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A Role for 12-lipoxygenase in Nerve Cell Death Caused by Glutathione Depletion

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Summary

An early and highly specific decrease in glutathione (GSH) in the substantia nigra is associated with Parkinson's disease, and low levels of GSH lead to the degeneration of cultured dopaminergic neurons. Using immature cortical neurons and a clonal nerve cell line, it is shown that a decrease in GSH triggers the activation of neuronal 12-lipoxygenase (12-LOX), which leads to the production of peroxides, the influx of Ca^{2+}, and ultimately to cell death. The supporting evidence includes: 1) inhibitors of arachidonate metabolism and 12-LOX block cell death induced by GSH depletion; 2) there is an increase in 12-LOX activity and a membrane translocation in HT22 cells, and an induction of the enzyme in primary cortical neurons following the reduction of GSH; 3) 12-LOX is directly inhibited by GSH; and 4) exogenous arachidonic acid potentiates cell death. These data show that the LOX pathway is a critical intermediate in at least some forms of neuronal degeneration.

Introduction

While physiologically programmed cell death (PCD) functions in the normal development of the nervous system, inappropriate PCD leads to the neuronal loss associated with various neuropathological disorders such as Alzheimer's disease (Su et al., 1994; Smale et al., 1995) and Parkinson's disease (PD) (Mochizuki et al., 1996). PCD in Alzheimer's disease and PD is thought to be linked to oxidative stress, for excessive lipid and protein oxidation occurs and cellular antioxidant systems are altered (reviewed by Jenner and Olanow, 1996; Simonian and Coyle, 1996). In the substantia nigra of PD patients, there is an early and highly specific decrease in glutathione (GSH) (Perry et al., 1982; Sofic et al., 1992; Sian et al., 1994), which may precede cell death (Dexter et al., 1994). In addition, the blocking of GSH synthesis by buthionine sulfoximine (BSO), an inhibitor of γ-glutamylcysteine synthetase, the rate-limiting synthetic step in GSH synthesis, results in the degeneration of cultured dopaminergic neurons (Jenner and Olanow, 1996). BSO also potentiates the toxicity to dopaminergic neurons of 6-hydroxydopamine, MPTP, and MPP^+ (Pileblad et al., 1989; Wullner et al., 1996). These observations suggest a pivotal role for GSH in the pathogenesis of PD (Jenner and Olanow, 1996).

In addition to γ-glutamylcysteine synthetase activity, the synthesis of GSH can be regulated by the transport of cystine, a precursor of GSH. This mechanism is exemplified by oxidative glutamate toxicity in neuronal cultures where toxicity is initiated by the competition of glutamate with the cystine uptake system. Glutamate inhibits cystine uptake, which in turn causes a decrease in GSH and the inability of the cell to deal with reactive oxygen species (ROS), ultimately leading to cell death (Murphy et al., 1989a). Oxidative glutamate toxicity is inhibited by antioxidants such as vitamin E (Murphy et al., 1989a; Schubert et al., 1992; Mayer and Noble, 1994). This cytotoxicity pathway is distinct from glutamate excitotoxicity, which is mediated by ionotropic glutamate receptors.

Neuronal cell death via the oxidative pathway has many of the characteristics of apoptosis, including nuclear condensation and cleavage of chromatin into oligonucleosomal fragments (Ratan et al., 1994b). Cell death depends on macromolecular synthesis (Ratan et al., 1994a, 1994b). The overexpression of *bcl-2* (Behl et al., 1993) or the activation of protein kinase C (Davis and Maher, 1994) prevents cell death. Growth factors such as epidermal growth factor also attenuate oxidative glutamate toxicity (Schubert et al., 1992). However, the signaling pathways that mediate oxidative glutamate toxicity remain largely unknown.

A number of biochemical pathways participate in the regulation of apoptosis (reviewed by Hale et al., 1996; McConkey and Orrenius, 1994). The sphingolipid metabolite ceramide mediates apoptosis in response to a variety of stimuli including tumor necrosis factor α and the Fas ligand (reviewed by Hannun, 1996). There is also recent evidence that other lipid metabolites, such as arachidonic acid–derived eicosanoids, may play a role in regulating cell survival (Korystov et al., 1996; Tang et al., 1996). Down-regulation of arachidonate lipoxygenase (LOX) results in apoptosis in a carcinosarcoma cell line (Tang et al., 1996), while irradiation-induced thymocyte apoptosis appears to be mediated by LOX metabolites (Korystov et al., 1996). Although the mechanisms by which arachidonic acid metabolites influence cell growth or survival are not known, arachidonic acid can activate several signaling pathways directly by itself or by its metabolites through LOX, cyclooxygenase, or cytochrome P450 pathways (reviewed by Shimizu and Wolfe, 1990).

LOXs are dioxygenases that incorporate molecular oxygen into specific positions of polyunsaturated fatty acids and, based on the site of insertion of the oxygen, are generally classified as 5-, 12-, or 15-LOXs (Shimizu and Wolfe, 1990). 12-LOX is the predominant LOX in the brain, and its mRNA is present in rat cortical neurons, oligodendrocytes, and astrocytes (Bendani et al., 1995). Cloned rat brain 12-LOX generates predominately 12-hydroxyeicosatetraenoic acid (12-HETE) and also some 15-hydroxyeicosatetraenoic acid (15-HETE) (Watanabe et al., 1993). 12-HETE and its derivatives are the major metabolites of LOXs in the brain (Sautebin et al., 1978; Shimizu et al., 1987). These metabolites may play an important role as second messengers in synaptic transmission and as retrograde messengers in learning

and memory (reviewed by Piomelli, 1994; Shimizu and Wolfe, 1990). They may also contribute to neuropathological events such as brain ischemia (Shimizu and Wolfe, 1990).

Several observations suggest that 12-LOX may play a role in oxidative glutamate toxicity. First, there is an early reduction in GSH levels in oxidative glutamate toxicity (Murphy et al., 1989b), and decreased GSH can trigger the activation of 12-LOX (Shornick and Holtzman, 1993; Hagmann et al., 1993). Second, peroxides are produced in oxidative glutamate toxicity (Maher and Davis, 1996), and peroxides induce the expression of c-Jun and c-Fos, which have been implicated in neuronal apoptosis through LOX pathways (Rao et al., 1993). Last, the activation of soluble guanylate cyclase via an NO-independent pathway is required for cell death in oxidative glutamate toxicity (Li et al., unpublished data), and arachidonate metabolites are activators of guanylate cyclase (Snider et al., 1984).

In this paper, evidence is presented for a requirement of 12-LOX in oxidative glutamate toxicity, and it is shown that glutamate-induced GSH depletion activates 12-LOX. The role of 12-LOX in linking GSH depletion to neuronal cell death is then demonstrated by establishing that BSO-induced cell death also requires 12-LOX activity. Finally, it is shown that 12-LOX is a prerequisite for the Ca^{2+} influx and peroxide production.

Results

Inhibitors of Arachidonate Metabolism Block Oxidative Glutamate Toxicity

HT22 is a hippocampal nerve cell line that is very susceptible to oxidative stress caused by exposure to glutamate. The addition of 5 mM glutamate to the growth medium results in cell lysis, which is initiated at 8 hr and is complete by 16 hr (Davis and Maher, 1994; Maher and Davis, 1996). Loss of cell viability was quantitated by the decrease in the ability of cells exposed to glutamate to metabolize the dye 3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Mosmann, 1983), compared to control (Figure 1A). In this system, the MTT is a direct measure of cell death since it correlates with trypan blue and colony formation viability assays (Davis and Maher, 1994). Because of its potential role in PCD, it was asked whether inhibitors of arachidonate metabolism alter toxicity. Figure 1A shows that glutamate toxicity was reduced in a dose-dependent manner by 5, 8, 11, 14-eicosatetraynoic acid (ETYA), a competitive analog of arachidonic acid, suggesting that arachidonic acid is involved in glutamate toxicity.

To determine which metabolites of arachidonic acid are involved in glutamate toxicity, inhibitors of oxygenases that use arachidonic acid as substrates were tested for their ability to reduce toxicity. Nordihydroguaiaretic acid (NDGA), a general LOX inhibitor, protected HT22 cells from glutamate toxicity (Figure 1B; see also, Murphy et al., 1989b). The concentration required for maximal protection was 0.5–1.0 μM. NDGA was toxic to cells at concentrations >5 μM (data not shown). In contrast, when indomethacin, a specific inhibitor of cyclooxygenase with an IC_{50} of 1 μM, was tested at concentrations up to 100 μM, no protective effect on glutamate toxicity was seen (Figure 1B). The epoxygenase inhibitor metyropane also did not protect at doses up to 1 mM (Figure 1B). Therefore, LOX enzymatic activity plays a role in glutamate toxicity.

Since there are several forms of LOX (Shimizu and Wolfe, 1990), selective inhibitors of 5- and 12-LOXs were used to determine the specific LOX required for glutamate toxicity. Two selective inhibitors of 12-LOX, baicalein (5, 6, 7-trihydroxyflavone) (Sekiya and Okuda, 1982) and cinnamyl-3, 4-dihydroxy-α-cyanocinnamide (CDC) (Cho et al., 1991), both conferred full protection from glutamate toxicity (Figure 1C). Three selective inhibitors of 5-LOX were also tested, and two of them, caffeic acid (Koshihara et al., 1984) and 5, 6-dehydro-arachidonic acid (DAA) (Sok et al., 1982), were not protective at any dose (Figure 1C, and data not shown). However, another 5-LOX inhibitor, 2, 3, 5-trimethyl-6-(12-hydroxy-5–10-dodecadiynyl)-1, 4-benzoquinone (AA861) (Yoshimoto et al., 1982), protected cells (Figure 1C). Although widely used as a 5-LOX inhibitor, AA861 also inhibits leukocyte 12-LOX (Yoshimoto et al., 1982). In view of the minimal effect of the other two inhibitors of 5-LOX and the similarity of brain 12-LOX to leukocyte 12-LOX (71% identity), the effect of AA861 is probably due to its inhibition of 12-LOX. These results suggest that 12-LOX, the predominant brain isoform of this enzyme, is involved in the pathway leading to oxidative glutamate toxicity.

Inhibitors of LOX Also Block the Oxidative Glutamate Toxicity in Primary Cortical Neurons

To determine whether the observations with the HT22 cells reflect the mechanism of oxidative glutamate toxicity in the brain, embryonic rat cortical neurons were prepared, and 1 day later, the cells were exposed to glutamate. These young cultures are devoid of functional glutamate receptors and are killed by glutamate-induced oxidative stress via an apoptotic pathway (Murphy and Baraban, 1990; Murphy et al., 1990; Ratan et al., 1994b). A 24 hr exposure to 5 mM glutamate resulted in the disappearance of neurites and the shrinkage of cell bodies (Figures 2A and 2B). The 12-LOX inhibitor baicalein almost completely rescued the cells from glutamate toxicity (Figure 2C). Other LOX inhibitors capable of protecting HT22 cells also blocked glutamate-induced cell death of cortical cells in a dose-dependent manner (Figure 1D). It was not possible to demonstrate an effect of ETYA on glutamate toxicity in primary cells since ETYA is very toxic to these cells. Consistent with the findings using HT22 cells, the 5-LOX inhibitors caffeic acid and DAA could not prevent glutamate-induced cell death in primary cortical cultures (Figure 1D, and data not shown).

Glutamate Activates 12-LOX and Causes Translocation to the Membrane in HT22 Cells, but Induces the Enzyme in Primary Neurons

The observation that 12-LOX inhibitors block glutamate toxicity suggests that this enzyme is a necessary step in the signaling pathway leading to cell death. LOX may simply be required in this pathway or it may be a limiting factor and demand activation during the cell death

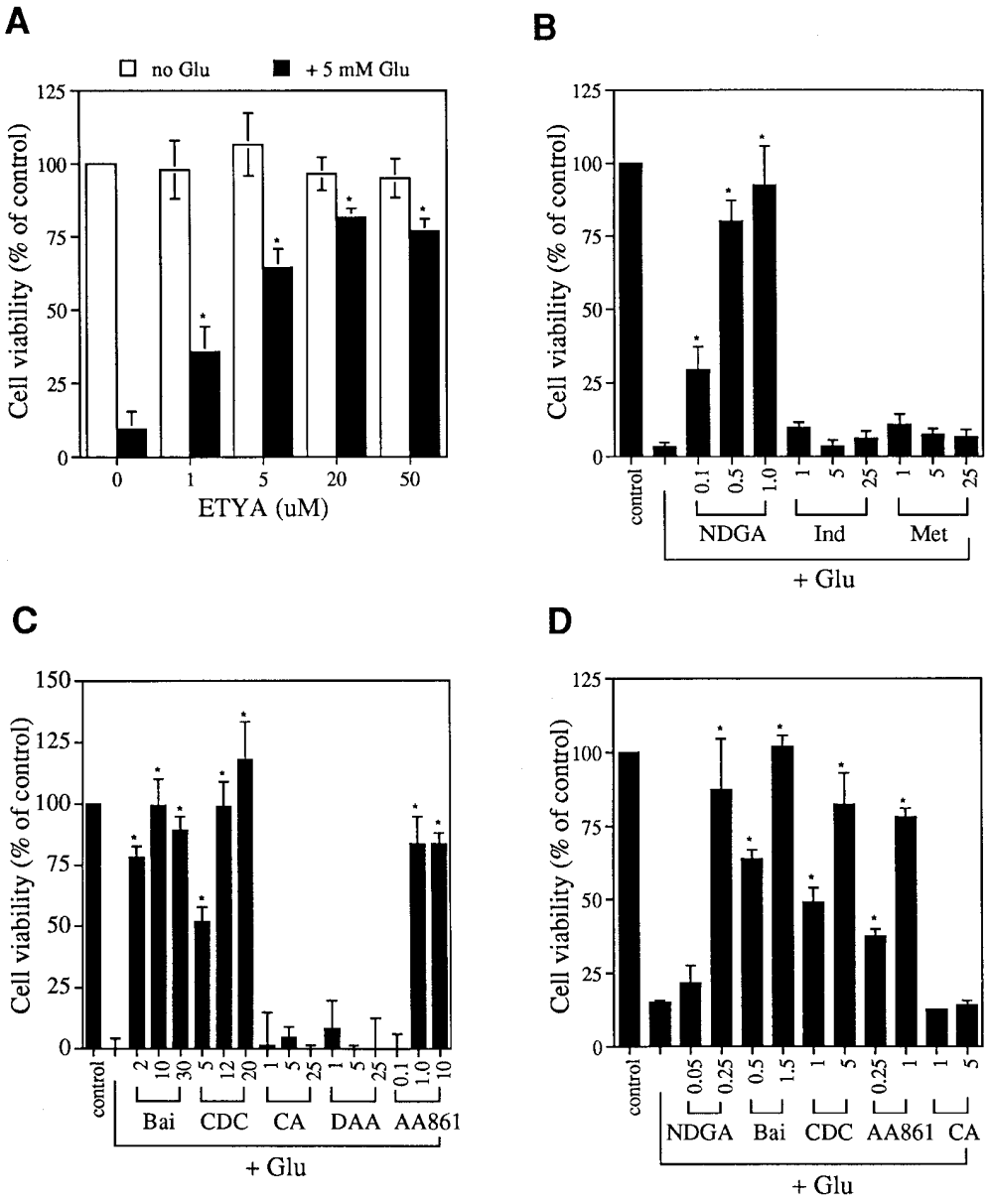

Figure 1. LOX Inhibitors Block Glutamate Toxicity

(A) Dose effect of the arachidonic acid analog. HT22 cells were seeded onto 96-well microtiter plates at 2.5×10^3 cells per well and the next day were treated with ETYA at the concentrations indicated 10 min before the addition of 5 mM glutamate. After 20 hr, the cell viability was determined by the MTT assay and confirmed by visual inspection. The MTT assay is, in this system, directly proportional to viable cell number (Davis and Maher, 1994). Results are expressed relative to controls with agents alone. The effect of the agents on dye reduction in the absence of cells, if any, was adjusted by subtracting the value for the medium control. The results shown are the mean ± SD of a typical experiment with five determinations. Similar results were obtained in three independent experiments. *, significantly different from glutamate treatment alone ($P < 0.005$, Student's *t*-test). The following experiments were subject to the same analysis described above unless otherwise stated.

(B) Dose effect of the LOX inhibitor NDGA, the cyclooxygenase inhibitor indomethacin (Ind), and the epoxygenase inhibitor metyropane (Met). The concentrations given for each inhibitor are in micromolars. The control did not receive glutamate.

(C) Dose effect of the 12-LOX inhibitors baicalein (Bai) and CDC and the 5-LOX inhibitors caffeic acid (CA), DAA, and AA861. The concentrations given for each inhibitor are in micromolars. The control did not receive glutamate.

(D) Dose effect of various LOX inhibitors in primary cortical neurons. Experiments were performed similarly to those described above except that 5×10^4 cells were seeded to each well of a 96-well plate. The concentrations given for each inhibitor are in micromolars. Error bars that are not visible are less than the width of the symbol.

process. Three assays were done to determine whether there is increased LOX activity following exposure to glutamate. LOX enzymatic activity was measured in a cell-free system, the expression and the translocation of LOX enzyme to the membrane were examined, and the rate of exogenous arachidonic acid uptake was determined as a function of time following glutamate exposure. Figures 3A and 3B show that all three assays indicate an increase in arachidonic acid metabolism between 4 and 8 hr after glutamate exposure in HT22 cells.

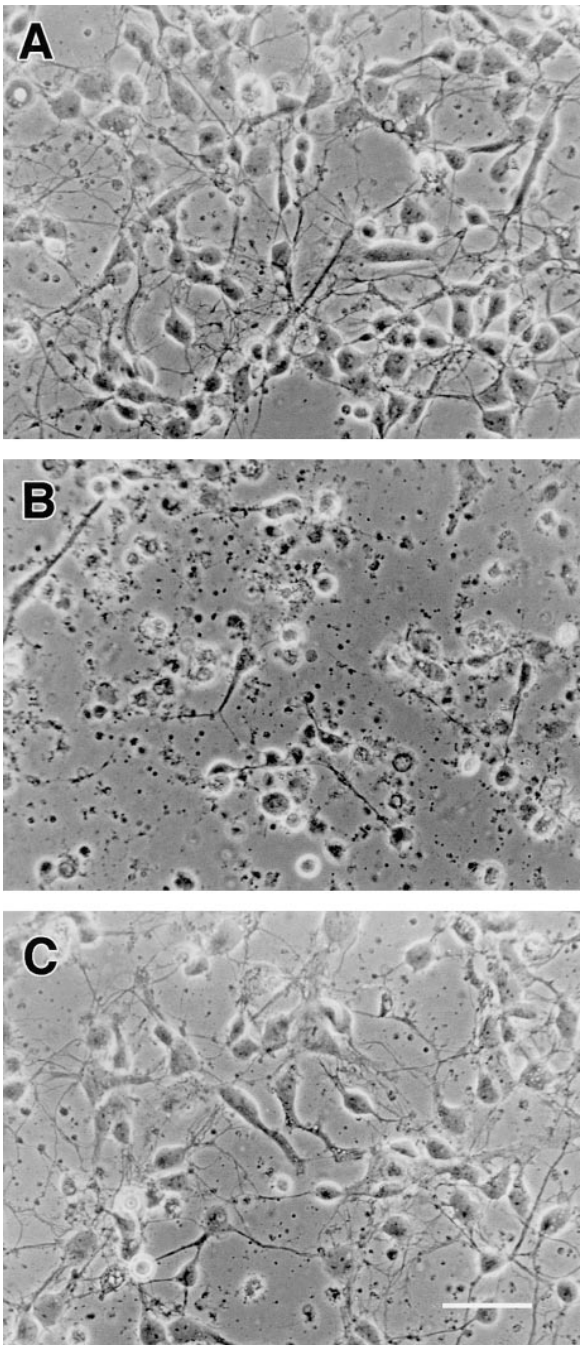

Figure 2. LOX Inhibitors Block Glutamate Toxicity in Rat Cortical Neurons

(A–C) Phase contrast images of immature cortical neurons mock treated (A), treated with 5 mM glutamate (B), or treated with 5 mM glutamate and 1.5 μM baicalein (C). One-day-old primary cultures prepared from embryonic day 17 rats were treated as described and photographed 24 hr later. Scale bar = 25 μM.

There is an increase in arachidonic acid uptake, and a 2- to 5-fold increase in the rate of 12-HETE formation in cell lysates (Figure 3A). LOX product formation and arachidonate uptake were blocked by NDGA and baicalein at concentrations that inhibit glutamate toxicity (data not shown). LOX activation is frequently accompanied by translocation to the membrane (Baba et al., 1989; Hagmann et al., 1993). Figure 3B shows that while there is no overall increase in the level of LOX protein in HT22 cells, which suggests that 12-LOX is constitutively expressed, there is a translocation of the larger form of the enzyme to the membrane fraction. Unlike HT22 cells, glutamate induces a 2- to 3-fold increase in 12-LOX protein in primary cortical neurons (Figure 3C). 12-LOX consists of two forms of about 76 kDa and 78 kDa. The same doublet pattern is also observed when lysates of HEK293 cells transfected with murine leukocyte-type 12-LOX are probed with the antibody used in our experiments (Kinzig et al., 1997).

The above experiments suggest that arachidonic acid may, in part, be limiting LOX activity in the presence of glutamate. If so, then arachidonic acid should potentiate glutamate toxicity. Exogenously added arachidonic acid indeed potentiated glutamate toxicity in a dose-dependent manner (Figure 4A). The potentiation effect was seen at a concentration as low as 2 μM, while arachidonic acid alone was not toxic at concentrations used.

Activation of 12-LOX Follows GSH Depletion

The activation of 12-LOX could be by increased substrate availability, the loss of an inhibitor, through a secondary activator, by increased enzyme synthesis, or by a combination of these factors. The above data show that there is no increase in LOX enzyme (Figure 3B) and that arachidonic acid is rate limiting in the presence of glutamate (Figures 3A and 4A). Since GSH directly inhibits LOX (Shornick and Holtzman, 1993; Hagmann et al., 1993), the temporal relationship between GSH concentration and LOX activity was examined. The exposure of HT22 cells to glutamate induces a depletion of intracellular GSH. Figure 3A shows that GSH drops to an almost undetectable level by 6 hr after the addition of 5 mM glutamate, which coincides with the observed increase in LOX activity and arachidonic acid uptake. LOX inhibitors did not alter the depletion of GSH (Figure 3A, and data not shown), suggesting that LOX acts downstream of GSH depletion. Glutamate also causes GSH depletion in primary cortical neurons (Figure 3C). To determine whether the 12-LOX activity in HT22 cells is regulated by GSH as with other cells, the effect of GSH on 12-LOX enzymatic activity was examined in lysates of HT22 cells treated with 5 mM Glu for 8 hr, at which time GSH has fallen to an undetectable level (Figure 3A). The formation of 12-HETE by 12-LOX was inhibited by increased GSH, with a half maximal inhibition of ~1 mM (Figure 5). This is well within the normal range of intracellular GSH (Halliwell and Gutteridge, 1989).

Depletion of GSH by BSO Induces Cell Death, Which Is Prevented by LOX Inhibitors

If the decreased level of GSH caused by glutamate exposure is the activator of 12-LOX and LOX-dependent cell death in HT22 cells, then decreasing GSH by methods independent of glutamate should also lead to a toxic response, which is inhibited by LOX inhibitors. BSO is an inhibitor of γ-glutamylcysteine synthetase, the rate-limiting enzyme in GSH biosynthesis. Exposure of HT22 cells to BSO reduces cellular GSH levels and causes

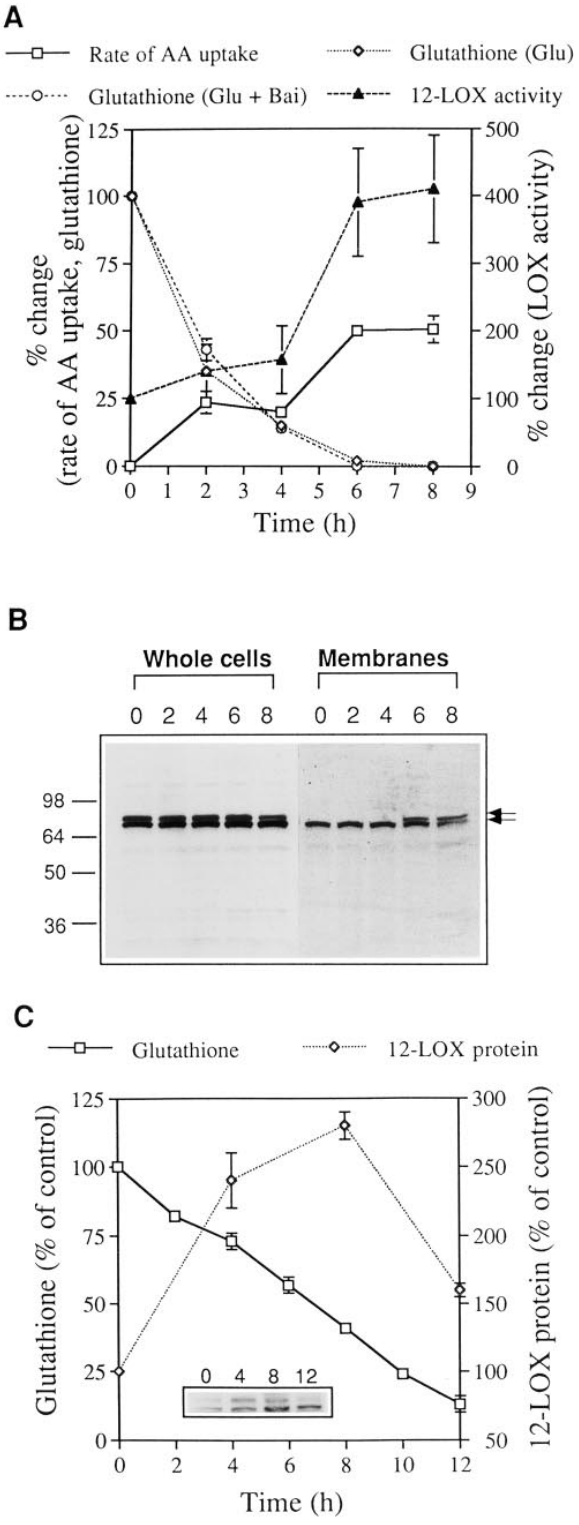

Figure 3. Time Course Analysis of GSH Levels, 12-LOX Activity, the Rate of Arachidonic Acid (AA) Uptake, and 12-LOX Expression and Translocation Following Glutamate Exposure

(A) HT22 cells were treated with 5 mM glutamate alone or with 10 μM baicalein (Bai) for various times and collected for the assay of GSH and 12-LOX activity as described in the Experimental Procedures. The rate of arachidonic acid uptake was determined by pulse-labeling cells for 1 hr with ^{3}H-arachidonic acid followed by analysis as described in the Experimental Procedures. Data are expressed relative to controls at 0 hr. Results for GSH are the mean ± SD of a typical experiment with three determinations. The level of GSH for the untreated control cells was 21.8 ± 1.4 nmol/mg protein and that of control 12-LOX activity was 0.11 ± 0.05 nmol/mg/15 min (n = 7). Results for 12-LOX activity and the rate of arachidonic acid uptake are the mean ± SD of four independent experiments.

(B) Equal amounts of total cell lysates (5 μg) or the membrane fraction (3.5 μg) from cells exposed to 5 mM glutamate for various times (hr) indicated above each lane were resolved in 10% SDS–polyacrylamide gels and transferred to a nitrocellulose. The nitrocellulose was probed with antiserum to human 12-LOX, HRP-conjugated secondary antibody, and developed with chemiluminescence. 12-LOX runs as a doublet of about 76 and 78 kDa (arrowheads). The protein molecular weights are given in kDa at the left.

(C) Time course analysis of GSH and 12-LOX in primary cortical neurons following glutamate treatment. Experiments were performed as described above. Insert, a representative Western blot of 12-LOX induction in primary cultures. The Western blots were quantitated (n = 3), and the results were expressed relative to control levels. Equal loading of protein (5 μg/lane) was verified by staining and with an anti-actin antibody. Error bars that are not visible are less than the width of the symbol.

cell death in a dose-dependent fashion (Figure 6A). Treatment of cells with 30 μM BSO for 20 hr resulted in total cell lysis. The toxicity of BSO was blocked by the arachidonic acid analog ETYA and other LOX inhibitors at concentrations similar to those that block glutamate toxicity (Figure 6B). BSO also depletes intracellular GSH and is toxic to primary cortical neurons (Figure 6A), and the toxicity is blocked by LOX inhibitors (data not shown). As with glutamate toxicity, arachidonic acid potentiates BSO toxicity, showing that the effect of arachidonic acid is not on amino acid transport (Figure 4B). These data suggest that 12-LOX activity is required for neuronal cell death due to GSH depletion independently of the mechanism of oxidative toxicity.

12-LOX Is Required for Ca^{2+} Influx

Ca^{2+} influx is required for oxidative glutamate toxicity, for 50 μM cobalt or Ca^{2+} depleted media block toxicity (Murphy et al., 1989a; Davis and Maher, 1994). Because in clonal cells 12-HETE can regulate ion channels (Stern et al., 1993), we asked whether 12-LOX activity is a prerequisite for the Ca^{2+} influx, which precedes cell lysis. The effect of LOX inhibitors on the level of intracellular Ca^{2+} following glutamate treatment was therefore examined. The intracellular Ca^{2+} response to glutamate was determined by flow cytometry using a ratiometric analysis with Indo-1 (June and Rabinovitch, 1991). The binding of Ca^{2+} to the fluorescent dye Indo-1 results in a spectral shift in Indo-1 excitation wavelength from 380–340 nm. The ratio of fluorescence intensities from the two emission peaks is proportional to the Ca^{2+} concentration (Grynkiewicz et al., 1985). HT22 cells were mock treated or treated with 5 mM glutamate in the presence or absence of LOX inhibitors for 12 hr, loaded with Indo-1, and Ca^{2+} influx was measured. Figure 7A shows that treatment with 5 mM glutamate for 12 hr resulted in a dramatic increase in overall Ca^{2+} levels. The rise of Ca^{2+} was prevented by the LOX inhibitors baicalein, NDGA, CDC, and AA861 as well as the arachidonic acid analog ETYA (Figure 7A). These inhibitors had no effect on Ca^{2+} levels in control cells (data not shown). Neither

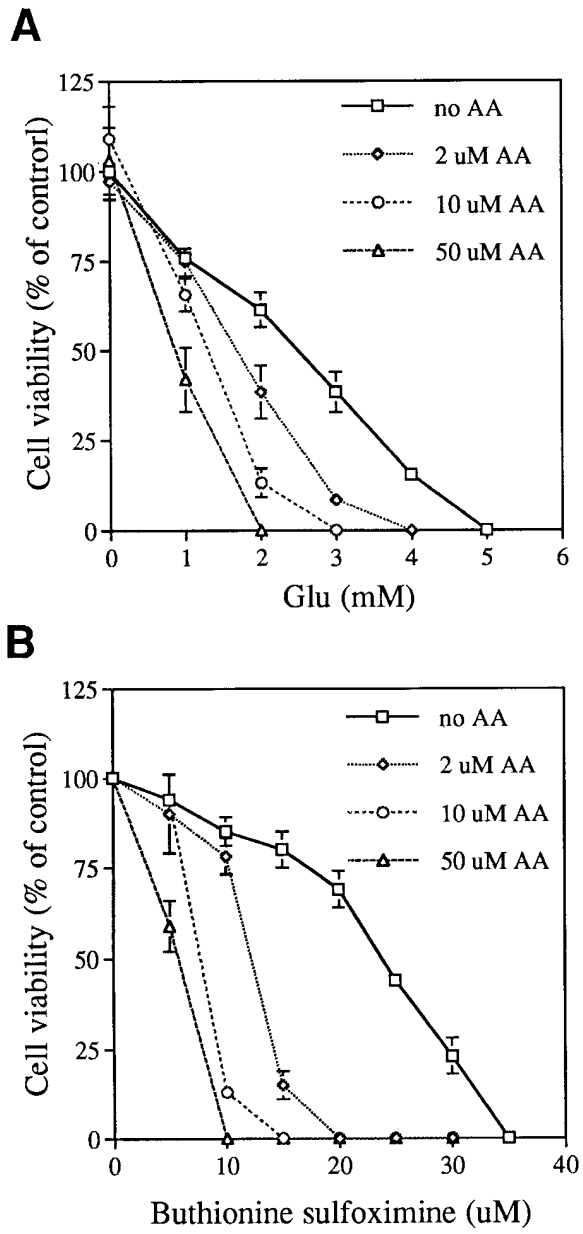

Figure 4. Exogenously Added Arachidonic Acid Potentiates Cell Death Induced by Glutamate (A) or BSO (B)

Experiments and data analysis were performed as described in the legends to Figure 1 or Figure 6, except that the HT22 cells were treated with glutamate or BSO and/or with arachidonic acid at the concentrations indicated.

(A) Results are expressed relative to control without any treatment. Note that arachidonic acid has no significant effect on cell growth at or under 50 μM (compare cell viabilities at 0 mM glutamate).

(B) Results are expressed relative to controls with agents alone. Repeated three times with similar results.

caffeic acid nor DAA, which could not block glutamate toxicity, could prevent the rise of Ca^{2+} (data not shown). It follows that 12-LOX activity is required for the Ca^{2+} influx. Similar experiments were not possible with primary cultures because intracellular calcium is not stabilized in young cultures where there is still spontaneous

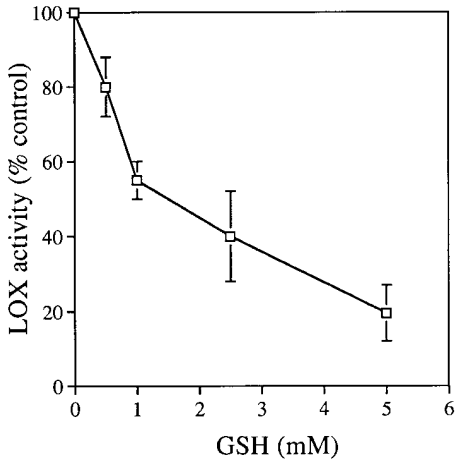

Figure 5. The GSH Concentration Regulates Neuronal 12-LOX Activity

Cell lysates of HT22 cells treated with 5 mM glutamate for 8 hr were used for an in vitro enzymatic assay for 12-LOX activity as described in the Experimental Procedures. GSH was added to the reaction mixtures at the indicated concentrations. 12-LOX activity, expressed as relative to controls without addition of GSH, is shown as the mean ± SD (n = 6).

cell death. Older cultures express ionotropic glutamate receptors, precluding these experiments.

12-LOX Activity Is Required for Peroxide Production

Peroxide formation is a necessary intermediate in oxidative glutamate toxicity (Davis and Maher, 1994; Maher and Davis, 1996). To determine whether LOX activation is required for peroxide formation, the effect of LOX inhibitors on the formation of peroxide was examined by flow cytometry using 2′,7′-dichlorofluorescin diacetate as a probe. The nonfluorescent dye 2′,7′-dichlorofluorescin diacetate is deacetylated by intracellular esterases after crossing cell membranes to give rise to dichlorofluorescin. The latter is trapped within cells and oxidized to the fluorescent compound dichlorofluorescein by peroxides (Royall and Ischiropoulos, 1993). Treatment of cells with 5 mM glutamate results in a dramatic increase in the level of peroxides (Figures 7B and 7C). The increase in the peroxide level was almost totally inhibited by treatment of cells with baicalein (Figures 7B and 7C), at a concentration that conferred full protection from glutamate toxicity (Figure 1). Other inhibitors of arachidonic acid metabolism and 12-LOX (but not 5-LOX) also reduced the accumulation of peroxides following treatment with glutamate (data not shown). Therefore, 12-LOX activation precedes peroxide production during oxidative glutamate toxicity.

Discussion

The above experiments demonstrate that 12-LOX is involved in the pathway of neuronal cell death, which is initiated by GSH depletion. This conclusion is based on the following data: 1) inhibitors of arachidonate metabolism and of 12-LOX prevent cell death (Figures 1 and 2);

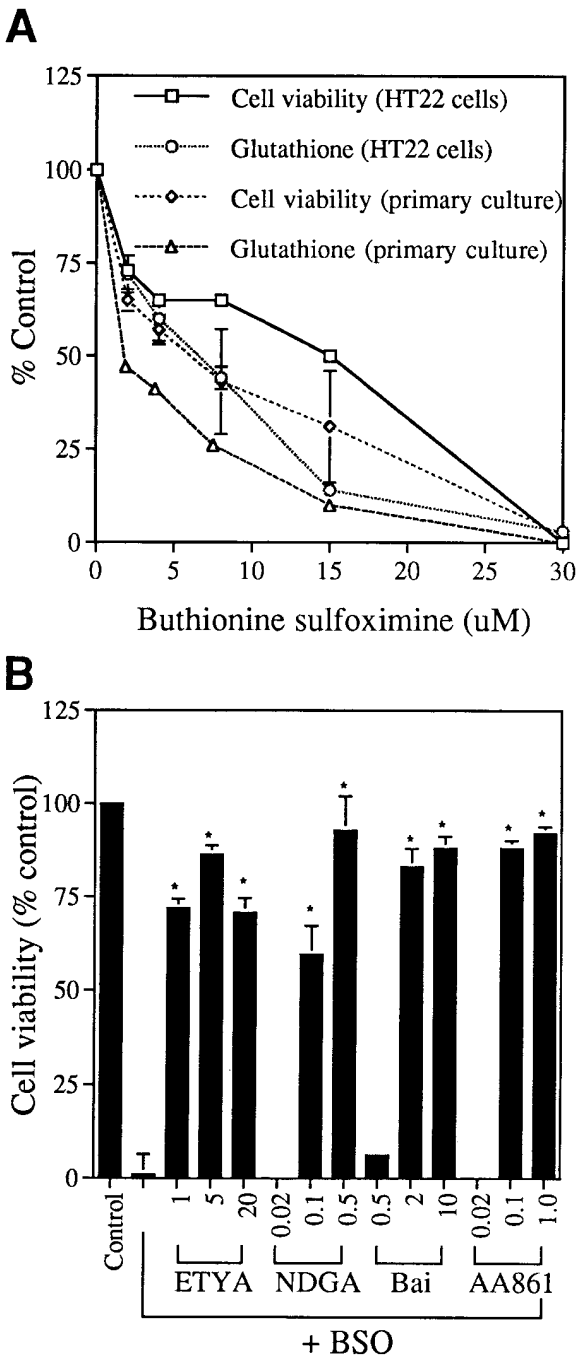

Figure 6. BSO Depletes Intracellular GSH and Induces Cell Death, Which Is Prevented by LOX Inhibitors

(A) Dose effect of BSO on intracellular GSH levels and cell viability. HT22 cells or primary cortical neurons were grown in DMEM containing 5% horse serum and treated with BSO at the concentrations indicated. Horse serum was used instead of FBS because the sensitivity of HT22 cells to BSO was much lower and more variable when cells were grown in FBS. BSO solutions were prepared just prior to use. GSH levels were measured as described in the Experimental Procedures at 12 hr after treatment. Results for GSH are the mean ± SD of a typical experiment with three determinations and expressed as the percentage of controls. Error bars that are not visible are less than the width of the symbol. The basal level of GSH was 21.8 ± 1.4 and 10.1 ± 0.5 nmol/mg protein for the untreated HT22 cells and primary cortical neurons, respectively. Cell viability was determined by the MTT assay at 20 hr after the treatment. Similar results were obtained in three independent experiments.

(B) Dose effect of LOX inhibitors on BSO toxicity. HT22 cells were treated with LOX inhibitors at the concentrations indicated (micromolars) for 1 hr followed by treatment with BSO at 30 μM for 20 hr. Cell viability was determined by the MTT assay and is expressed as the percentage of control. The experiment was repeated three times with similar results. *, significantly different from BSO treatment alone ($P < 0.005$, Student's t-test).

2) the activity of 12-LOX is increased following glutamate exposure, and there is a shift of one LOX isoform from the cytoplasm to the plasma membrane in HT22 and increased enzyme production in primary cultures (Figure 3); and 3) exogenously added arachidonic acid potentiates cell death (Figure 4). Since the metabolism of arachidonic acid by 12-LOX yields a number of eicosanoids (Shimizu and Wolfe, 1990), it is likely that one or more of these, which remain to be identified, play critical roles in the induction of the neuronal cell death via the oxidative pathway.

Despite being the major LOX isoform in the brain (Sautebin et al., 1978; Shimizu et al., 1987), the biological roles of 12-LOX have not been well characterized. However, arachidonic acid metabolism has been implicated in neuronal function (see, for example, Murphy et al., 1989b; Shimizu and Wolfe, 1990; Piomelli, 1994). 12-LOX metabolites are messengers for FMRFamide (Phe-Met-Arg-Phe amide, a molluscan peptide) induced hyperpolarization in Aplysia sensory neurons (Piomelli et al., 1987; Buttner et al., 1989), and the activation of the NMDA receptor causes an increase in Ca^{2+} influx and a subsequent release of arachidonic acid (Dumuis et al., 1988, 1993). In contrast, the above data show that the metabolism of arachidonic acid is increased by glutamate (Figure 3) and that Ca^{2+} influx is downstream of the requirement for 12-LOX (Figure 7A).

Although a variety of potential mechanisms may activate 12-LOX in the neuronal cell death pathway, it is likely that the enzyme is initially activated by GSH depletion. This is supported by the following experiments: 1) the time course analysis, which shows that increased 12-LOX activity follows the depletion of GSH (Figure 3A); 2) the in vitro enzymatic assay, which shows that an increase in the GSH concentration inhibits 12-LOX activity (Figure 5); and 3) the artificial depletion of GSH by BSO, which activates a LOX-dependent cell death pathway that is indistinguishable from that of glutamate toxicity (Figure 6). The regulation of 12-LOX activity by GSH has also been demonstrated in several other systems (Shornick and Holtzman, 1993; Hagmann et al., 1993).

The data presented above are summarized in the following model of GSH depletion-induced neuronal cell death. Competition of cysteine uptake by glutamate or direct inhibition of a GSH-synthesis enzyme by BSO results in the depletion of intracellular GSH, which then triggers the activation of 12-LOX and therefore an increased production of 12-LOX metabolites such as 12-HETE. The activation of 12-LOX is an event upstream of Ca^{2+} influx and ROS production in HT22 cells. Although LOX activation is also upstream of ROS production in primary cultures and is required for cell death, it

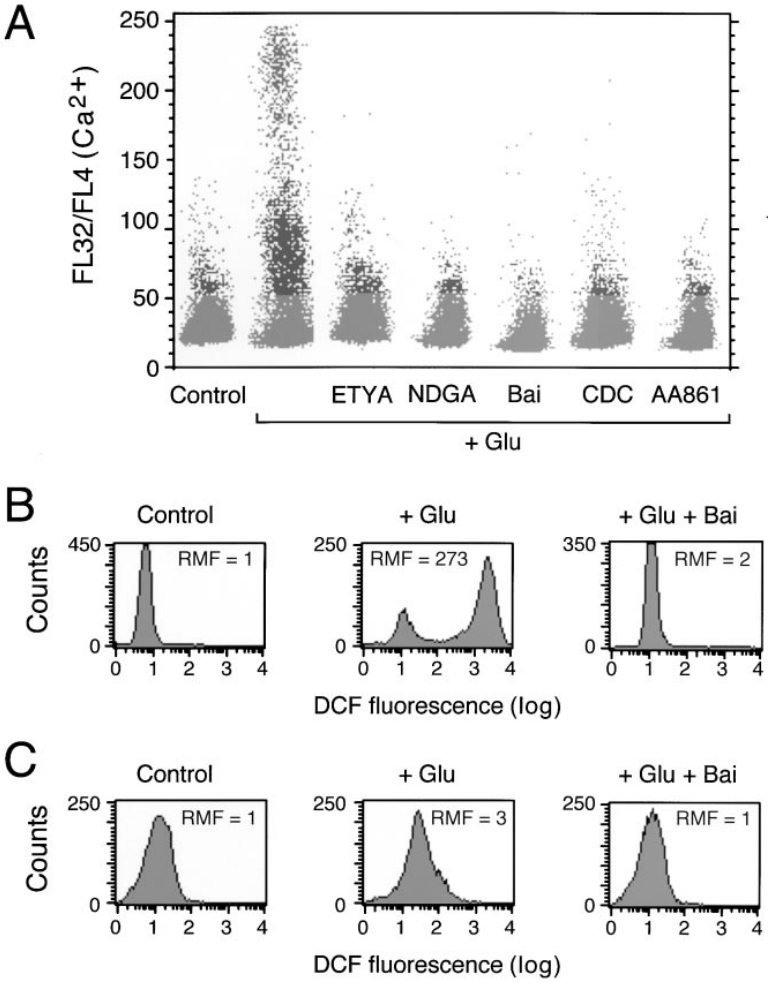

Figure 7. Inhibition of LOX Prevents Glutamate-Induced Ca^{2+} Influx and Peroxide Production

(A) HT22 cells were mock treated or treated with 5 mM glutamate in the absence or presence of other reagents for 12 hr; loaded with Indo-1 for 30 min; and the fluorescence from the two emission peaks of Indo-1, 410 nm (FL32) and 485 nm (FL4), was collected, and the ratio of the FL32 to FL4 was obtained. Scatter plots displaying the side angel scatter (SSC-height, which is proportional to the gravity of cells) versus the FL32:FL4 ratio of intracellular indo-1 emitted fluorescence (proportional to Ca^{2+}) were transferred to an Adobe Photoshop program and combined (SSC-height on the x-axis is not marked). Each plot represents a total of 10,000 viable cells. Similar results were obtained in three independent experiments, each with two repeated samples. The concentrations of LOX inhibitors were 20 μM for ETYA, 1 μM for NDGA, 10 μM for Bai, 15 μM for CDC, and 1 μM for AA861. Control, no glutamate treatment.

(B) Experiments were performed in HT22 cells as above except that intracellular peroxide level was measured by FACS analysis described in the Experimental Procedures. A typical set of histograms showing cell counts versus DCF fluorescence are presented. Results were from 10,000 viable cells. The relative medium fluorescences (RMF) are given for each histogram. Control, no treatment; glutamate, 5 mM; baicalein, 10 μM. Similar results were obtained in two independent experiments.

(C) Experiments were performed as in (B) except that primary cortical neurons were used. Baicalein was used at 1.5 μM.

has not been formally established that it is upstream of calcium influx in that system. This model predicts that the activation of 12-LOX by other means will also contribute to neuronal cell death.

The cascade linking GSH depletion, 12-LOX activation, and neuronal cell death has implications in neuropathological conditions such as PD. GSH plays a central role in the GSH peroxidase–mediated breakdown of H_2O_2 and organic peroxides, thus protecting cells from oxidative damage (Meister and Anderson, 1983). In PD, a marked nigral GSH depletion occurs primarily in non-neuronal cells, yet the majority of the cell death occurs in neurons. A role for GSH depletion in regulating 12-LOX activity as shown in this study may help to resolve the above discrepancy, since 12-LOX is also abundant in neuroglial cells (Bendani et al., 1995). Eicosanoids such as 12-HETE are membrane permeable (but GSH is not) and can act as extracellular messengers. Therefore, GSH depletion and LOX activation in glia may generate 12-LOX metabolites, which can act on nerve cells. Neuronal cells may be more susceptible to 12-LOX metabolites than non-neuronal cells, and 12-LOX metabolites released from neuroglial cells may initiate or potentiate neuronal cell death. A potential target for 12-LOX metabolites is Ca^{2+} channels (Stern et al., 1993), and the above experiments and data to be presented elsewhere show that LOX metabolites indirectly activate Ca^{2+} influx.

In conclusion, our studies establish a role for 12-LOX in coupling neuronal cell death to GSH depletion. These findings underscore the significance of the early loss of GSH observed in PD and further strengthen the oxidative stress hypothesis for the pathogenesis of PD. An early loss of GSH in PD may initiate a series of events, including the activation of 12-LOX, leading to the production of ROS. The loss of GSH also renders cells unable to efficiently remove the ROS even though the levels of GSH peroxidase are normal. The observation of a causal role for GSH depletion in the production of ROS suggests that an early correction of intracellular GSH concentration in PD warrants consideration as a therapeutic approach to the disease.

Experimental Procedures

HT22 Cell Culture and Toxicity Studies

The HT22 nerve cell line is a subclone of HT4 (Morimoto and Koshland, 1989), which was derived from the mouse hippocampus. The HT22 clone was selected for its sensitivity to glutamate toxicity. The cells do not possess active ionotropic glutamate receptors and are not subject to excitotoxicity (Maher and Davis, 1996). HT22 cells were propagated in Dulbecco's Modified Eagle Medium (DMEM; Vogt and Dulbecco, 1963) supplemented with 10% fetal bovine serum (FBS). Cell survival was determined by the MTT assay as described (Schubert and Behl, 1993), which in this cell system correlates with cell death as determined by trypan blue exclusion and a

colony-forming assay (Davis and Maher, 1994). Briefly, HT22 cells are dissociated with pancreatin (GIBCO/BRL, Bethesda, MD) and seeded onto 96-well microtiter plates in 5% dialyzed FBS at a density of 2.5×10^3 cells per well in 100 μl medium. The next day, cells are treated with various reagents according to the experimental design and 5 mM glutamate. Twenty hours after the addition of glutamate, 10 μl of the MTT solution (5 mg/ml) is added to each well and incubated for 4 hr. Solubilization solution (100 μl: 50% dimethylfomamide, 20% SDS [pH 4.8]) is then added to the wells, and the next day, the absorption values at 570 nm are measured. The results are expressed relative to the controls specified in each experiment, and were subjected to statistical analysis (Student's *t*-test).

Primary Cortical Culture and Toxicity Studies

Primary cortical neurons were prepared from embryonic day 17 Sprague-Dawley rats as described (Abe et al., 1990). Cells were dissociated from the cortex and maintained in minimal essential medium supplemented with 30 mM glucose, 2 mM glutamine, 1 mM pyruvate, and 10% fetal calf serum. For toxicity studies, cells were plated on polylysine-coated 96-well microtiter dishes at 50,000 cells/100 μl in each well and subjected to the described treatments 24 hr after the initial plating. The effect of various reagents on glutamate toxicity was determined by the MTT assay and also assessed visually through cell counting. Results are expressed relative to the controls treated with reagents alone.

LOX Assays

LOX activity was measured in cell lysates by the method of Natarajan et al. (1993). Briefly, cell lysates were prepared in 25 mM Tris–HCl (pH 7.7). The reaction was initiated by the addition of 10 μM arachidonic acid together with 2 μCi/ml [^{3}H] arachidonate (New England Nuclear, Boston). After 15 min at 37°C, the reaction mix was extracted and analyzed for 12 [^{3}H]-HETE by reverse phase high pressure liquid chromatography (Shornick and Holtzman, 1993). The uptake of arachidonic acid in whole cells was determined by incubating 1.5×10^5 cells/60 mm tissue culture dish with 10 μM arachidonate plus 5 μCi/ml [^{3}H] arachidonate for 1 hr at various times after adding glutamate. The culture medium and cells were then collected and assayed for radioactivity.

GSH Measurement

Cellular GSH content was determined by the method of Tietze (1969). HT22 cells (5×10^5) or 8×10^6 primary cortical neurons were collected in 125 μl phosphate-buffered saline, and 62 μl 10% sulfosalicylic acid was added. After 15 min on ice, the solution was centrifuged, and the supernatant was added to 25 μl 50% triethanolamine. The GSH content was assayed in a 96-well microtiter plate as follows: 50 μl samples or standards were placed in each well and then 50 μl 1.5 mM 5, 5′-dithiobis-2-nitrobenzoic acid and 50 μl solution containing 0.6 mg/ml NADPH and 8.3 U/ml GSH reductase were sequentially added. Reaction kinetics were determined by repeatedly measuring OD_{405} of the samples at an interval of 20 s. The GSH content was determined by comparison to a standard, and the protein content of the pellet was measured by a commercial kit (Pierce, Rockford, IL).

Subcellular Fractionation and Western Blot Analysis of 12-LOX

Cells were collected either directly in 1× Laemmli buffer (Laemmli, 1970) or in lysis buffer followed by subcellular fractionation by the method of Hagmann et al. (1993). Briefly, cells were sonicated and then centrifuged at 10,000 g for 10 min. The resulting supernatant was then recentrifuged at 100,000 g for 1 hr. The 100,000 membrane fraction (pellet) was finally dissolved in 1× Laemmli buffer.

For Western blot analysis, equal amounts of cell lysates or membrane fractions were resolved in 10% polyacrylamide gel containing sodium dodecyl sulfate and electrophoretically transferred to polyvinylidene difluoride hybridization membrane (Micron Separations Inc., Westboro, MA). The membrane was first probed with a rabbit antiserum against human platelet 12-LOX (Cayman Chemical, Ann Arbor, MI), which cross-reacts with several isoforms of 12-LOX, at a dilution of 1:2000 and then with horseradish peroxidase–conjugated goat anti-rabbit immunoglobulin G secondary antibody at a dilution of 1:20,000. The antibody conjugates were detected using a chemiluminescence Western blot kit (Amersham, Buckinghamshire, England).

Ca^{2+} Measurement

The intracellular ionized calcium concentration was determined using Indo-1 acetoxymethylester and ratiometric analysis through flow cytometry. HT22 cells were treated for a specific time and loaded with 1 μM Indo-1 in the presence of 0.005% Pluronic F-127 by adding the dye to the loading medium (DMEM plus 10% fetal calf serum). After an incubation of 30 min at 37°C, cells were then collected, washed, and resuspended in room temperature phenol red-free HEPES buffered DMEM supplemented with 2% dialyzed FBS and 7 μM propidium iodide to determine cell viability. Cells were allowed a 15 min recovery period to hydrolyze the ester bond before being analyzed with a FACStarplus flow cytometer (Becton Dickinson). The fluorescence from the two emission peaks of Indo-1, 410 nm (FL32) and 485 nm (FL4), was collected, and the ratio of the FL32 to FL4 was obtained. Ca^{2+} concentration is proportional to the ratio of FL32 to FL4 (Grynkiewicz et al., 1985), which was presented at arbitrary units. Each assay was based on the analysis of 10,000 viable cells.

Peroxide Measurement

Intracellular peroxide levels were measured by the flow cytometric method (Royall and Ischiropoulos, 1993). Briefly, HT22 cells were seeded at a density of 0.5×10^6 and primary neurons at 5×10^6 cells/100 mm dish and, 12 hr later, were either mock treated or treated with 5 mM glutamate and other reagents. After another 12 hr, cells were dissociated from plates by incubating in 5 ml pancreatic solution. 2′,7′-dichlorofluorescin diacetate (10 μM) was added during cell dissociation for 10 min. Cells were then collected, washed, and resuspended in room temperature phenol red-free HEPES buffered DMEM supplemented with 2% dialyzed FBS. We found that washing cells using warm medium instead of cold medium is extremely critical to the reduction of the background. This washing step explains the much higher increase in peroxides than reported previously (Maher and Davis, 1996). Viable cells (10,000) were subjected to FACScan (Becton Dickinson) with excitation set at 488 nm and emission at 525 nm.

Reagents

Tissue culture reagents were purchased from GIBCO (Grand Island, NY), and the minimal essential medium used for cortical neurons was from Sigma (St. Louis). The fluorescence dyes 2′,7′-dichlorofluorescin diacetate and indo-1 acetoxymethylester (indo-1) were from Molecular Probes (Eugene, OR). 5, 8, 11, 14-ETYA was from LC Laboratories (Woburn, MA). AA861, baicalein, CDC, and DAA were from Biomol (Plymouth, PA). Arachidonic acid, DL-BSO, caffeic acid, GSH, indomethcain, metyrapone, MTT, and NDGA were from Sigma.

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