Inhibition of Astrocyte Glutamate Uptake by Reactive Oxygen Species: Role of Antioxidant Enzymes

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ABSTRACT

Background: The recent literature suggests that free radicals and reactive oxygen species may account for many pathologies, including those of the nervous system.

Materials and Methods: The influence of various reactive oxygen species on the rate of glutamate uptake by astrocytes was investigated on monolayers of primary cultures of mouse cortical astrocytes.

Results: Hydrogen peroxide and peroxynitrite inhibited glutamate uptake in a concentration-dependent manner. Addition of copper ions and ascorbate increased the potency and the efficacy of the hydrogen peroxide effect, supporting the potential neurotoxicity of the hydroxyl radical. The free radical scavenger dimethylthiourea effectively eliminated the inhibitory potential of a mixture containing hydrogen peroxide, copper sulphate, and ascorbate on the rate of glutamate transport into astrocytes. The inhibitory effect of hydrogen peroxide on glutamate uptake was not altered by the inhibition of glutathione peroxidase, whereas the inhibition of catalase by sodium azide clearly potentiated this effect. Superoxide and nitric oxide had no effect by themselves on the rate of glutamate uptake by astrocytes. The absence of an effect of nitric oxide is not due to an inability of astrocytes to respond to this substance, since the same cultures did respond to nitric oxide with a sustained increase in cytoplasmic free calcium.

Conclusion: These results confirm that reactive oxygen species have a potential neurotoxicity by means of impairing glutamate transport into astrocytes, and they suggest that preventing the accumulation of hydrogen peroxide in the extracellular space of the brain, especially during conditions that favor hydroxyl radical formation, could be therapeutic.

INTRODUCTION

During the last decade, an increasing number of pathologies have been attributed at least in part to the actions of reactive oxygen species (ROS). These unstable and highly reactive intermediates originate from the reaction of the dioxygen molecule in its triplet state ($^3O_2$) with elements of biological tissues. The first molecule to be formed in the reduction of triplet dioxygen is always the superoxide radical (O_2^-). This relatively unstable intermediate can dismutate spontaneously to form hydrogen peroxide (H_2O_2), and the reaction between these species can lead to the formation of hydroxyl radical (· OH), the molecule possessing the highest oxidant potential that can be found in biological tissues (1). Superoxide dismutase, associated with catalase or glutathione peroxidase (GPX), provides the most efficient endogenous means to scavenge superoxide and hydrogen or organic peroxides before they have time to react and produce hydroxyl radicals

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(1,2). Recently, various groups (such as Volterra et al. [3] and Piani et al. [4]) have shown that hydrogen peroxide–generating systems interact with the active transport of glutamate (Glu) into astrocytes, resulting in a significant inhibition of Glu uptake and leading to yet another means to produce neurotoxicity. Glu is considered to be the most prevalent excitatory neurotransmitter in the brain (5). Extracellular Glu is normally kept at low concentrations by the ability of astrocytes to accumulate this amino acid efficiently (6). However, high concentrations of Glu in the extracellular space can be neurotoxic (7–9).

In this study, our purpose was (1) to compare the ability of specific oxygen-derived intermediates to interfere with the Glu transport of primary mouse astrocyte cultures, and (2) to evaluate the importance of the possible protective mechanisms provided by catalase and glutathione peroxidase.

MATERIALS AND METHODS

Cell Culture

Primary cultures of cerebral cortical astrocytes were prepared from C57BL/6JxSJL new-born mice (1–2 days old) as previously described (10). Briefly, forebrains were removed aseptically from the skulls, the meninges were excised carefully under a dissecting microscope, and the neocortex was dissected. The cells were dissociated by passage through needles of decreasing gauges (16G1, 19G1, 25G1) two to three times with a 10-ml syringe. Trypsin was not used for tissue dissociation. The cells were seeded at a density of 10^5 cells per cm^2 on 6-well plates in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) and 25 mM glucose in a final volume of 2 ml per well and incubated at 37°C in an atmosphere containing 5% CO_2 at 95% humidity. The culture medium was renewed 3–4 days after seeding and subsequently twice per week. These conditions yield astrocyte cultures containing over 90% glial fibrillary acidic protein (GFAP) immunoreactive cells (11).

^{3}H-Glu Uptake Assay

Experiments on ^{3}H-Glu uptake were performed on primary cultures of cerebral cortical astrocytes at about 14 days in vitro (DIV) after they reached confluence. Reagents were added for a defined period of time prior to ^{3}H-Glu uptake (see corresponding figure legends). The medium was replaced by 1 ml fresh medium containing 50 μM Glu and 18.5 kBq (9.25 pmol) of ^{3}H-Glu. The uptake assay was terminated 15 min later by aspiration of the assay medium and washing the cells three times with 4 ml ice-cold phosphate-buffered saline (PBS) containing 5 mM Glu. Astrocytes were then lysed by 2 ml of 10 mM NaOH containing 0.1% Triton X-100 and a 500-μl aliquot was assayed for ^{3}H by liquid scintillation counting. The protein content was measured by the method of Bradford (12) in 100 μl of the remaining lysate. Results are expressed as the mean ± SEM of three separate experiments. For Figs. 1, 3, and 4, ANOVA analyses were performed, using Bonferroni (Figs. 1–4) or Dunnett (Tables 1, 2, and 4) post-tests; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Nitric Oxide Monitoring

Control medium was stored in 40 ml HPLC-screw cap vials sealed by a Teflon septum (National Scientific Company, Lawrenceville, GA). To prevent nitric oxide (NO) oxidation, deoxygenated medium was performed by extensive bubbling with nitrogen for 15 min (Air Liquide, San Diego) through a long cannulae penetrating the septum. NO containing medium (NO-medium) was prepared by passing a gas stream of 5% NO in 95% N_2 (Air Liquide) through the medium for at least 15 min. NO-medium was always prepared fresh immediately before use and diluted 1:10 with deoxygenated control medium. To ensure identical NO concentration during different experiments, the NO concentration was monitored continuously with an ISO-NO meter (World Precision Instruments [WPI], Sarasota, FL) connected to a computerized on-line data acquisition system (Duo 18, WPI, Sarasota, FL). The electrode was inserted into a T-adapter which was placed in the medium flow between the peristaltic pump and tissue chamber.

Calcium Imaging

Calcium concentrations in astrocytes were estimated using the Fura-2 method as described earlier (13,14). In brief, calcium imaging experiments were performed on astrocytes in confluent monolayers cultured on glass coverslips at 14 DIV. Cell cultures were incubated in 3 μM Fura-2 AM (Molecular Probes, Eugene, OR) for 45 min at 37°C in physiological saline (composition in mM: NaCl, 140; KCl, 3.5; KH_2PO_4, 0.4;

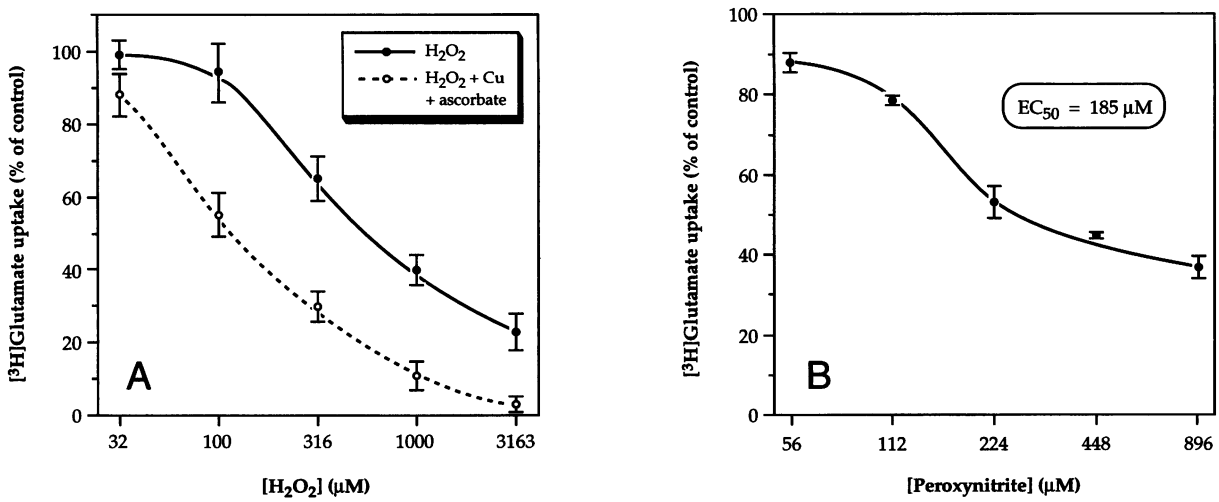

FIG. 1. Concentration-dependent inhibition by hydrogen peroxide and peroxynitrite of Glu uptake: potentiation by copper ions and ascorbate

(A) H_2O_2, $CuSO_4$ (25 μM), and ascorbate (100 μM) were added 10 min before incubation with ^{3}H-Glu. (B) Peroxynitrite was added immediately after addition of the ^{3}H-Glu solution. The symbols ● and ○ indicate the uptake levels for the curves of H_2O_2 alone and H_2O_2 + $CuSO_4$ + ascorbate, respectively (A).

Na_2HPO_4, 1.25; $CaCl_2$, 2.2; $MgSO_2$, 2; glucose, 10; HEPES-NaOH, 10 at pH 7.3) and washed for 30 min before mounting onto a perfusion chamber stage of an inverted Nikon Microscope equipped with epifluorescence, a silicon-intensified target (SIT) camera (Dage) and an LEP filter wheel (LUDL Electronic Products) fitted with appropriate fluorescence filters (Omega Optical). Real-time digitized display, image acquisition, and Ca^{2+} measurements were made with MCID imaging software (Imaging Research, Inc.). Dye loading and all experiments were performed at room temperature (23°C). Calibration of the system was performed as described in detail earlier (15,16).

Physiological saline in sealed Erlenmeyer beakers was deoxygenated by extensive bubbling with nitrogen (20 min). NO-stock solution was prepared by bubbling physiological saline with pure NO in sealed containers. Dilutions were made by transfer of NO-stock physiological saline to deoxygenated physiological saline. The diluted NO-solution was superfused onto the cell cultures. NO concentrations in the bath were monitored by a NO sensitive electrode (ISO-NO meter, WPI).

$[Ca^{2+}]_i$ was monitored while astrocytes were superfused with physiological saline for 8 min followed by a 20 min-period of superfusion with NO-containing physiological saline, which was replaced by physiological saline during a subsequent period of 30 min. During the NO-application, the concentration of NO in the superfusion chamber increased up to 10 μM. One other subset of cell cultures (controls) was superfused with NO-containing physiological saline that had been bubbled with oxygen to metabolize the NO and subsequently deoxygenated by bubbling with N_2.

Reagents

Superoxide was produced either by mixing 1 mM xanthine with 200 mU/ml xanthine oxidase 5 min prior to addition to the cultures, or by direct application of its potassium salt (Sigma), as mentioned in the figure and table legends. The former yields 6.5 μmol superoxide per liter and per minute, as assayed by the reduction of nitroblue tetrazolium salt by superoxide (not shown) (17). L-[G-^{3}H] glutamic acid (^{3}H-Glu) was purchased from Amersham (Little Chalfont, U.K.), N,N'-dimethylthiourea (DMTU) from Janssen Chimica USA (Los Angeles, CA), peroxynitrite from Alexis Corporation (San Diego, CA), and the other reagents from Sigma Chemical (St. Louis, MO). The concentration of the stock solution of H_2O_2 was determined spectrophotometrically using a value for ε_{240} of 44 [M^{-1} cm^{-1}] (18).

RESULTS

The uptake of ^{3}H-Glu in untreated cultures is time-dependent and is maximal between 10 and 15 min (not shown); thus the incubation time for all uptake experiments was chosen to be 15 min.

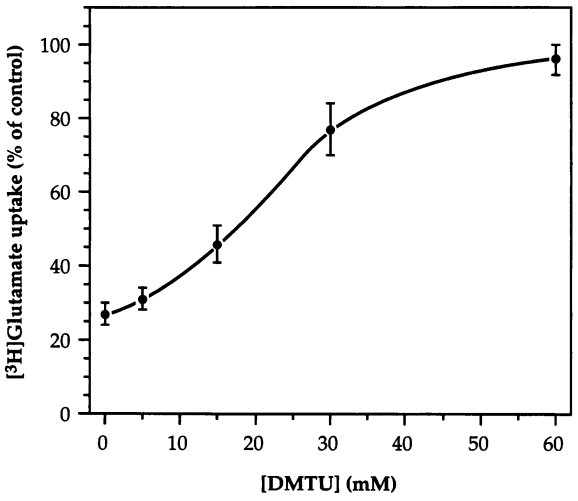

FIG. 2. Hydrogen peroxide–induced inhibition of Glu uptake: protection by DMTU

Various concentrations of DMTU were added in the presence of 0.5 mM H_2O_2 + 25 μM $CuSO_4$ + 100 μM ascorbate. DMTU was added 20 min before incubation with ^{3}H-Glu.

TABLE 1. Absence of effect of superoxide on glutamate uptake

Condition	Glutamate Uptake (% of Control)
H_2O_2	46 ± 2**
H_2O_2 + catalase	100 ± 3
Xanthine + xanthine oxidase	48 ± 1**
Xanthine + xanthine oxidase + catalase	101 ± 3
Xanthine + xanthine oxidase + SOD	46 ± 1**
Potassium superoxide	103 ± 2

Reagents were applied at the following concentrations: H_2O_2, 2 mM; xanthine, 1 mM; xanthine oxidase, 200 mU/ml; catalase, 800 U/ml; SOD, 100 U/ml; potassium superoxide, 1 mM. H_2O_2, the mixture containing xanthine and xanthine oxidase, and potassium superoxide were added 10 min before incubation with ^{3}H glutamate.

** $p < 0.01$.

In 36 separate assays (used as controls for the experiments), the rate of total Glu uptake into astrocytes has been averaged to 4.48 ± 0.26 (nmol/mg protein/min).

Effect of Hydrogen Peroxide and Peroxynitrite on Glu Uptake

Hydrogen peroxide inhibited Glu uptake into primary astrocyte cultures in a concentration-dependent manner (Fig. 1A). This inhibitory effect was clearly potentiated by the addition of 25 μM $CuSO_4$ and 100 μM ascorbate to the incubation medium. For example, hydrogen peroxide at 3.16 mM decreased the rate of Glu uptake to 23% of that of untreated cells and the uptake of Glu was almost completely inhibited by the addition of copper and ascorbate to the incubation medium (Fig. 1A). An examination of astrocytes under the light microscope showed a granulation, indicating a stress condition in the presence of 3.16 mM of hydrogen peroxide.

We then determined if the potent and labile oxidant peroxynitrite, which is known for its ability to promote irreversible oxidations of thiol groups (19,20), was effective in inhibiting Glu uptake into astrocytes. As shown in Fig. 1B, peroxynitrite induced a concentration-dependent inhibition of Glu uptake, with an estimated EC_{50} of 185 μM.

Protection by DMTU

DMTU, a sulphydryl antioxidant able to react with either hydrogen peroxide or hydroxyl radicals (21), prevented the reactive oxygen species–induced inhibition of Glu uptake in a concentration-dependent manner. In the presence of 0.5 mM hydrogen peroxide, 25 μM $CuSO_4$, and 100 μM ascorbate, the rate of Glu uptake was 27% that of untreated cells, but a 20-min pretreatment with 60 mM DMTU restored the rate of Glu transport to 96% that of untreated cells (Fig. 2).

Effect of Superoxide

We next examined the superoxide radical anion, another well-known reactive oxygen species, to evaluate its effect on glutamate uptake. Addition of 1 mM potassium superoxide had no effect on the rate of Glu uptake, while a mixture containing 1 mM xanthine plus 200 mU/ml xanthine oxidase (a superoxide- and hydrogen peroxide-generating system [22]) inhibited Glu uptake by 52%, a level of inhibition similar to that induced by 2 mM hydrogen peroxide (54%) (Table 1).

Involvement of Antioxidant Enzymes

Catalase, a specific hydrogen peroxide scavenger, completely prevented the inhibition of Glu uptake by either hydrogen peroxide or the xanthine

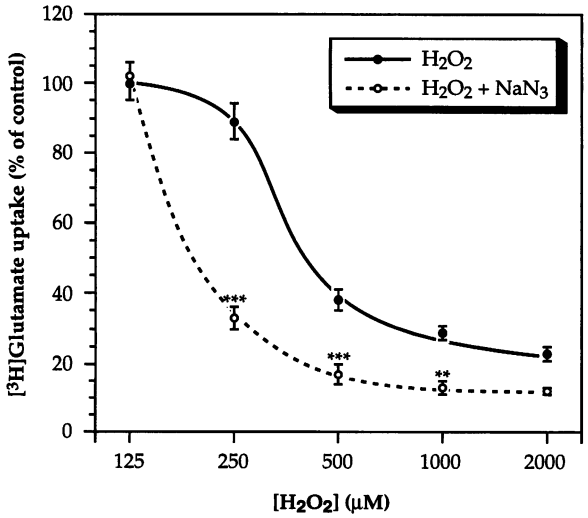

FIG. 3. Hydrogen peroxide–induced inhibition of Glu uptake: potentiation by sodium azide

NaN_3 (5 mM) was added 20 min before incubation with ^{3}H-Glu. The symbols ● and ○ indicate the uptake levels for the curves of H_2O_2 alone and H_2O_2 + NaN_3, respectively.

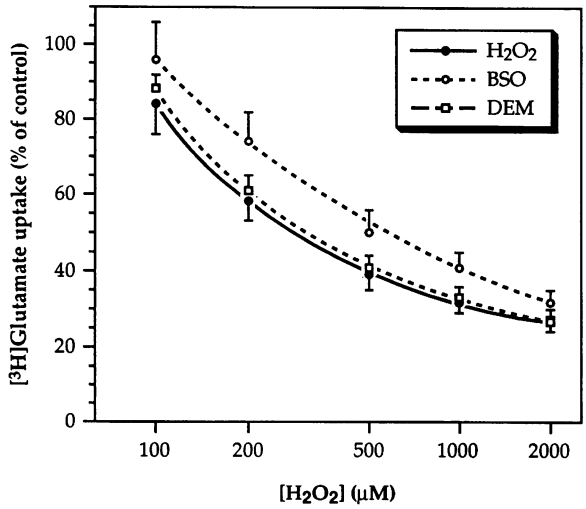

FIG. 4. Hydrogen peroxide–induced inhibition of Glu uptake: effect of reagents inducing a decrease in cellular glutathione

Various concentrations of hydrogen peroxide were added either alone (●) or in the presence of 500 μM BSO (○) or 100 μM DEM (□). BSO and DEM were added 18 hr before incubation with ^{3}H-Glu.

and xanthine oxidase mixture, whereas the specific superoxide scavenger superoxide dismutase was without effect (Table 1). These observations indicate that superoxide by itself does not affect the uptake of Glu by astrocytes. We used sodium azide, a nonspecific catalase inhibitor (23), which was not cytotoxic in our cultures for the period of time required for Glu uptake assay, as demonstrated by the absence of significant change in extracellular lactate dehydrogenase activity following 35 min in the presence of 5 mM NaN_3 (not shown). Sodium azide proved to be effective as a catalase inhibitor in our system (not shown) and displaced to the left the curve of hydrogen peroxide–induced inhibition of Glu uptake (Fig. 3).

Glutathione peroxidase (GPX) represents a well known endogenous hydrogen peroxide scavenger, which also catalyses the reduction of organic hydroperoxides (24). We used two different means to decrease GPX activity in astrocyte cultures. First, we pre-incubated the cells with L-buthionine-[S,R]-sulfoximine (BSO) or diethylmaleate (DEM), two reagents that have been shown (25,26) to induce a dramatic fall in astrocyte cytoplasmic glutathione, the cosubstrate (or cofactor) of GPX. As shown in Fig. 4, the addition of DEM did not influence the effect of hydrogen peroxide on Glu uptake, while BSO showed only a slight (but insignificant) tendency to reduce the effect of hydrogen peroxide. We then used two substances that directly inhibit the GPX activity, *N*-ethylmaleimide (NEM) and mercaptosuccinic acid (MSA). NEM potentiated hydrogen peroxide–induced inhibition of Glu uptake and also had a significant effect on Glu uptake by itself (Table 2). On the other hand, when MSA was applied at concentrations up to 1 mM and pre-incubated for 1 hr, it had no significant effect on either basal or hydrogen peroxide–induced levels of Glu uptake (Table 2).

Nitric Oxide

Another ubiquitous oxygen-derived reactive substance is the presumptive intercellular messenger, nitric oxide (NO). NO is not stable in aqueous solutions in the presence of oxygen (27,28). Thus, we used either NO donors or a NO-gassed aqueous solution previously deoxygenated with N_2 to remove dissolved O_2 molecules. The actual NO concentrations measured under each condition are shown in Table 3. Neither the NO donors sodium nitroprusside (SNP) or S-nitroso-*N*-acetylpenicillamine (SNAP) (up to 1 mM) nor NO-containing aqueous solution (5 μM of free NO) significantly changed the level of basal Glu uptake (Table 4).

The lack of effect of NO on Glu uptake could indicate that our astrocytes were simply insensi-

TABLE 2. Hydrogen peroxide–induced inhibition of glutamate uptake: effect of glutathione peroxidase inhibitors

H_2O_2 (mM)	MSA (mM)	NEM (mM)	Glutamate Uptake (% of Basal)
0	0	0	100 ± 2
0.5	0	0	34 ± 3**
0.5	0.1	0	36 ± 2**[a]
0	1	0	108 ± 5
0.5	1	0	35 ± 1**[a]
0	0	0.25	65 ± 5**
0.5	0	0.25	17 ± 1**[b]

MSA was added 4 hr, H_2O_2 and NEM 10 min before incubation with ^{3}H-glutamate.

[a]Not significantly different from H_2O_2 alone ($p > 0.05$).

[b]Significantly different from H_2O_2 alone ($p < 0.01$).

** $p < 0.01$.

TABLE 3. Concentration of free NO in solution in various conditions producing NO

NO Source	Effective NO Concentration (μM)
SNP 0.1 mM	0.09
SNP 1 mM	0.66
SNAP 0.25 mM	107.00
SNAP 1 mM	428.00
NO-gased solution dilution 1:10	5.00

tive to NO. However, our cultured astrocytes were able to show (Fig. 5) that the NO-containing solution, but not the NO-metabolite containing solution, led to a significant and sustained increase in $[Ca^{2+}]_i$, demonstrating that these astrocyte cultures are able to respond to NO, but not in terms of Glu accumulation.

DISCUSSION

Since the discovery of the enzyme superoxide dismutase in 1969 by McCord and Fridovich (29), superoxide, hydrogen peroxide, and oxygen-derived free radicals, now all included under the term

TABLE 4. Effect of NO and NO donors on glutamate uptake

Reagent (% of Control)	Glutamate Uptake
H_2O_2 0.2 mM	66 ± 5**
H_2O_2 2 mM	39 ± 1**
NO	91 ± 5
SNP 0.1 mM	103 ± 6
SNP 1 mM	96 ± 5
SNAP 0.25 mM	98 ± 3
SNAP 1 mM	100 ± 3

All reagents were added immediately before addition of ^{3}H-glutamate.

** $p < 0.01$.

reactive oxygen species (ROS), have been incriminated regularly in multiple pathological processes (1,30–32), including carcinogenesis (33–35), ischemia and reperfusion injury (36–38), and neurodegenerative disorders (39–41). Volterra et al. (3) and Piani et al. (4) recently demonstrated that hydrogen peroxide could inhibit Glu uptake by primary astrocyte cultures. Our results are consistent with this observation.

In this study, we first demonstrated the concentration-dependence of the hydrogen peroxide effect on the inhibition of Glu uptake by astrocytes. It was not possible to establish an EC_{50} value for this effect of hydrogen peroxide, as the maximally effective concentration would likely have been cytotoxic. The granulation of astrocytes, suggesting stress to the cells, was observed within 25 min in the presence of 3.16 mM of hydrogen peroxide, even though this concentration was unable to block Glu uptake completely (data not shown). However, as an index of hydrogen peroxide potency, a concentration of 630 μM results in a 50% decrease in basal Glu uptake level (Fig. 1A). The more than 10-fold potentiation of this hydrogen peroxide effect by addition of copper ions and ascorbate strongly argues for an involvement of hydroxyl radicals (Fig. 1A). This highly reactive substance could be produced by a Fenton-type reaction involving a regeneration of Cu^+ ions by ascorbate (42). The high concentration of DMTU (60 mM) required to provide a good protection of Glu detoxification by astrocytes also accounts for the high reactivity of the deleterious substance(s) scavenged by this nontoxic sulphydryl compound (Fig. 2).

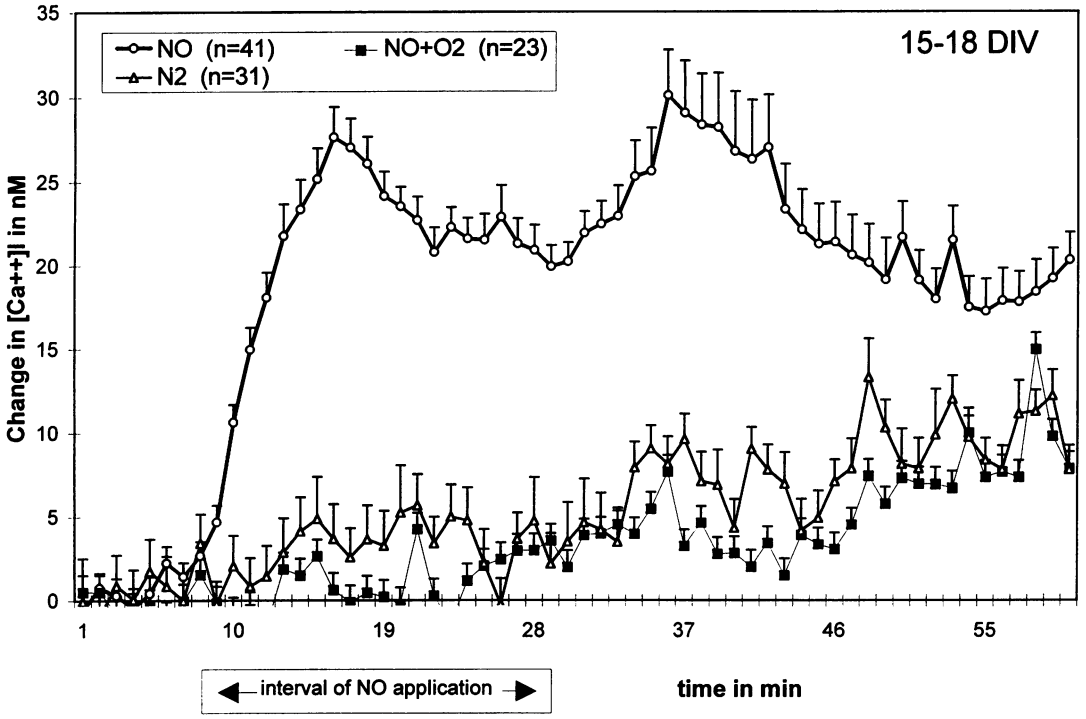

FIG. 5. NO-induced increase in cytoplasmic Ca^{2+}

NO was applied 8 min after the beginning of $[Ca^{2+}]_i$ recording, for a 20-min period of time. $[Ca^{2+}]_i$ was recorded up to 30 min after NO removal. The graph shows the change in $[Ca^{2+}]_i$ compared with the basal level, in function of time.

We also showed that superoxide, the intermediate of all reactions involving dioxygen reduction (43), has no effect by itself on Glu uptake, as demonstrated by the absence of superoxide effect of the generating system consisting of xanthine, xanthine oxidase, and catalase, or by direct application of potassium superoxide (Table 1). Furthermore, the lack of effect of NO, another oxygen-derived molecule, cannot be explained by the absence of responsiveness of our astrocyte model to this second messenger, as our cultures did respond to aqueous NO solution with a sustained increase in intracellular calcium (Table 3, Fig. 5). As mentioned above, superoxide and NO by themselves have no effect on Glu uptake into astrocytes, but peroxynitrite, a potent oxidant produced by the addition of NO to superoxide (20,44–46), promoted a concentration-dependent inhibition of Glu uptake, with an estimated EC_{50} of 185 μM (Fig. 1B). Because of its instability at physiological pH (7.35), peroxynitrite was not preincubated for 10 min but added immediately following the addition of ^{3}H-Glu solution. This could explain a lower maximal effect than that of hydrogen peroxide. These results are consistent with those of Trotti et al. (47), who reported a concentration-dependent inhibition by peroxynitrite of Glu uptake by purified or recombinant glutamate transporters in liposomes. This inhibition seemed to be due to a direct interaction of peroxynitrite with the transporters (47).

Among the two well-known endogenous ROS scavengers, catalase and glutathione peroxidase, only catalase seems to be able to reduce the potential neurotoxicity of hydrogen peroxide (Fig. 3). Sodium azide, a nonspecific catalase inhibitor, potentiated the inhibition of Glu uptake by H_2O_2. Sodium azide is also known to inhibit oxydative phosphorylation, resulting in a energy deficit affecting Na^+-K^+ ATPase, which in turn inhibits the glutamate transporter system. Therefore, the observed effect of sodium azide on Glu uptake may also be attributed in part to altered energy metabolism. Conditions leading either to (*1*) a decrease in cytoplasmic glutathione, the cosubstrate of GPX (Fig. 4), or (*2*) a selective inhibition of GPX (Table 2) were unable to potentiate the inhibition of Glu uptake by hydrogen peroxide. NEM, tested as a nonselective GPX

inhibitor, is a well-known alkylating agent that reacts with nucleophilic groups (48) and could also be expected to alkylate free sulphydryl groups participating in Glu transport, as suggested by Volterra et al. (3). Therefore, the high potency of NEM on hydrogen peroxide–mediated inhibition of Glu uptake could be independent of the inhibition of GPX but related only to the involvement of reduced cysteine amino acids linked to the Glu transporters. On the other hand, mercaptosuccinate (MSA) has been shown to be highly specific for selenolate groups such as the selenocysteine present at the active site of GPX subunits (49). Thus, the lack of effect of MSA on the inhibition of Glu uptake suggests that GPX does not directly participate in the elimination of ROS involved in the Glu-mediated neurotoxicity. The lack of effect of GPX inhibition on H_2O_2-induced decrease in Glu uptake could be explained by recent findings that rather than regenerating the GPX/GSH/GS reductase system, NADPH may play a role in maintaining the intergrity and functionality of the catalase, although NADPH is not directly involved in the catalase activity (50). This is consistent with our results that catalase is able to prevent excitotoxicity by scavenging efficiently the excess of the hydrogen peroxide formed in the brain.

One of the enzymes activated by calcium is phospholipase A_2, which catalyses the release of arachidonate by phospholipid membranes. This polyunsaturated fatty acid in turn is able to inhibit Glu uptake by astrocytes (50–52). Furthermore, calpains, which transform xanthine dehydrogenase into xanthine oxidase, thus leading to ROS production—especially in the case of an ischemia (53,54)—are also activated by calcium. Finally, the hypothesized accumulation of extracellular Glu might not be the only phenomenon occurring during events like ischemia or carcinogenesis. There could also be an increased neurotransmitter release (55) or a hypersensitivity of NMDA receptors. In light of these possibilities, the diminished uptake capacity of astrocytes would still potentiate the pathological Glu levels even more (e.g., a 50% uptake inhibition of a 10-fold increased basal Glu release would result in a 20-fold increased extracellular Glu level). These results confirm the involvement of ROS in excitotoxicity by allowing Glu to accumulate in the extracellular space, leading to a continuous depolarization of neurons, followed by high and sustained calcium influxes (56,57).

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