

The Role of Monoamine Metabolism in Oxidative Glutamate Toxicity

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Glutamate kills neuronal cells by either a receptor-mediated pathway or the inhibition of cystine uptake, the “oxidative pathway.” Antioxidants can block cell death initiated by either pathway, suggesting that toxicity is dependent on the production of free radicals. We provide evidence that in a neuronal cell line, glutamate toxicity via the oxidative pathway requires monoamine metabolism as a source of free radicals. Glutamate toxicity is inhibited by monoamine oxidase (MAO) type-A-specific inhibitors, but only at concentrations much higher than those required to inhibit classical type-A MAO. Toxicity is not inhibited by MAO type-B-specific inhibitors at any concentra-

tion. Furthermore, treatment of cells with agents that block monoamine uptake inhibits glutamate toxicity. These results suggest that an enzyme distinct from MAO is involved in monoamine metabolism and demonstrate a relationship between glutamate toxicity and monoamine metabolism. These data also have implications for the understanding and treatment of neurodegenerative disorders in which glutamate toxicity is thought to be involved.

Key words: glutamate toxicity; free radicals; monoamine oxidase; neuronal cells; Parkinson's disease; hydrogen peroxide; dopamine

Free radicals are postulated as mediators of an increasing number of neurodegenerative diseases (Ames and Shigenaga, 1992; Jenner, 1994); however, the pathways that lead to their production are incompletely described. One potential mechanism for their generation is via the excitatory amino acid glutamate. Glutamate is thought to be a major cause of neuronal cell death in a number of different neurodegenerative diseases (Greenamyre et al., 1985; Choi, 1992; Lees, 1993). Two pathways for glutamate toxicity have been described: excitotoxicity (Olney, 1969), which occurs through the activation of glutamatergic receptors (Choi, 1988; Michaels and Rothman, 1990), and oxidative glutamate toxicity, which is mediated via a series of disturbances to the redox homeostasis of the cell (Murphy et al., 1989). These pathways are incompletely characterized, but both result in the production of free radicals (Murphy et al., 1989; Choi, 1992).

The oxidative pathway has been described in primary neuronal cell cultures (Murphy et al., 1989, 1990; Oka et al., 1993; Davis and Maher, 1994), in neuronal cell lines (Miyamoto et al., 1989; Murphy et al., 1989; Davis and Maher, 1994), and in tissue slices (Vornov and Coyle, 1991) and may be a major source of glutamate-induced cell damage *in vivo* (Greene and Greenamyre, 1995; Gwag et al., 1995). The early steps in the oxidative glutamate toxicity pathway have been characterized. Exposure of cells to glutamate results in an inhibition of cystine transport into the cell (Murphy et al., 1989), which gives rise to an inability to

maintain intracellular glutathione levels. The low levels of intracellular glutathione lead to a reduced ability to protect against oxidative reactions within the cell and, ultimately, cell death. The accumulation of excess free radicals seems to be responsible for the toxicity, because cell death can be prevented by the administration of antioxidants (Miyamoto et al., 1989; Davis and Maher, 1994). Despite these observations, the sources of free radicals that contribute to neuronal cell death are not known.

A major source of free radicals is hydrogen peroxide (H_2O_2). H_2O_2 is continually generated within cells as a result of metabolic activity. If not efficiently removed, H_2O_2 is converted to molecules that may irreversibly damage the cell (Halliwell and Gutteridge, 1993). The enzymes responsible for detoxifying H_2O_2 are the glutathione peroxidases and catalase. In neuronal cells, the burden falls on the glutathione pathway, because these cells have lower levels of catalase (Mavelli et al., 1982). We observed that different neuronal cell lines exhibit varying sensitivity to glutamate toxicity, indicating that a specific set of metabolic functions may predispose certain types of neuronal cells to toxicity. In nerve cells, a source of H_2O_2 results from the metabolism of catecholamines and indoleamines by monoamine oxidase (MAO) (monoamine: O_2 oxidoreductase EC 1.4.3.4) (for reviews, see Weyler et al., 1990; Singer and Ramsay, 1995). It is likely, therefore, that MAO activity could predispose certain types of neuronal cells to glutamate toxicity. This potential role of MAO in glutamate toxicity was examined in a neuronal cell line that is particularly sensitive to glutamate. It is shown that the H_2O_2 that kills the cells is derived, at least in part, from the oxidation of monoamines by an enzymatic activity with unique properties.

MATERIALS AND METHODS

Materials. Cultureware was from Costar (Pleasanton, CA); tissue culture products were from Life Technologies (Gaithersburg, MD); [3H]-tryptamine hydrochloride and [^{14}C]-tyramine hydrochloride were from NEN; anti-rat neuron-specific enolase was from PolySciences (Warrenton, PA); clorgyline, deprenyl, pargyline, RO16-6491, RO41-1049, dox-

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Table 1. Toxicity of glutamate and its analogs

Reagent	% Survival
Control	100
Glutamate (5 mM)	3 ± 1
Kainate (5 mM)	95 ± 5
Aspartate (10 mM)	100 ± 2
AMPA (5 mM)	98 ± 4
ACPD (100 μM)	102 ± 3
NMDA (10 mM)	96 ± 5
NMDA (10 mM) + glycine (5 mM)	97 ± 6
Quisqualate (0.5 mM)	6.1 ± 0.9
Glutamate (5 mM) + APV (10 mM)	1
Glutamate (5 mM) + MK-801 (20 μM)	0
Cystine (1 mM)	100 ± 5
Glutamate (5 mM) + cystine (1 mM)	95 ± 8

HT-22 cells were incubated with the above reagents for 24 hr, and then cell viability was assessed using the MTT assay. Data are expressed as percent survival relative to untreated controls and are the means of triplicate determinations ± SD. The results were confirmed by visual inspection of the cells. APV, 2-amino-5-phosphonovaleate; ACPD, 1-aminocyclopentane-1,3 dicarboxylic acid. ACPD is a selective metabotropic glutamate receptor agonist.

epin, indatraline, imipramine, clomipramine, alaproclate, quinacrine, NMDA, AMPA, quisqualate, kainate, 2-amino-5-phosphonovaleate (APV), MK-801, and 1-aminocyclopentane-1,3 dicarboxylic acid (ACPD) were obtained from Research Biochemicals (Natick, MA); TC715 [N-(2-aminoethyl)-3-iodobenzamide HCl] and TC724 (pirlindole mesylate) were obtained from Tocris Cookson (Bristol, UK); glutamate, aspartate, tryptamine, tyramine, reserpine, harmine, semicarbazide, and other reagent grade chemicals were from Sigma (St. Louis, MO). Diphenylene iodinium (DPI) was from Dr. A. Cross, The Scripps Research Institute.

Cell characterization. HT-4 cells, an immortalized mouse hippocampal cell line, were obtained from B. H. Morimoto and D. E. Koshland (Morimoto and Koshland, 1990) and subcloned. The HT-22 clone was the most sensitive to glutamate of the 25 clones tested and was used in the experiments described herein. The HT-22 subclone has been characterized in detail with respect to ionotropic glutamate receptors and monoamine synthesis. It is negative for both according to the following criteria. NMDA, aspartate, AMPA, kainate, and ACPD were not toxic at concentrations up to 10 mM (Table 1). Quisqualate is toxic but via the nonreceptor-mediated oxidative pathway (Schubert et al., 1992). In addition, the glutamate receptor antagonists APV and MK-801 do not block glutamate toxicity in the HT-22 cells; however, cystine blocks glutamate toxicity. Furthermore, PCR and Northern blot analysis of the HT-22 cells using NMDA, kainate-type non-NMDA, quisqualate-type non-NMDA, and metabotropic receptor primers and/or probes, obtained from Dr. J. Boulter, The Salk Institute, were negative (unpublished observations). Finally, the HT-22 cells do not make norepinephrine, DOPA, dopamine, or epinephrine, as determined by gas chromatographic analysis. The PC12 cell line was used as a positive control for these analyses, because these cells express NMDA receptor mRNA (but not functional receptor protein) and synthesize dopamine (Schubert et al., 1980, 1992).

Cytotoxicity assays. The HT-22 cells were maintained in DMEM/10% FCS and passaged by trypsinization. Cell viability was assayed using the MTT assay (Hansen et al., 1989) or a colony-forming assay (Cook and Mitchell, 1989). For the MTT assay, cells were plated into 96-well plates at 5×10^3 cells/well in complete medium, and 24 hr later the experimental agents were added. The ability of cells to reduce MTT was assayed 24 hr after the addition of the experimental agents, exactly as described previously (Davis and Maher, 1994). To complement the MTT assay, a colony-forming assay was used that measures the ability of cells to divide (Cook and Mitchell, 1989). After treatment, cells were dissociated and diluted serially into complete medium, and the number of colonies that formed was determined 7 d later.

Primary cultures. Cultures of rat cortical neurons were prepared from Sprague Dawley embryonic day 18 fetal rats, using the dissociation method of Huettner and Baughman (1986), and they were cultured essentially as described (Murphy et al., 1990). The purity of neuronal cells versus glial cells was assessed by morphology and staining with neuron-specific enolase. Cultures contained <20% glial cells at the time of assay.

The toxic effect of glutamate on neurons and glial cells within the cultures was assessed visually and quantified by counting a raster of 0.5 mm² fields across a dish and using the MTT assay as described above. It was apparent that the contaminating glial cells were unaffected by the glutamate, negating the possible error in MTT measurements attributable to glial cell death. Results are expressed as the percentage ± SD of the glutamate-treated well compared with the untreated control.

Measurement of peroxide levels. Peroxide levels within cells were measured using a method developed (Bass et al., 1983) from the original method of Ames and colleagues (Cathcart et al., 1983) using flow cytometry of cells stained with the dye dichlorofluorescein diacetate (DCFH-DA). The diacetate is deesterified and trapped within cells, where it reacts further with hydroperoxides to form the fluorescent product dichlorofluorescein. Cells (25×10^4) were plated in 60 mm dishes in growth medium. After exposure to glutamate and agents as required, the cells were incubated with 10 mM DCFH-DA for 10 min. Cells were then harvested by trypsinization and washed in phenol red-free DMEM, 2% FCS, at 4°C, and analyzed using a FACStar Plus with visible excitation and emission at 520 ± 17 nm. Because quantitative measurements are subject to error attributable to the nonratiometric emission of the dye, data were expressed as a percentage change relative to control cells set arbitrarily at 100%.

Measurement of MAO activity. The activity of MAO in intact HT-22 cells was determined by using a slight modification of the method of Edelstein and Breakefield (1981). Briefly, cells in 24-well dishes were rinsed with PBS and incubated in PBS containing either 22.5 μM [³H]tryptamine or 37.8 μM [¹⁴C]tyramine and 3.7 μM ascorbic acid. After 30 min at 37°C, the reactions were stopped by the addition of 20 μl of 10 N NaOH. After incubation for 2 hr at room temperature to dissolve the cells, the lysates were acidified by the addition of 80 μl of 10 N HCl. Two hundred microliters of this mixture were added to 4 ml of toluene-based Econofluor in a 5 ml liquid scintillation vial. The vials were shaken for 5 min on an automated shaker, and the phases were allowed to separate for several hours before they were counted in a liquid scintillation counter. The activity of MAO in cell homogenates was measured by the method of Wurtman and Axelrod (1963), using either [³H]tryptamine or [¹⁴C]tyramine.

Labeling of mitochondria with ³H-clorgyline. ³H-clorgyline was synthesized according to Fowler (1978). The product had a specific activity of 1.8 Ci/mmol, was >99% pure by HPLC analysis, and was fully biologically active according to the criteria used in this paper. The product was stored in ethanol at −80°C and dried in a speed-vac before use. HT-22 mitochondria were prepared by the digitonin method (Moreadith and Fiskum, 1984) and labeled with ³H-clorgyline in 50 mM Tris-HCl, pH 7.4, as described by Cawthon et al. (1981).

Statistical analysis. The data were analyzed by either an unpaired Student's *t* test or ANOVA, as appropriate.

RESULTS

MAO inhibitors inhibit glutamate toxicity

We recently showed that the HT-22 hippocampal cell line is killed by glutamate via the oxidative pathway (Davis and Maher, 1994). Thus, treatment with glutamate causes a decrease in glutathione levels and a concomitant increase in H₂O₂ levels before the overt manifestation of cell death. The HT-22 cells do not contain ionotropic glutamate receptors (see Table 1 and Materials and Methods). To further characterize the steps leading to cell death, we set out to identify the sources of H₂O₂ in cells that contribute to the toxicity of glutamate. A series of amine oxidase inhibitors was tested to determine whether any might block glutamate toxicity in the HT-22 cells. Figure 1A shows that the irreversible MAO-A inhibitor clorgyline and the reversible inhibitors harmine, RO41-1049, and TC724 provide good protection from glutamate toxicity. The inhibitors that were tested cover a range of different chemical structures, indicating that the protection from glutamate toxicity provided by the MAO-A inhibitors was not limited to one class of compounds. MAO-A inhibitors also protected HT-22 cells from quisqualate and L-oxalyl-α,β-diaminopropionic acid toxicity (data not shown). Hydralazine, an irreversible nonsubtype-specific MAO inhibitor also protected the cells from glutamate toxicity (Fig.

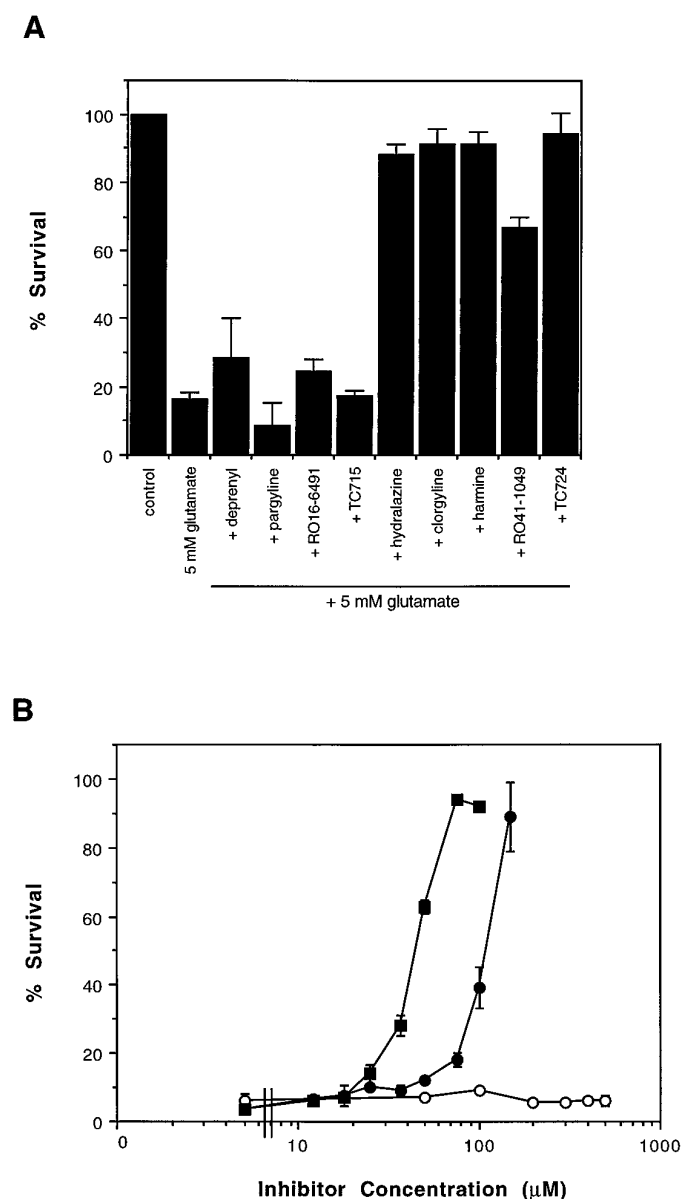


Figure 1. Rescue of neuronal cells from glutamate toxicity by MAO inhibitors. *A*, HT-22 cells were incubated with 100 μ M deprenyl, 100 μ M pargyline, 100 μ M RO16-6491, 100 μ M TC715, 100 μ M hydralazine, 100 μ M clorgyline, 50 μ M harmine, 100 μ M RO41-1049, or 100 μ M TC724 for 8 hr in the presence of 5 mM glutamate. After 24 hr, cell viability was assessed using the MTT assay. Data are expressed as % survival relative to controls treated with inhibitor alone and are the mean of triplicate determinations \pm SD. Statistical analysis of the results showed that the MAO-A inhibitors provided significant protection ($p < 0.0001$) from glutamate toxicity, whereas the MAO-B inhibitors did not. Similar results were obtained in five separate experiments. *B*, HT-22 cells were exposed to an increasing dose of harmine (■), clorgyline (●), or deprenyl (○) in the absence or presence of 10 mM glutamate. After 24 hr, cell viability was assessed using the MTT assay. Data are expressed as % survival relative to controls treated with inhibitor alone and are the mean of triplicate determinations \pm SD. Similar results were obtained in three separate experiments.

1A). In contrast, the MAO-B-specific inhibitors deprenyl, pargyline, RO16-6491, and TC715 have no rescuing effect (Fig. 1A), nor does the amine oxidase inhibitor semicarbazide (not shown). Figure 1B shows the dose requirement for the MAO inhibitors harmine, clorgyline, and deprenyl, and demonstrates that a high dose of inhibitor

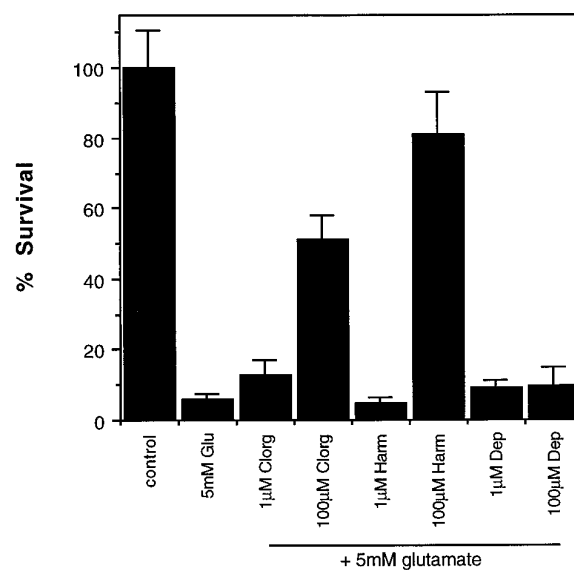


Figure 2. Primary cortical cells were exposed to 5 mM glutamate (Glu) and clorgyline (Clorg), harmine (Harm), or deprenyl (Dep) at the indicated concentrations. After 24 hr, surviving neurons were counted as described (Murphy et al., 1990). Survival after glutamate exposure is expressed as the % of the mean number of neurons counted in control cultures treated with inhibitor alone. Values represent the mean of triplicate determinations \pm SD. Statistical analysis of the results showed that clorgyline and harmine provided significant protection ($p < 0.0001$) from glutamate toxicity, whereas deprenyl did not. All experiments were repeated at least twice with similar results.

is required. Furthermore, a combination of the MAO-A inhibitor clorgyline, at a concentration that does not protect the cells from glutamate toxicity, and either deprenyl or pargyline (10–100 μ M) do not block glutamate-induced cell death (not shown). Colony-forming assays and viable cell counts confirmed the protective effect of the drugs on glutamate toxicity that was observed when the MTT assay was used (not shown).

To extend the results obtained with the HT-22 cells, neurons in primary rat cortical cultures were tested for the effect of MAO inhibitors on glutamate toxicity. Short-term culture conditions in which receptor-mediated toxicity does not function (Murphy et al., 1990) were used in these studies. The type of glutamate toxicity operating in these neurons was confirmed by experiments demonstrating the toxicity of glutamate and quisqualate but not NMDA or kainate (not shown). The primary cortical neurons were exposed to glutamate in the absence or presence of MAO inhibitors for 24 hr. The neurons were protected from glutamate toxicity only by MAO-A inhibitors such as clorgyline and harmine (Fig. 2), confirming our results in the HT-22 cell line.

Despite the significant protection from glutamate toxicity afforded by the MAO-A inhibitors in both the neuronal cell line and the primary neuronal cultures, the inhibitor concentrations that were required were significantly higher than those reported to block MAO activity. Indeed, when added to cell homogenates, MAO inhibitors lose their type-specificity at concentrations >1 μ M (Salach et al., 1979; Edelstein and Breakefield, 1981). To determine whether the dose of MAO inhibitor required to block activity correlated with the concentration of MAO inhibitor that protected the HT-22 cells from glutamate toxicity, MAO activity in whole cells was assayed (Edelstein and Breakefield, 1981) in the presence of increasing concentrations of the inhibitors. Tryptamine or tyramine were used as substrates, because they are metabolized by

Table 2. Effect of MAO inhibitors on MAO activity in HT-22 cells

Treatment	% MAO activity
Clorgyline (1 μM)	12.8 \pm 4.3
Clorgyline (10 μM)	15.2 \pm 2.5
Clorgyline (100 μM)	18.9 \pm 2.8
Pargyline (1 μM)	46.5 \pm 15.3
Pargyline (10 μM)	19.7 \pm 2.9
Pargyline (100 μM)	17.6 \pm 2.5
Harmin (1 μM)	10.3 \pm 4.2
Harmin (10 μM)	10.9 \pm 4.9
Harmin (100 μM)	18.9 \pm 6.0
Hydralazine (1 μM)	74.9 \pm 3.2
Hydralazine (10 μM)	40.8 \pm 7.1
Hydralazine (100 μM)	13.6 \pm 8.2
DPI (1 μM)	92.2 \pm 4.6

The activity of MAO in intact HT-22 cells was determined by using a slight modification of the method of Edelstein and Breakefield (1981). The results are presented as the percentage of the value obtained with the untreated control ($300\text{--}400\text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}\text{ protein}$) \pm SD. Similar results were obtained in two to three separate experiments.

both MAO-A and MAO-B. As shown in Table 2, doses of the inhibitors that had little or no effect on glutamate toxicity lead to a complete inhibition of MAO activity in intact cells. These data do confirm, however, that the major MAO in the HT-22 cells is MAO-A, because the MAO-B-specific inhibitor pargyline was more effective at concentrations $>1\text{ }\mu\text{M}$.

Agents that block monoamine uptake protect cells from glutamate toxicity

Despite the lack of correlation between MAO inhibition and inhibition of glutamate toxicity, the finding that the efficacy of the MAO inhibitors held true to their MAO-type subdivisions, even at the high concentrations used (i.e., pargyline does not protect at high concentrations at which it blocks MAO-A activity in whole cells), suggested that monoamine metabolism was involved in glutamate toxicity. If this were the case, then a reduction in the intracellular levels of monoamines should also protect cells from glutamate toxicity; however, because we have been unable to detect monoamine synthesis in the HT-22 cells, endogenous production is unlikely to be a significant source of monoamines in these cells.

Another potential source of monoamines is the medium in which the cells are grown. Thus, agents that block monoamine uptake were assayed to determine whether they could decrease glutamate-induced cell death. As shown in Figure 3A, all of the uptake inhibitors that were tested significantly reduced glutamate toxicity in the HT-22 cells. The group of monoamine uptake inhibitors that was tested included a range of different chemical structures, indicating that protection from glutamate toxicity was not restricted to one class of uptake inhibitors. All of these agents are capable of inhibiting the uptake of norepinephrine, serotonin, and dopamine, although with varying potencies (Richelson, 1994).

To extend the results obtained with the HT-22 cells, neurons in primary rat cortical cultures were tested for the effect of monoamine uptake inhibitors on glutamate toxicity. The primary cortical neurons were exposed to glutamate in the absence or presence of the monoamine uptake inhibitors for 24 hr. The neurons were protected from glutamate toxicity by all of the uptake inhibitors tested (Fig. 3B), in agreement with our results in the HT-22 cell line.

To confirm that the monoamine uptake inhibitors were indeed

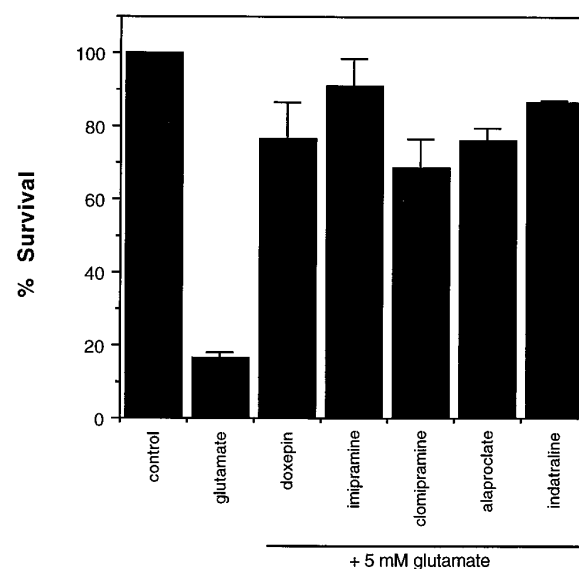
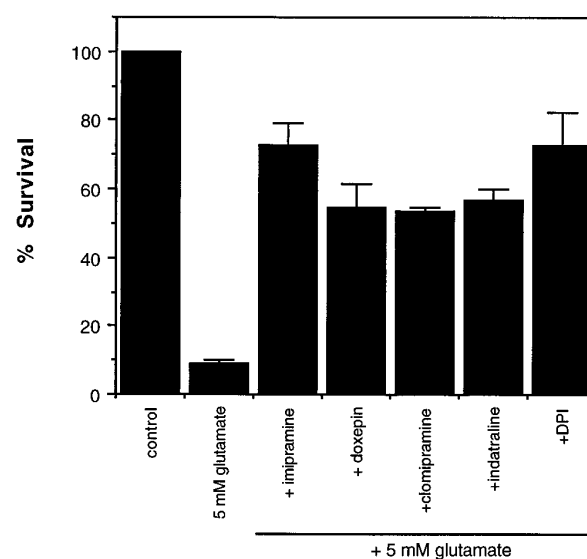
A**B**

Figure 3. *A*, Rescue of neuronal cells from glutamate toxicity by monoamine uptake inhibitors. HT-22 cells were incubated with 75 μM doxepin, 75 μM imipramine, 30 μM clomipramine, 200 μM alaproclate, or 10 μM indatraline for 8 hr in the presence of 5 mM glutamate. % survival was measured after 24 hr by the MTT assay. Data are expressed as % survival relative to controls treated with inhibitor alone. The concentrations of inhibitors used in this experiment afforded maximal protection without causing significant cell death. At higher concentrations the uptake inhibitors were all extremely toxic to the cells. Values represent the mean of quadruplicate determinations \pm SD. Statistical analysis of the results showed that the uptake inhibitors provided significant protection ($p < 0.0001$) from glutamate toxicity. Similar results were obtained in five separate experiments. *B*, Primary cortical cells were exposed to 5 mM glutamate and 25 μM imipramine, 25 μM doxepin, 10 μM clomipramine, 10 μM indatraline, or 0.1 μM DPI. After 24 hr, surviving neurons were counted as described (Murphy et al., 1990). Survival after glutamate exposure is expressed as the % of the mean number of neurons counted in control cultures treated with inhibitor alone. Values represent the mean of triplicate determinations \pm SD. Statistical analysis of the results showed that both the uptake inhibitors and DPI provided significant protection ($p < 0.0001$) from glutamate toxicity. All experiments were repeated at least twice with similar results.

Table 3. Effect of monoamine uptake inhibitors on MAO activity in whole cells versus cell homogenates

Treatment	% MAO activity
Intact cells	
Imipramine (10 μ M)	89.4 \pm 18.0
Imipramine (75 μ M)	32.9 \pm 11.0
Doxepin (10 μ M)	91.9 \pm 10.0
Doxepin (75 μ M)	42.5 \pm 3.6
Clomipramine (10 μ M)	82.7 \pm 10.0
Clomipramine (30 μ M)	41.5 \pm 15.0
Cell extracts	
Imipramine (10 μ M)	95.4 \pm 3.2
Imipramine (75 μ M)	75.0 \pm 0.1
Doxepin (10 μ M)	96.8 \pm 2.3
Doxepin (75 μ M)	77.5 \pm 2.7
Clomipramine (10 μ M)	90.8 \pm 5.0
Clomipramine (30 μ M)	79.0 \pm 4.0

The activity of MAO in intact HT-22 cells was determined by using a slight modification of the method of Edelstein and Breakefield (1981). The activity of MAO in cell extracts was determined by the method of Wurtman and Axelrod (1963). The results are presented as the percentage of the value obtained with the untreated control \pm SD. Similar results were obtained in two to three separate experiments.

acting to block monoamine uptake into the HT-22 cells, the effects of the inhibitors on MAO activity in whole cells was compared with the effects on enzyme activity in cell homogenates by using treatment conditions similar to those used to assay the effects of these agents on glutamate toxicity. The uptake inhibitors would be expected to block MAO activity in whole cells through inhibition of substrate uptake but have little or no effect on MAO activity in cell homogenates. We used this indirect assay to monitor monoamine uptake by the HT-22 cells because direct assays generally require pretreatment of the cells with MAO inhibitors (Michel and Hefti, 1990), which could complicate the interpretation of the results. For each of the uptake inhibitors tested, two concentrations were used: one at which little or no protection from glutamate toxicity was seen and one at which maximal protection from glutamate toxicity was seen. As shown in Table 3, none of the uptake inhibitors had a large effect on MAO activity in cell homogenates. In contrast, at the concentrations that protected the HT-22 cells from glutamate toxicity, the uptake inhibitors significantly reduced MAO activity in whole cells, whereas lower concentrations of the uptake inhibitors had little or no effect. These results, as well as those shown in Table 2 and Figure 4, also demonstrate that the HT-22 cells are capable of monoamine uptake. Although the concentrations of the uptake inhibitors that inhibited glutamate toxicity did not block monoamine uptake completely, the data are consistent with the possibility that these inhibitors decreased intracellular monoamine levels, and thereby oxidase activity, enough to reduce the amount of H_2O_2 production to a level that did not overwhelm the capacity of the cells for eliminating it.

The results with the monoamine uptake inhibitors suggested that the culture medium was a source of monoamines that could potentiate cell damage in the presence of glutamate. Because the medium itself (DMEM) does not contain monoamines, the most likely source is the serum. To test this possibility, HT-22 cells were treated with glutamate in either a defined medium without serum (N2 medium; Bottenstein and Sato, 1979) or medium with serum that was pretreated with charcoal to remove monoamines and

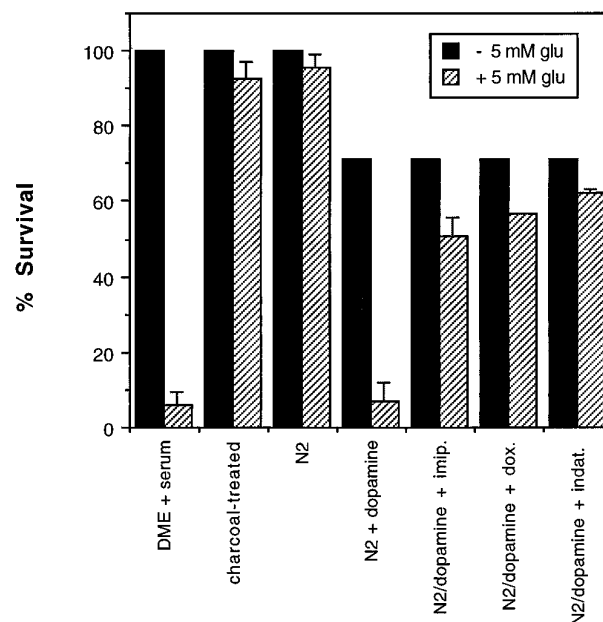


Figure 4. Effect of culture medium on glutamate toxicity in neuronal cells. HT-22 cells were incubated for 8 hr plus (hatched bars) or minus (solid bars) 5 mM glutamate in DMEM containing 10% FCS (DME + serum), N2 medium (N2), DMEM containing 10% charcoal-treated serum (charcoal-treated) (Vrana et al., 1993), N2 medium supplemented with 100 μ M dopamine (N2 + dopamine), N2 medium supplemented with 100 μ M dopamine and 75 μ M imipramine (N2/dopamine + imip.), N2 medium supplemented with 100 μ M dopamine and 75 μ M doxepin (N2/dopamine + dox.), or N2 medium supplemented with 100 μ M dopamine and 20 μ M indatraline (N2/dopamine + indat.). % survival was measured after 24 hr by the MTT assay, except in the case of the experiments with dopamine, in which survival was measured by the colony-forming assay because dopamine interfered with the MTT assay. Data are expressed as % survival relative to controls treated with the complete medium alone (DME + serum). Values represent the mean of quadruplicate determinations \pm SD. Statistical analysis of the results showed that both N2 medium and charcoal-treated serum provided significant protection from glutamate toxicity ($p < 0.0001$). Dopamine eliminated this protection, but the further addition of the uptake inhibitors provided significant protection ($p < 0.001$) from the toxicity seen in the presence of glutamate and dopamine. Similar results were obtained in three separate experiments.

other small molecules (Vrana et al., 1993). As shown in Figure 4, little or no cell death was observed in glutamate-treated cells when these modified media preparations were used; however, addition of 100 μ M dopamine to the N2 medium eliminated the protection afforded by the removal of serum (Fig. 4). This concentration of dopamine is only slightly toxic to the cells (Fig. 4), in agreement with previous studies (Michel and Hefti, 1990), and is within the range of physiological concentrations for this monoamine (0.1–1 mM) (Jonsson, 1971; Michel and Hefti, 1990). Although dopamine can be directly toxic to cells (Michel and Hefti, 1990), this occurs at higher concentrations than those that were required to facilitate glutamate toxicity in our system. Monoamine uptake inhibitors blocked the cell death seen in the N2 medium in the presence of dopamine (Fig. 4), providing further evidence that the protective effect of these inhibitors is attributable to their ability to inhibit monoamine uptake. In addition, the protection from glutamate toxicity in the presence of dopamine afforded by the uptake inhibitors indicates that dopamine is acting intracellularly to promote glutamate toxicity. Similar results were obtained using serum-free medium with no additions (except dopamine where appropriate), but overall cell survival under all conditions

Table 4. Effect of MAO inhibitors on the stimulation of H₂O₂ production by glutamate

Treatment	– Glutamate	+ Glutamate
Control	100 ± 17	139 ± 23*
Clorgyline (100 μM)	76 ± 12	77 ± 12
Deprenyl (100 μM)	120 ± 18	149 ± 23*
Imipramine (75 μM)	142 ± 15	135 ± 16
DPI (1 μM)	192 ± 31	210 ± 31
Quinacrine (10 μM)	151 ± 12	157 ± 12

HT-22 cells were incubated for 6–8 hr in the presence or absence of 5 mM glutamate with the MAO inhibitors clorgyline and deprenyl, the monoamine uptake inhibitor imipramine, or the NADPH oxidase inhibitors diphenylene iodonium (DPI) and quinacrine. The levels of intracellular peroxides were estimated by FACS analysis as described in Materials and Methods. Because quantitative measurements are subject to error attributable to the nonratiometric emission of the dye, data are presented as a percentage change relative to control cells arbitrarily set at 100%. Forward scatter measurements determined that cell volume remained constant across the experimental procedures. The data are expressed as the mean ± SD of 10,000 independent measurements. With the exception of the points marked with an asterisk, all values for glutamate-treated cells were not significantly different from those for untreated cells. **p* < 0.005. Repeated three times with similar results.

was reduced. Taken together, these results provide additional support for a role of monoamine metabolism in glutamate-induced cell death.

MAO inhibitors reduce glutamate-induced peroxide formation

Glutamate treatment leads to an increase in H₂O₂ within cells (Choi, 1992). If an oxidase activity is the primary source of this oxidative stress, then the various inhibitors should be able to block its formation. Both the MAO-A inhibitors and the monoamine uptake inhibitors block glutamate-induced increases in H₂O₂ (Table 4). In contrast, the MAO-B inhibitor deprenyl fails to inhibit the glutamate-induced increase in H₂O₂ formation (Table 4). Although the increases in H₂O₂ are relatively small, similar changes in H₂O₂ concentrations are sufficient to cause cell death in other cell culture systems (Behl et al., 1994).

Oxidase inhibitors protect cells from glutamate toxicity

DPI was originally characterized as an inhibitor of flavoprotein-dependent oxidases such as NADPH oxidase (Cross, 1990). As shown in Figure 5, 1 μM DPI almost completely inhibited glutamate toxicity. DPI also blocked glutamate toxicity in primary cortical neurons (Fig. 4*B*); however, DPI did not significantly inhibit MAO activity in either whole cells (Table 2) or cell extracts (data not shown). Quinacrine is also known to be a direct inhibitor of NADPH oxidase (Cross, 1990), although it can act at several other sites in cells as well (Cross, 1990). Similar to DPI, quinacrine significantly reduced glutamate toxicity in the HT-22 cells (Fig. 5). In addition, both DPI and quinacrine blocked glutamate-induced increases in H₂O₂ (Table 4). These findings provide further evidence for the role of an oxidase activity that is distinct from MAO-A in glutamate toxicity.

Identification of a novel clorgyline binding protein in HT-22 cells

To verify that a novel oxidase with the pharmacological properties of the enzyme-mediating glutamate toxicity exists in the HT-22 cells, mitochondria were prepared and labeled with 5 and 100 μM ³H-clorgyline. Figure 6 shows that at the low concentration of clorgyline, only a single band, corresponding to the size of MAO (63 kDa) (Cawthon et al., 1981), is observed. In contrast, at 100 μM clorgyline, an additional protein with an apparent molecular

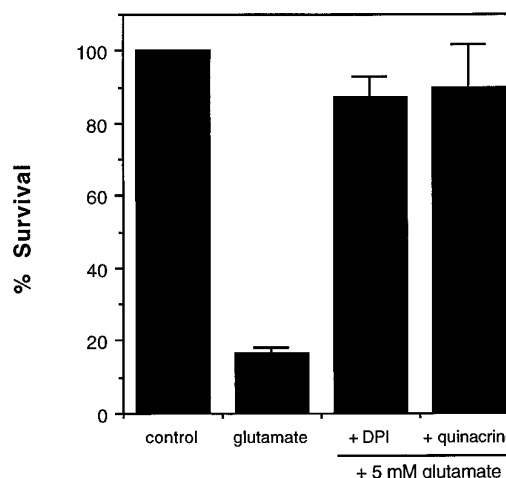


Figure 5. Oxidase inhibitors protect neuronal cells from glutamate toxicity. HT-22 cells were incubated with 1 μM DPI or 10 μM quinacrine for 8 hr in the presence of 5 mM glutamate. % survival was measured after 24 hr by the MTT assay. Data are expressed as % survival relative to controls treated with inhibitor alone. The concentrations of inhibitors used in this experiment afforded maximal protection without causing significant cell death. Values represent the mean of quadruplicate determinations ± SD. Statistical analysis of the results showed that both DPI and quinacrine provided significant protection (*p* < 0.0001) from glutamate toxicity. Similar results were obtained in five separate experiments.

weight of ~40 kDa was labeled. Deprenyl, which is a specific inhibitor of MAO-B at 1 μM but inhibits both MAO-A and MAO-B at 100 μM (Salach et al., 1979; Edelstein and Breakefield, 1981), completely blocks the binding of ³H-clorgyline to the 63 kDa protein band but not to the lower molecular weight band. This result is in precise agreement with our observation that glutamate toxicity is not inhibited by deprenyl (Figs. 1, 2) but is inhibited by clorgyline. Excess cold clorgyline completely blocks the binding of ³H-clorgyline to both bands. These data, as well as the studies with the MAO inhibitors, suggest that the novel oxidase activity is possibly related to MAO-A but clearly to a different enzyme.

DISCUSSION

The above data show that the metabolism of monoamines is involved in oxidative glutamate toxicity in HT-22 cells and in rat

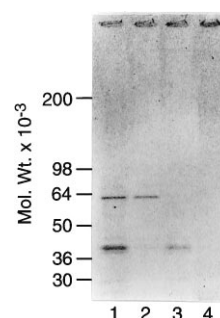


Figure 6. ³H-clorgyline binding to HT-22 mitochondria. Mitochondria were prepared from low-density cultures of HT-22 cells and labeled with different concentrations of ³H-clorgyline in the absence or presence of the indicated antagonists, as described in Materials and Methods. Lane 1, 100 μM ³H-clorgyline; lane 2, 5 μM ³H-clorgyline; lane 3, 100 μM ³H-clorgyline plus 100 μM deprenyl; and lane 4, 100 μM ³H-clorgyline plus 10 mM unlabeled clorgyline. Molecular weights (in kilodaltons) are indicated at left. Similar results were obtained in two independent experiments.

primary cortical neurons. The enzyme that seems to mediate this metabolism is inhibited by a number of specific, but chemically distinct, MAO-A inhibitors. Although higher concentrations of these inhibitors than are necessary to block MAO-A enzymatic activity are required to inhibit glutamate toxicity, high concentrations of MAO-B inhibitors, which also block MAO-A enzymatic activity at high doses, do not inhibit glutamate toxicity. Direct evidence for an enzyme with these characteristics comes from the studies with ^3H -clorgyline, which show the presence of a protein in the HT-22 cells that only binds clorgyline when the drug is present at high concentrations (Fig. 6). Furthermore, the binding of ^3H -clorgyline to this protein is not inhibited by deprenyl. In addition to this pharmacology, a large body of other data supports the hypothesis that monoamine metabolism is responsible for the oxidative stress that leads to cell death after treatment with glutamate. These data include the observation that an array of structurally unrelated agents that prevent the accumulation of monoamines within cells protect the cells from glutamate toxicity. Furthermore, dopamine can replace the toxic factor in serum when it is rendered nonfunctional, in terms of promoting cell death, by charcoal filtration. A dependence on serum for glutamate toxicity was observed previously in both primary neuronal cell cultures (Erdo et al., 1990) and neuronal cell lines (Froissard and Duval, 1994). Our results demonstrating a role for monoamine metabolism in glutamate toxicity provide an explanation for these data.

A critical role for monoamine metabolism in glutamate toxicity is consistent with the toxicity being mediated via an oxidative pathway, because monoamine breakdown generates a potential free radical, H_2O_2 . Cells normally use glutathione peroxidase to remove H_2O_2 , but the addition of glutamate leads to an inability to synthesize glutathione and hence a failure to neutralize the H_2O_2 via glutathione peroxidase (Murphy et al., 1989). Also consistent with this pathway are the data showing that the H_2O_2 level in the cells increases in the presence of glutamate but is reduced by MAO-A inhibitors and other treatments that prevent the accumulation of monoamines within cells. Thus, monoamine metabolism seems to play a decisive role in oxidative glutamate toxicity. Because receptor-mediated glutamate toxicity is associated with the production of free radicals as well (Choi, 1992), it is possible that monoamine metabolism is also important in this form of glutamate toxicity.

Treatment of cells with glutamate causes a depletion of cellular glutathione that precedes cell death (Murphy et al., 1989). Glutathione depletion by glutamate is not sufficient to cause cell death, however, because many protective agents, including low Ca^{+2} , lipoxygenase inhibitors, antioxidants (Miyamoto et al., 1989; Murphy et al., 1989), and the protein kinase C activator TPA (Davis and Maher, 1994), fail to block glutamate-induced depletion of glutathione levels, although they inhibit glutamate toxicity. The results presented here suggest that some of these agents either may act directly on the novel oxidase described above to reduce its activity or may indirectly affect the enzyme activity by decreasing the supply of substrate. Because the activity of this enzyme may be a major source of H_2O_2 in neuronal cells, inhibition of the enzyme would lead to a reduction of H_2O_2 , which in turn could significantly reduce the impact of glutathione depletion. Consistent with this idea is the recent report (Kitayama et al., 1994) that the protein kinase C activator TPA inhibits dopamine uptake into cells through a direct effect on the dopamine transporter.

A similar disruption to redox homeostasis *in vivo* might result in

neuronal cell death in neurodegenerative diseases such as Parkinson's disease (PD). Oxidative hypotheses for age-related disorders and PD encompass not only radical generation attributable to toxins (Hasegawa et al., 1990; Przedborski et al., 1992) but also those produced by cellular metabolism, such as catecholamine metabolism (Spina and Cohen, 1989). Supporting an oxidant hypothesis for PD are the observations of depleted glutathione levels and increases in lipid peroxidation in the substantia nigra of PD patients (Jenner, 1994). To account for the tissue specificity of PD, however, other factors must be considered. For example, there is evidence that glutamate plays a role. The onset of degeneration of the substantia nigra elevates glutamatergic input to the substantia nigra from the subthalamic nucleus, which seems to be essential for the progression of PD, because severance of these tracts can halt the progression (Bergman et al., 1990). Although glutamate receptor antagonists have some protective effects in models of PD (Sonsalla et al., 1989; Turski et al., 1991; Lees, 1993), several recent studies suggest that glutamate toxicity *in vivo* may not be mediated entirely by glutamate receptors (Greene and Greenamyre, 1995; Gwag et al., 1995) but rather through other effects of glutamate, such as those described in this report. It follows that the pressures of handling both catecholamine transmitters and glutamatergic input may combine to make the substantia nigra particularly susceptible to neurotoxic insults. The data presented above formally demonstrate this direct link between glutamatergic dysfunction and monoamine metabolism in neurotoxicity.

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