayer confluence. For electrophysiological experiments cells were plated at low density and grown *in vitro* for only 3–5 d (i.e., before reaching confluence) to avoid formation of gap junctions between cells and consequent space-clamp problems. These cultures contain more than one cellular type. For our studies

we selected only cells with a quite uniform, flattened morphology (type 1-like astrocytes) that responded to the application of glutamate (30 μ M) with an inward current representing electrogenic uptake (see Results and Wyllie et al., 1991).

Glutamate uptake assay in astrocytes. Cortical astrocytes plated as a monolayer culture (200–300 μ g protein/35 mm Petri dish) were incubated in the presence of different agents at 25°C under circular shaking conditions for 10 min (unless otherwise specified) with oxygenated Krebs/bicarbonate buffer containing (in mM) 124 NaCl, 4.6 KCl, 1.2 CaCl₂, 1.3 MgCl₂ $\times$ 6H₂O, 0.416 KH₂PO₄ $\times$ 2H₂O, 26.75 NaHCO₃, 10 glucose, adjusted to pH 7.4, and 40 μ M ³H-glutamate (63.5 Ci/mmol; NEN, Dreieich, Germany; isotopic dilution 1:25,000). Uptake assay was stopped by addition of ice-cold buffer with 100-fold excess cold glutamate and cells scraped from the culture dishes with NaOH (0.2 N) and counted by liquid scintillation for incorporated radioactivity. Protein content was measured in aliquots of the solubilized cells following the method of Lowry et al. (1951). ³H-glutamate concentration utilized is close to the K_m value of the high-affinity glutamate uptake in our astrocytic preparation (Volterra et al., 1992). Mean uptake values expressed as nmol/mg protein/10 min are reported in Figure 1. Elsewhere uptake is expressed as percentage of control, to facilitate comparisons among different experiments. In the experiments where uptake was measured after exposure to either xanthine + xanthine oxidase (X/XO) or H₂O₂ for 10 min, the reaction generating the radicals was rapidly stopped by substituting the cell supernatant with one containing a cocktail of superoxide dismutase (SOD) (90 U/ml) + catalase (CAT) (3000 U/ml) and repeating the wash three times before adding Krebs buffer. To start glutamate uptake assay, ³H-glutamate was directly added together with the buffer at this point or after a further period of incubation (see legends for Figs. 2B, 4). In some of the experiments with antioxidants or SH reagents, different protocols were utilized: in most cases astrocytes were incubated for 20 min with any such agents and ³H-glutamate uptake assay was run in the last 10 min of incubation. The irreversible SH chelator *p*-chloromercuribenzoate (pCMB) was added for 5 min to the cells and, after wash, uptake was tested in the next 5 min (see legends to Figs. 3, 5).

Electrophysiological patch-clamp recordings. Whole-cell recordings were performed with a List EPC 7 patch-clamp amplifier. Patch pipettes were made from borosilicate glass capillary tubes and had a resistance of 3–5 M Ω when filled with the following (intracellular) solution (in mM): KCl, 120; NaCl, 5; CaCl₂, 1; MgCl₂, 7; EGTA-KOH, 5; HEPES-KOH, 10; ATP(K), 5; pH 7.2. Cells in the bath were continuously superfused with a solution of the following composition (in mM): NaCl, 140; KCl, 5.4; CaCl₂, 1.8; MgCl₂, 1; HEPES-NaOH, 5; pH 7.4. In some experiments BaCl₂ (5 mM) was added in order to block most of the K⁺ conductances, giving rise to a large depolarization (20–30 mV over the resting potential; see also Brew and Attwell, 1987). Drugs were directly applied onto cells via a fast multiple-way microperfusion system. Resting potentials (V_m) were generally around $-65/-70$ mV; the holding potential (V_h) was kept close to V_m . Experiments consisted of a continuous monitoring of the holding current and of the effects produced on its level by application of glutamate [30 μ M in the presence of 10 μ M 2-amino-5-phosphonovalerate (APV) + 100 μ M 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX)] and/or H₂O₂ (1–5 mM), while changes in V_m produced by these agents were revealed by rapid switching to the current-clamp mode. When the effect of H₂O₂ on glutamate uptake current was tested, a solution containing 1 or 5 mM H₂O₂ + 30 μ M glutamate was prepared and perfused onto the cells on top of the glutamate response by switching the microperfusion line. Thin-layer chromatography of glutamate present in such solution excluded oxidative damage to the transmitter by H₂O₂.

Lactate dehydrogenase activity assay. Lactate dehydrogenase (LDH) activity, as a marker of cell membrane disruption (Koh and Choi, 1987), was measured in samples (0.15 ml) taken from the supernatant medium of astrocytic cultures exposed for different times to oxygen radicals. A diagnostic LDH kit from Sigma (procedure 228-UV) was utilized, with a calibration curve constructed from LDH standards (Sigma procedure 340-UV). Data are expressed as U/liter/min/mg protein, following the calculation protocol of the kit manual.

Measurement of the formation of thiobarbituric acid-reactive material. Formation of thiobarbituric acid-reactive material (TBAR), as an index of lipid peroxidation processes, was assessed by spectrophotometric measurement. After incubation (10–60 min) of astrocytes (1.2 mg protein) with X/XO (50 mU/ml) the reaction was stopped by the addition of 1.5 ml ice-cold HCl (0.8 N) containing 12.5% trichloroacetic acid

Figure 2. Glutamate uptake inhibition by free radicals is progressive during exposure (*A*) and persistent after inactivation and removal of radicals (*B*). *A*, Time course study of ³H-glutamate uptake in astrocytic cultures during exposure to either X (500 μ M)/XO (50 mU/ml) (●) or H₂O₂ (500 μ M) (▲). For each time point, uptake in the presence of free radicals was compared to uptake in control conditions and plotted as percentage of inhibition $\pm$ SD. Data points represent the average of two or three experimental values in triplicate. *B*, Study of the duration of free radical-induced inhibition. Cells were first exposed to either X/XO or H₂O₂ for 10 min, and then washed three times with SOD/CAT-containing buffer and incubated in Krebs buffer for postexposure periods of variable duration. ³H-glutamate assay was performed in the last 10 min of each postexposure period and compared to uptake in control groups, which were initially exposed to Krebs buffer instead of radicals and then treated with an identical protocol. Data points are obtained from two or three different experiments in triplicate and calculated as above.

and the cells were scraped from Petri dishes. We then added 1.2 ml of TBAR (0.72%) to 300 μ l aliquots and the samples were boiled for 20 min. After cooling and centrifugation at 1500 $\times$ *g* for 15 min, absorbance of supernatants at 532 nm was measured. Quantitation of TBAR was based on a standard curve constructed with malonyldialdehyde (0.2–30 nM).

Materials. Xanthine oxidase (0.4–1.1 U/mg) was obtained from both Sigma and Fluka. The two enzyme preparations display different electrophoretic profiles: the Fluka preparation has a major protein band at $\approx$ 130 kDa, while the Sigma lacks such a band, showing a predominant band of $\approx$ 85 kDa. Some batches of Sigma XO at 50 mU/ml induced slight inhibition ($\approx$ 10%) of glutamate uptake in the absence of xanthine, which was blocked by aprotinin, a protease inhibitor. The Fluka enzyme did not modify basal uptake and was selected for the studies here reported. The other enzymes superoxide dismutase (3250–3570 U/mg) and catalase (48,700 U/mg), and reagents xanthine, reduced glutathione, DL-dithiothreitol (DTT), *p*-chloromercuribenzoic acid, 6 α -methylprednisolone-21-emissuccinate (MP), α -tocopherol (VIT E; free and acetate ester), and ouabain were purchased from Sigma; *N*-ethylmaleimide (NEM), 5-5-dithio-bis-nitrobenzoic acid (DTNB), thiobarbituric acid, and malondialdehyde diethylacetate were from Aldrich; ascorbate was from Merck; and H₂O₂ was from Farmitalia-Carlo Erba. EAA receptor inhibitors APV and CNQX were from Tocris. Compound U74500A was kindly provided by Upjohn Co.

Results

The possible effect of oxygen free radicals on the high-affinity Na⁺/K⁺-dependent glutamate transport in glial cells was first explored by adding the free radical-generating system X (500 μ M)/XO (50 mU/ml) to primary cultures of astrocytes from rat cerebral cortex, while running glutamate uptake assay (see Materials and Methods). We found that within 10 min X/XO induced $\approx$ 40% reduction of glutamate uptake (Fig. 1). Neither X nor XO alone proved able to affect glutamate uptake, indicating that inhibition was due to the products of X/XO reaction. Moreover, X/XO-induced inhibition could be almost completely prevented by addition of the cocktail of oxygen radical scavenger enzymes SOD (90 U/ml) and CAT (3000 U/ml; see Fig. 1). Neither SOD nor CAT modified glutamate uptake in control conditions and SOD alone was largely unable to prevent X/XO

effect (not shown). Analysis of the kinetic parameters of uptake indicates that inhibition by X/XO involves modification of both *K_m* and *V_{max}* values (*K_m*: $+22.8 \pm 5.6\%$ vs control; *p* < 0.05, paired *t* test; *V_{max}*: $-37.3 \pm 12\%$ vs control; *p* < 0.01). We then tested the effect of H₂O₂, another important oxidant formed in living cells and a source of OH[•] via iron catalysis (Siesjö et al., 1989). Similar to X/XO, H₂O₂ reduced glutamate uptake in a dose-dependent manner: we mostly utilized 500 μ M H₂O₂, which inhibits uptake by 20%. Inhibition by H₂O₂ was completely abolished in the presence of CAT (3000 U/ml; Fig. 1). The different efficacy of X/XO and H₂O₂ in inhibiting glutamate uptake correlates with the respective potency as oxidants in our experimental medium. Reduction of glutamate uptake with either agents was observed in experiments on synaptosomes from rat cortex, suggesting that radicals may affect also neuronal high-affinity glutamate transport (data not shown).

We then studied the time course of the inhibitory action of oxygen radicals. In the presence of either X/XO or H₂O₂, inhibition displayed a similar temporal pattern, appearing within a few minutes and then progressively increasing with time (up to 30 min of assay) without reaching a steady plateau level (Fig. 2*A*). Uptake function was then studied at various times after exposure of the cells to either X/XO or H₂O₂ followed by inactivation and removal of the radicals (Fig. 2*B*). Inhibition outlasted the presence of radicals without significantly changing with time. The same level of inhibition induced by 10 min of exposure to X/XO or H₂O₂ was observed 30 min and 1 hr later. In one experiment with X/XO it persisted for at least 4 hr (not shown).

Free radicals are well-established toxic species for the cells. Therefore, uptake inhibition might simply reflect a general damaging action, such as disruption of the cell membrane integrity and loss of the ionic gradients. To investigate this possibility we measured the release of cytosolic LDH activity in the supernatant of astrocytic cultures after exposure to radicals as an index of membrane damage (Table 1). After 10 min in the

Table 1. Extracellular LDH activity in astrocytic cultures after exposure to free radical-generating systems

	LDH activity (U/L/min/mg protein $\pm$ SD)	Number of deter- minations
10 min		
Control	7.7 $\pm$ 2.4	11
X/XO	7.5 $\pm$ 1.9	8
H ₂ O ₂	6.1 $\pm$ 0.8	8
30 min		
Control	8.3 $\pm$ 1.9	10
X/XO	24.5 $\pm$ 2.9**	8
H ₂ O ₂	15.8 $\pm$ 6.9	6
Triton X-100 (0.1%)	701.4 $\pm$ 71.6	16

Astrocytic cultures were incubated for 10 or 30 min in control conditions or in the presence of X/XO (500 μ M/50 mU/ml) or H₂O₂ (500 μ M). Cells' supernatants were then tested for LDH activity. Each determination was performed in duplicate and results of different experiments averaged together. In each experiment a separate group of cells was exposed for 10 min to the detergent Triton X-100 to acquire a comparative value of maximal cell damage. One-way ANOVA performed on control, X/XO, and H₂O₂ groups revealed significant treatment effect only at 30 min. In particular, exposure of the cells to X/XO induced a significantly higher LDH release with respect to control conditions, while H₂O₂ treatment was not reliably different from control.

** $P < 0.01$, Tukey method for multiple comparisons.

presence of H₂O₂ or X/XO at the same concentrations that significantly inhibit glutamate uptake, we were unable to detect any increase in the extracellular LDH with respect to controls. Treatment of the cells with the detergent Triton X-100 (0.1%) gave an LDH value $\approx$ 100-fold higher, which we considered the "maximal cell damage." Long exposures to free radicals, leading to high degrees of uptake inhibition (see Fig. 2A), correlated with little LDH release. After 30 min of incubation of the cells in control conditions, with X/XO, or with H₂O₂, LDH values were 1.18%, 3.49%, and 2.25% of "maximal cell damage," respectively.

We then wanted to explore the molecular mechanism by which oxygen radicals inhibit glutamate uptake. As a first step, we evaluated the protective efficacy of various types of antioxidants. We first tested lipophilic molecules that preferentially insert into the membranes and are particularly suited for protection against lipid peroxidation chain reactions (Witting, 1980). These included the prototype lipophilic antioxidant VIT E (20 or 200 μ M), MP (100 μ M), a corticosteroid exhibiting antioxidant properties at high concentrations (Demopoulos et al., 1982), and U74500A (10 μ M), a 21-aminosteroid derivative that blocks lipid peroxidation reactions by chelating the iron catalyst (Braugher et al., 1987). Compounds were added to the cells 10 min prior to uptake assay to let them incorporate into the membranes, and also during the assay together with X/XO. All three lipophilic antioxidants, even if tested at high concentrations, were completely ineffective in preventing uptake inhibition (Fig. 3). In the same experimental conditions (10 min exposure to X/XO), we were unable to detect lipid peroxidative processes in astrocytes by monitoring the formation of TBAR products. A signal of peroxidation was observed only with a 1 hr exposure. We then went on to test hydrophilic antioxidants, such as ascorbic acid (VIT C; 2 mM) and GSH (4 mM). A dramatic difference in the effects of the two compounds was observed: while VIT C completely failed to prevent inhibition by X/XO, GSH blocked it by $>90\%$ (Fig. 3). GSH is an endogenous antioxidant that can act either in conjunction with the enzyme glutathione

Figure 3. Different effectiveness of various antioxidants in protecting glutamate uptake from free radical-induced inhibition. Astrocytic cultures were incubated for 20 min without (solid bars) or with antioxidants: lipophilic compounds (striped bars); hydrophilic compounds (cross-hatched bars). In the first 10 min cells were exposed to buffer $\pm$ antioxidants, while in the next 10 min free radical-generating systems X/XO (500 μ M/50 mU/ml) or H₂O₂ (500 μ M) $\pm$ antioxidants were added and ³H-glutamate assay carried out. Lipophilic antioxidants were dissolved in dimethyl sulfoxide (DMSO) vehicle, in particular, VIT E (20 μ M), MP (100 μ M), and U74500A (10 μ M) in 1% DMSO (v/v), and VIT E (200 μ M) in 2% DMSO (giving a cloudy solution). Control uptake and uptake inhibition by free radicals in the absence of antioxidants were tested in DMSO for adequate comparison. In fact, X/XO inhibition is slightly attenuated in DMSO (DMSO 1%: see first bar; DMSO 2%: -31.2 $\pm$ 4.6%, not shown), possibly due to antioxidant properties of the S $\rightarrow$ O group. Hydrophilic compounds VIT C (2 mM), GSH (4 mM), and DTT (2 mM) did not require vehicle and were compared to X/XO or H₂O₂ in regular buffer. Bars show the percentage $\pm$ SD of change induced in glutamate uptake by X/XO or H₂O₂ in the absence or presence of various antioxidants with respect to the corresponding control value, and are calculated from at least three different experiments in triplicate. Statistical evaluation of the protective effect of each antioxidant was performed by comparing the percentage of uptake inhibition induced in the same experiment by the free radical-generating system without and with that antioxidant. Asterisks indicate that only disulfide-reducing agents modify inhibition by X/XO or H₂O₂ in a statistically significant manner (**, $P < 0.01$, and *, $P < 0.05$, paired Student's *t* test).

peroxidase as an H₂O₂ scavenger, or directly as a thiol substrate or disulfide-reducing agent (Meister and Anderson, 1983). To establish whether the SH redox properties of GSH were involved in the protection mechanism, we utilized another compound, DTT (2 mM), which is a synthetic agent with selective disulfide-reducing properties (Cleland, 1964). DTT not only completely protected glutamate uptake from X/XO inhibition but also slightly enhanced it above control value. A cautionary point regarding the interpretation of the above results is that XO contains SH groups in its structure, whose redox state might affect enzyme function (Della Corte and Stirpe, 1972). Therefore, the capacity of GSH and DTT to protect uptake could be trivially explained by a reducing attack to XO resulting in decreased formation of free radicals. To rule out this possibility

Figure 4. DTT largely reverses free radical-induced glutamate uptake inhibition. Astrocytic cultures were exposed for 10 min to either X/XO (500 μ M/50 mU/ml; $\circ$ and $\bullet$) or H₂O₂ (500 μ M; $\triangle$ and $\blacktriangle$). Removal of radicals was followed by a postexposure period (20 min for X/XO group and 10 min for H₂O₂ group) in the absence ($\blacktriangle$, $\bullet$) or presence of 2 mM DTT ($\triangle$, $\circ$). ³H-glutamate uptake was tested in the last 10 min of the postexposure period and groups $\pm$ DTT compared to controls (for details see Fig. 2B legend). Data points represent the average $\pm$ SD of three different experiments in triplicate. Groups incubated with DTT in the postexposure period show a statistically significant reduction of uptake inhibition with respect to groups without DTT, both in the case of X/XO and of H₂O₂ exposure (**, $P < 0.02$, and *, $P < 0.05$, respectively; paired Student's *t* test).

we repeated the protection experiments using H₂O₂ instead of X/XO and found that DTT also completely blocked the H₂O₂ effect (Fig. 3).

At this point we wanted to obtain further evidence that oxidation of thiol groups is involved in the inhibitory action of free radicals. Therefore, DTT was now administered after exposure of the cells to either X/XO or H₂O₂, after uptake was inhibited and free radical-generating systems were no longer present. Within 10–20 min of incubation, DTT (2 mM) proved able to reverse at least 50–60% of the inhibition induced by either source of radicals (Fig. 4).

Finally, we exposed glial cells to different SH reagents in the absence of radicals (Fig. 5). Interestingly, control uptake was stimulated in the presence of the reducing agents GSH (4 mM) or DTT (2 mM) ($\approx +15\%$). On the contrary, a 30–40% reduction (without LDH release) was observed upon application of chemical SH modifiers such as pCMB (10 μ M) or NEM (1 mM), while no relevant changes were seen with DTNB (500 μ M), a thiol oxidant that promotes formation of disulfide bridges between SH groups of vicinal cysteines.

As a next step, we asked which molecular substrates of free radical oxidation might be responsible for inhibition of glutamate uptake. At first we focused on the Na⁺/K⁺ ATPase pump for two reasons: (1) oxygen radicals have been reported to inhibit brain ATPase (Hexum and Fried, 1979), and (2) distinct purified isoforms of the protein contain reactive SH groups (Sweadner,

Figure 5. Glutamate uptake function is sensitive to SH reagents. Astrocytic cultures were exposed to distinct types of SH reagents: reducing agents GSH (4 mM) and DTT (2 mM) (open bars), SH modifiers pCMB (10 μ M) and NEM (1 mM) (crosshatched bars), and oxidant DTNB (500 μ M) (solid bar). In all cases, except with pCMB, cells were incubated for 20 min with the SH reagent and uptake assay was run in the last 10 min by addition of ³H-glutamate to the medium. pCMB, dissolved in 1% DMSO, was applied to the cells for 5 min and then removed. After three washes, uptake was tested in the next 5 min. In this case, controls were incubated with 1% DMSO for 5 min, before wash and uptake assay. The effect of each SH reagent is expressed as percentage of variation ($\pm$ SD) with respect to uptake in the corresponding control conditions. Each value represents the average of at least three different experiments in triplicate. Asterisks indicate that all compounds, except DTNB, induce a statistically significant modification of glutamate uptake (**, $P < 0.01$, and *, $P < 0.05$, respectively, vs control; two-tailed paired Student's *t* test).

1979). To start we checked the relevance of ATPase inhibition on glutamate uptake function in astrocytes by measuring uptake in the presence of ouabain, a blocker of the pump. Indeed, incubation for 10 min with ouabain (0.2–2 mM) resulted in a dose-dependent inhibition of glutamate uptake (see Fig. 6 legend for quantitative data). The effect of ouabain on uptake saturated at 1 mM, a concentration reported to block glial ATPase completely (Sweadner, 1979). We then tested the effect of H₂O₂ on glutamate uptake in the presence of ouabain. Coadministration of H₂O₂ (0.5 mM) and 1 mM ouabain within 10 min led to 44.5% uptake inhibition, a significantly higher effect than those of either H₂O₂ or ouabain alone and amounting to $\approx 80\%$ of their sum (Fig. 6). A time course study of uptake inhibition by ouabain revealed an increasing effectiveness of the ATPase blocker over time, similar to free radicals (Fig. 6; see also Fig. 2). However, at all times inhibition by ouabain was significantly lower than by H₂O₂ + ouabain. Moreover, the degree of additivity between H₂O₂ and ouabain effects did not change with time, being $86 \pm 7\%$ at 5 min, $78 \pm 6\%$ at 10 min, and $81 \pm 9\%$ at 20 min.

We then decided to undertake an electrophysiological study to establish whether radicals act directly on the electrogenic glutamate uptake process. We first characterized the current related to astrocytic glutamate transport (see Brew and Attwell, 1987). Cells were whole-cell clamped at resting potential ($-65/-70$ mV) and glutamate (30 μ M) was applied in the presence of inhibitors of ionotropic EAA receptors (10 μ M APV + 100

Figure 6. Time course of glutamate uptake inhibition induced by ouabain, H_2O_2 , and their coadministration. Glutamate uptake was tested at different times (5, 10, 20 min) in the presence of ouabain (1 mM; ■), H_2O_2 (500 μ M; ▲), or ouabain + H_2O_2 (same concentrations; ●). At 10 min, 0.2, 1, and 2 mM ouabain inhibited uptake by $24.3 \pm 4.9\%$ ($n = 3$), $31.7 \pm 3.8\%$ ($n = 4$), and $31.2 \pm 7.5\%$ ($n = 4$), respectively, and 1 mM was selected as the saturating concentration. For each time point, uptake in the presence of the above agents was compared to uptake in control conditions and plotted as percentage of inhibition ($\pm$ SD). Data points represent the average of two or three experiments in triplicate. Statistical analysis reveals that coadministration of ouabain + H_2O_2 results in a significantly higher inhibition of uptake with respect to the exposure to either ouabain or H_2O_2 alone (**, $P < 0.01$, and *, $P < 0.05$, one-way ANOVA followed by the Tukey method for multiple comparisons).

μ M CNQX). Most of the cells responded to this treatment with an inward current, often > 100 pA, which showed clear voltage dependence, did not reverse at positive potentials, was apparently not accompanied by noise increase, and could be abolished by substitution of choline for sodium (Fig. 7A). Such features identify electrogenic glutamate uptake (see Wyllie et al., 1991). Only those cells displaying the type of current above described in response to glutamate were selected for further studies. In 8 of 19 such cells brief application of H_2O_2 (1–5 mM) caused an inward current accompanied by slight membrane depolarization, which was insensitive to 5 mM Ba^{2+} and partially reversed upon washout of the compound (Fig. 7B; see legend for quantitative data). However, in the majority of the cells, H_2O_2 had no detectable effects on holding current or membrane potential (Fig. 7C, left). In these cells, we went on to test the effect of H_2O_2 on glutamate uptake current. Figure 7C (right) illustrates one such experiment representative of seven (see legend for quantitative data). At first, glutamate was applied to elicit inward uptake current, and then, when peak current was reached, H_2O_2 (1 mM, three cells; 5 mM, four cells) was added together with glutamate (see Materials and Methods). In six cells glutamate current was substantially reduced by H_2O_2 within 30–40 sec ($-71.4 \pm 22.2\%$), while in one cell it was only slightly modified. When H_2O_2 was removed, glutamate current only partially recovered, while upon washout of glutamate, membrane current came back to its original holding level.

Discussion

The data here presented indicate that reactive oxygen species, generated by either X/XO reaction or H_2O_2 administration, significantly reduce the capacity of high-affinity transport systems to take up and remove glutamate from the extracellular space. No release of cytosolic LDH from astrocytes was seen in

parallel to uptake inhibition of 30–40%, suggesting that the reduction observed in glutamate transport was not due primarily to cell damage, but to some more specific mechanism. As long as the cells were exposed to oxygen radicals, uptake inhibition increased progressively. When the reactive species were inactivated by addition of scavenger enzymes and removed from the cells, such increase stopped, but the established level of inhibition persisted for at least 1 hr. These observations suggest that free radicals act by inducing a long-lasting chemical modification of some substrate related to uptake function, possibly a protein or a lipid component of astrocytic membranes. In our studies three distinct lipophilic antioxidants, at concentrations that completely block lipid peroxidation in brain homogenates (Braugher et al., 1987), failed to prevent uptake inhibition by oxygen radicals. Moreover, no detectable peroxidation was observed in astrocytes in parallel to uptake inhibition with X/XO. On the contrary, the disulfide-reducing agents GSH and DTT completely abolished the effect of free radicals and DTT largely restored uptake function when applied after exposure to radicals. These data indicate that oxidation of protein thiol groups rather than lipid peroxidation is likely to represent the mechanism leading to glutamate uptake inhibition by free radicals. Since reversal of uptake inhibition with DTT is substantial but not complete, at present we cannot rule out other mechanisms in the action of radicals (Davies et al., 1987). However, the observation that, in the absence of radicals, GSH and DTT enhance while pCMB or NEM reduce control uptake reinforces the idea that sulfhydryl groups play a key role in controlling glutamate uptake function.

The opposing action exerted by oxidizing radical species and disulfide-reducing agents on glutamate uptake is reminiscent of the redox modulation of NMDA current (Aizenman et al., 1989, 1990). In that case, oxidation results in reduced Ca^{2+} entry through the NMDA channel. The functional consequences expected in the case of glutamate uptake are opposite: oxidation would increase the extracellular levels of glutamate, which in turn would enhance receptor activation. The overall outcome of these redox effects on glutamatergic transmission remains to be established with more integrated models.

Previous studies indicate that free radicals inhibit Na^+/K^+ ATPase in the brain (Hexum and Fried, 1979). Such inhibition would account for a disturbance of the physiological ion gradients and result in reduced driving force for glutamate uptake. Therefore, we investigated whether inactivation of Na^+/K^+ ATPase is the mechanism by which free radicals reduce glutamate transport. Application of H_2O_2 to astrocytes whole-cell clamped at resting potential induced a Ba^{2+} -insensitive inward current accompanied by small membrane depolarization, which might reflect Na^+/K^+ ATPase inhibition (see Fleming, 1980; Johnson et al., 1992). However, this effect was observed only in 40% of the cells, while in the remaining ones no appreciable change in holding current and membrane potential occurred with H_2O_2 . Heterogeneity of ATPase isoforms present in glial cells might account for different cell sensitivity to H_2O_2 (McGrail et al., 1991). Biochemical experiments with ouabain indicate that pump inhibition progressively reduces glutamate uptake. However, in the presence of 1 mM ouabain, H_2O_2 further reduces uptake (by $\approx 80\%$ of its expected effect). A possible explanation is that radicals and ouabain together block ATPase faster than ouabain alone, thereby affecting glutamate uptake in a more drastic way. Alternatively, the inhibitory mechanism of radicals may include a component other than ATPase inactivation. This

Figure 7. Electrophysiological analysis of H₂O₂ actions on holding current and glutamate uptake current in astrocytes. *A: Left*, Large inward current (−300 pA) in an astrocyte whole-cell clamped at −67 mV ($V_h = V_m$) in response to brief application of glutamate (30 μ M) via fast microperfusion. The bathing solution is a normal saline containing 140 mM NaCl + inhibitors of ionotropic glutamate receptors: APV (10 μ M) and CNQX (100 μ M). *Right*, Recording from the same cell $\approx$ 5 min later, after switching bathing solution to a Na⁺-free medium, containing 140 mM choline Cl. Longer application of glutamate is unable to elicit inward current in such medium. *B*, Effect of H₂O₂ perfusion (5 mM for $\approx$ 15 sec) onto a cell clamped at −62 mV. Partial recovery ($\approx$ 45%) is observed upon washout of the compound. Such experiment was repeated in eight cells responding to glutamate as above and displaying an average V_m of -65.9 ± 4 mV. Inward currents in response to H₂O₂ varied among different cells (-62 ± 48.3 pA), as did the degree of reversibility of the effect ($65 \pm 36\%$). In some cells V_m at peak of H₂O₂ was read by rapid switch to the current-clamp mode and found depolarized by 3.4 ± 3.6 mV ($n = 5$). In three cells, where the experiment was performed in the presence of 5 mM Ba²⁺, to abolish all astrocytic K⁺ conductances (Newman, 1985), H₂O₂ still induced inward currents. The effect of DTT (2 mM) was also tested in seven cells: in four, DTT did not modify holding current; in two, elicited a small outward current (34 ± 6 pA); in one, a small inward current (−15 pA) (not shown in the figure). *C: Left*, Absence of effect of H₂O₂ on astrocytic holding current in another cell. V_m is −68 mV. Note that application of H₂O₂ is significantly longer than in *B*. Similar result was obtained in 11 cells, which did not display gross differences from the ones in *B* with respect to morphology, V_m , or entity of glutamate uptake current. *Right*, In the same cell, $\approx$ 3 min after test on holding current, H₂O₂ (5 mM) inhibits glutamate uptake current. Response to glutamate (+ APV/CNQX) in this cell is −195 pA at peak before H₂O₂, −70 pA after 40 sec in the presence of H₂O₂, and −135 pA at peak after H₂O₂. On average, in six experiments H₂O₂ reduced glutamate uptake current from -118 ± 111 pA to -42 ± 54 pA, with a statistically significant effect ($P < 0.05$, two-tailed paired Student's *t* test).

latter possibility is supported by the observation that H₂O₂, in most of the cells where it does not modify membrane current, significantly inhibits glutamate uptake current, suggesting that free radicals directly interfere with the glutamate transport process. At present, no information exists about chemical reactivity of glutamate transporters to oxygen radicals. However, a report that pCMB affects Na⁺-dependent glutamate binding to synaptic membranes (thought to represent binding to the trans-

porter) suggests that glutamate transporters might contain functionally relevant SH groups (Ogita and Yoneda, 1986). Indeed, a recently cloned glial glutamate transporter carries both a single cysteine in its major external loop and two close cysteines in a membrane-spanning domain (Pines et al., 1992). Therefore, it will be of great interest to see whether free radicals may react at those sites with the transporter protein resulting in functional inhibition of the uptake process.

Uncontrolled formation of oxygen radicals as well as abnormal elevation of the extracellular levels of EAA have been shown to occur as a consequence of traumatic or ischemic insults (see Siesjö et al., 1989). Glutamate uptake inhibition by free radicals could be an important mechanism linking the two phenomena and possibly contributing to their neurotoxic consequences. At present the basic mechanisms leading to pathological EAA accumulation in ischemia/reoxygenation are still controversial. Both Ca^{2+} -dependent exocytotic release of glutamate (Bosley et al., 1983; Drejer et al., 1985) and Ca^{2+} -independent phenomena, such as impaired or reversed uptake function (Ikeda et al., 1989; Katayama et al., 1991; Rubio et al., 1991), have been implicated. Interestingly, Pellegrini-Giampietro et al. (1990) found that "*in vitro* ischemia" in hippocampal slices leads to massive Ca^{2+} -independent release of EAA that is blocked by radical scavengers and reproduced by radical-generating systems. Our present finding that oxygen radicals inhibit glutamate uptake could well explain such phenomenon.

To our knowledge, only a few studies directly checked neurotransmitter reuptake function under hypoxic/ischemic conditions. All of them reported reduced glutamate or D-aspartate uptake (Weinberger and Cohen, 1982; Hauptman et al., 1984; Silverstein et al., 1986; Swanson, 1992). Moreover, even during postischemic reflow, when normal metabolic and ion conditions are reestablished, glutamate uptake did not promptly recover (Hauptman et al., 1984; Silverstein et al., 1986). In particular, Silverstein et al. (1986) found normal uptake 24 hr but not 1 hr after transient ischemia and concluded that reduced uptake was due to some specific persistent inhibitory mechanism. They implicated arachidonic acid, which is massively released during ischemia and potentially inhibits glutamate uptake *in vitro* (Barbour et al., 1989; Volterra et al., 1992). Based on the present results, formation of oxygen free radicals could also take part in the process. In fact, the arachidonate effect is strictly dependent on the presence of the free fatty acid and is mostly reversed upon its removal (Volterra et al., 1992). In many brain areas this occurs within the first 15–30 min of reperfusion (Yoshida et al., 1982). On the contrary, the effect of radicals presented here is independent of their lifetime and, in our *in vitro* conditions, lasts at least 1 hr after their neutralization. Interestingly, a slow, progressive wave of extracellular accumulation of EAA, which develops during postischemic reflow, has been observed by some authors (e.g., Andinè et al., 1991). At parallel times, hyperactivity of hippocampal glutamatergic fibers preceding functional death was reported (Andinè et al., 1988; Urban et al., 1989). Long-lasting inhibition of glutamate uptake induced by free radicals might play a role in the development of this late component of EAA accumulation and damage.

Amotrophic lateral sclerosis (ALS) is another degenerative neurologic disorder where disturbance of glutamate uptake function as well as oxidative stress might play a central role. Thus, recent data show that glutamate uptake is decreased selectively in the brain and spinal cord areas associated with motor neuron degeneration (Rothstein et al., 1992). Moreover, genetic linkage between a familial variant of ALS and defects in the gene encoding for a cytosolic form of the SOD enzyme (SOD-1) was recently described (Rosen et al., 1993). In view of a better understanding of the mechanisms leading to neurotoxicity in this disease, it will be of importance to investigate whether oxidative stress and excitotoxicity are linked by free radical-induced inhibition of glutamate uptake.

In conclusion, we have shown that glial glutamate transport

is a functional target in the action of reactive oxygen species. This evidence indicates an additional mechanism for oxygen radical toxicity and reinforces the concept that free radicals and EAA may cooperate in the genesis of neurotoxic injury. Protein oxidation more than lipid peroxidation seems relevant in this case. This observation may prove important for the antioxidant therapeutic approach against excitotoxic neuronal damage. In fact, while lipophilic antioxidants already demonstrated an ability to attenuate excitotoxicity (Miyamoto et al., 1989; Monyer et al., 1990), new agents able not only to interfere with lipid peroxidation reactions but also to preserve or restore the physiological redox state of proteins could result in a more complete neuroprotection.

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