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Antioxidants and Herbal Extracts Protect HT-4 Neuronal Cells against Glutamate-Induced Cytotoxicity

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Antioxidant therapy has been shown to be beneficial in neurological disorders including Alzheimer's disease and cerebral ischemia. Glutamate-induced cytotoxicity in HT-4 neuronal cells has been previously demonstrated to be due to oxidative stress caused by depletion of cellular glutathione (GSH). The present study demonstrates that a wide variety of antioxidants inhibit glutamate-induced cytotoxicity in HT-4 neuronal cells. Low concentrations of α-tocopherol and its analogs were highly effective in protecting neuronal cells against cytotoxicity. Purified flavonoids and herbal extracts of *Gingko biloba* (EGb 761) and French maritime pine bark (Pycnogenol®) were also effective. We have previously shown that pro-glutathione agents can spare GSH and protect cells from glutamate insult in a C6 glial cell model. The protective effects of nonthiol-based antioxidants tested in the HT-4 line were not mediated via GSH level modulation. In contrast, protective effects of thiol-based pro-glutathione agents α-lipoic acid (LA) and N-acetyl cysteine (NAC) corresponded with a sparing effect on GSH levels in glutamate-treated HT-4 cells. Glutamate-induced cytotoxicity in HT-4 cells is a useful model system for testing compounds or mixtures for antioxidant activity.

Keywords: Oxidative stress, neurodegeneration, antioxidant, *Ginkgo biloba*, Pycnogenol®, glutathione

INTRODUCTION

Oxidative stress has been implicated in numerous neurological disorders, including Parkinson's and Alzheimer's disease and cerebral ischemia.[1] Although the exact mechanisms of oxidative damage are not completely characterized, the evidence suggests that antioxidant treatment may have therapeutic potential in neurodegenerative disorders. Cerebral ischemia studies have demonstrated that antioxidants such as α-lipoic acid (LA)[2] and superoxide dismutase[3] provide protection against brain injury. Supplementation with vitamin E[4] or *Ginkgo biloba* extract (EGb 761)[5] has been shown to delay the progression of Alzheimer's disease. The free radical spin trap, phenyl-N-*t*-butylnitrone (PBN) has been shown

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to delay the age-related accumulation of oxidized protein in gerbil brains.[6] Taken together, the evidence suggests that antioxidant treatment may have therapeutic potential in neurodegenerative disorders.

In vitro studies with cultured neurons have demonstrated that antioxidants provide protection against various cytotoxic agents, such as glutamate. Although glutamate is a neurotransmitter, elevated levels are associated with several neurological disorders, and can lead to rapid cell death.[1] In neurons expressing glutamate receptors (NMDA, kainate, and AMPA receptors), glutamate treatment leads to a hyperstimulation of the glutamate receptors, and cells die by "excitotoxicity".[7] Treatment of neurons with antioxidants such as vitamin E[8] or LA[9] has been shown to decrease excitotoxic death. In neuronal cells lacking glutamate receptors (immature cortical neurons, HT-4, C6, PC12, HT-22, and oligodendroglia), glutamate treatment has been shown to inhibit cystine transport, causing glutathione (GSH) depletion and ultimately cell death.[10–14] Since GSH is a major cellular antioxidant, its depletion can leave cells more vulnerable to reactive oxygen species. Exogenously administered antioxidants such as LA[15] and vitamin E[10] completely prevent this nonreceptor-mediated glutamate-induced cytotoxicity.

The efficacy of antioxidants such as LA and vitamin E in completely inhibiting nonreceptor-mediated glutamate-induced cytotoxicity may be primarily because oxidative stress, possibly lipid peroxidation, is a central event leading to cytotoxicity. Due to depletion of cellular GSH by glutamate, neuronal cells become more vulnerable to oxidative damage, leading to cell death. Previously, we demonstrated that thiol-based antioxidants, such as LA, protect C6 glial cells from glutamate-induced cytotoxicity by modulating cellular GSH levels.[15] The protective effect of vitamin E, on the other hand, may be mediated through its ability to inhibit lipid peroxidation. In the present study, the ability of a wide variety of antioxidants, such as tocopherol and its analogs, flavonoids (quercetin and rutin), thiol-based antioxidants, and herbal extracts (EGb 761 and Pycnogenol®) to protect neuronal cells against glutamate-induced cytotoxicity was evaluated.

MATERIALS AND METHODS

Reagents

α-Tocopherol, the flavonoids quercetin and rutin, butylated hydroxytoluene (BHT), GSH, N-acetyl cysteine (NAC), bovine serum albumin (BSA), lactate dehydrogenase (LDH) activity assay kit, and desferrioxamine mesylate were purchased from Sigma Chemical Co. (St. Louis, MO). *Ginkgo biloba* extract (EGb 761) was obtained as a gift from the IPSEN Institute (Paris, France) and French maritime pine bark extract (Pycnogenol®) obtained as a gift from Horphag Research (Geneva). LA was provided by ASTA Medica (Frankfurt, Germany). Trolox was purchased from Aldrich (Milwauke, WI). Dulbecco's Modified Eagle's Medium (DMEM), trypsin–EDTA and Dulbecco's Phosphate Buffer Saline without calcium or magnesium, pH 7.4 (PBS) were obtained from Life Technologies (Gaithersburg, MD). Fetal bovine serum (FBS) was obtained from Gemini (Calabasas, CA). Sodium pyruvate and penicillin–streptomycin were obtained from UCSF Cell Culture Facility (San Francisco, CA). HPLC-grade solvents were purchased from Fisher Scientific (Fair Lawn, NJ). L-glutamate (sodium salt) was purchased from Sigma, and glutamate (HCl) was purchased from RBI (Natick, MA). Both forms of glutamate exhibited similar effects in all experiments.

Cells and Cell Culture

HT-4 cells (rat hippocampal cell line) were obtained as a gift from Dr. Daniel Koshland, University of California, Berkeley, CA, USA. In

previous work where glutamate-induced cytotoxicity experiments were conducted with C6 cells, it was observed that C6 cell death was greatly influenced by the type of serum used.[15] HT-4 cells were found to be less affected by serum type and thus more favorable for glutamate-induced cytotoxicity studies. Maher and Davis have further subcloned HT-4 cells (HT-22), which have also been widely used in glutamate-induced cytotoxicity studies.[13] HT-4 cells were routinely grown in DMEM supplemented with 10% FBS and 100 units/ml penicillin–streptomycin at 37°C in a humidified atmosphere containing 95% air and 5% CO_2.

All experiments were performed in triplicate using the following protocol: cells at 75–90% confluency were trypsinized, and subcultivated in culture plates (80,000 cells/ml). After 24 h, the medium was changed and cells were treated with glutamate and other agents simultaneously. Substances were added and dissolved in the appropriate vehicle; flavonoids and herbal extracts in DMSO, trolox, ascorbate, BHT, NAC and lipoate in PBS and α-tocopherol in ethanol. The cells were continually cultured at 37°C in a humidified air containing 5% CO_2 until the conclusion of experiments.

Cell Viability

Cell viability was assayed by measuring LDH leakage as previously described by Murphy *et al.*[10] After the completion of the experiments, culture medium was removed from plates and centrifuged (500*g*, 5 min). The detached cells were separated from medium, and 0.5 ml of the medium was mixed with 0.5 ml of BSA (5% w/v in PBS) for storage. Detached cells were washed once with PBS, centrifuged, and mixed with 0.5 ml Triton X-100 (0.5% v/v in PBS) and 0.5 ml of BSA (5% w/v in PBS). Attached cells were washed with PBS and lysed with 0.5 ml 0.5% Triton X-100 and mixed with 0.5 ml of BSA (5% w/v in PBS) for storage. LDH activity was measured spectrophotometrically[16] using an LDH activity assay kit within one day of harvesting. Viability was determined by the following formula: viability = LDH activity of attached cells/Total LDH activity (medium LDH activity + detached cell LDH activity + attached cell LDH activity). Detached cell LDH activity was found to be negligible in all cases. Values shown in figures are mean ± standard deviation.

HPLC Determination of GSH

At the end of experiments, HT-4 cells were washed with ice cold PBS, treated with 2% monochloroacetic acid (w/v in water) or 5% *m*-phosphoric acid (w/v in water) and scraped. All the samples were immediately frozen in liquid nitrogen and stored at −80°C until HPLC analysis. Immediately before measurement, samples were thawed, vortexed, then centrifuged at 16,000*g* for 3 min at +4°C. The clear supernatant was removed and injected into the HPLC system. GSH measurements were performed with an HPLC system using electrochemical detection with either a BAS (West Lafayette, IN) amperometric detector (2% monochloroacetic acid extract)[17] or ESA (Chelmsford, MA) coulometric detector (5% *m*-phosphoric acid extract).[18] An Alltech (Deerfield, IL) Altima C-18 (150 mm × 4.6 mm, 5 µm particle size) column was used for GSH separation in both methods. GSH values were expressed as nanomoles per milligram of protein. Precipitated protein was resolubilized in 0.2 N NaOH, and protein values were determined using the Biorad DC protein assay kit (Hercules, CA).

Malondialdehyde (MDA) Determination

Malondialdehyde (MDA) levels were measured by a fluorescent HPLC method. The use of HPLC allows for the specific detection of an MDA–TBA complex, and thus avoids nonspecific results that can be obtained by standard TBA tests.[19] MDA measurements were performed based on the method described previously by Wong *et al.*[20]

After treatment, culture medium was removed from cells and discarded. The cells were scraped in a 1% (w/v) BHT solution in methanol and PBS (1 : 1, v/v). TBA (50 mM) and phosphoric acid (2 mM) were added and the samples were heated at 90°C for 1 h. The samples were then neutralized (1 : 1, v/v) with NaOH dissolved in a mixture of water and methanol (1 : 10, v/v) and centrifuged at 16,000*g* for 3 min. The supernatant was injected into the HPLC system. An Alltech (Deerfield, IL) Altima C-18 (150 mm × 4.6 mm, 5 µm particle size) column was used for sample separation using a mobile phase consisting of 50 mM sodium phosphate buffer (pH 6.8) and ethanol (60 : 40, v/v). An excitation wavelength of 525 nm was used, and emission recorded at 550 nm.

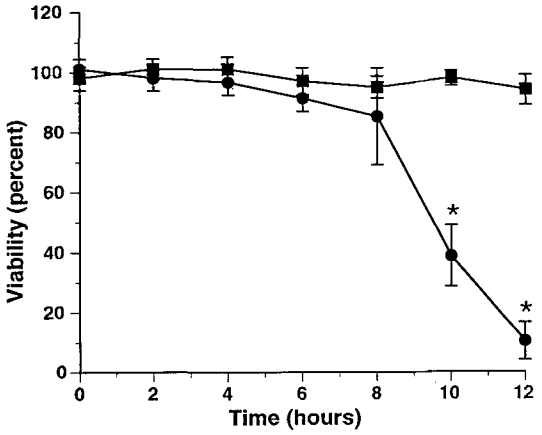

FIGURE 1 Glutamate-induced cytotoxicity is characterized by loss of cell viability. HT-4 cells were treated with (●) or without (■) glutamate (10 mM). *$P < 0.05$ compared with the control cells not treated with glutamate.

Data Presentation

Results are expressed as mean ± SD of at least three independent experiments. Differences were determined by Student's *t*-test and analysis of variance with $P < 0.05$ as the minimum level of significance.

RESULTS

Glutamate-Induced Cytotoxicity in HT-4 Neuronal Cells

Treatment of HT-4 cells with 10 mM glutamate resulted in cell death within 12 h (Figure 1). At 8 h, the time before most cell death occurs, GSH levels were observed to be decreased to less than 20% of that of control (Figure 7) and MDA levels, a measure of lipid peroxidation, increased two-fold (data not shown). These results demonstrate that oxidative stress and GSH depletion preceded cell death.

Tocopherol Derivatives Protect HT-4 Cells from Glutamate-Induced Cytotoxicity

Treatment of HT-4 cells with various tocopherol analogs completely protected cells against glutamate-induced cytotoxicity. At 5 µM concentrations, α-tocopherol, β-tocopherol and pentamethylchromane (PMC) completely protected HT-4 cells against glutamate-induced cytotoxicity, whereas a concentration of 50 µM was required for protection by trolox (Figure 2). A dose comparison between the lipophilic tocopherols and their water-soluble counterpart trolox demonstrates that the lipophilic analogs were effective at much lower doses compared to the water-soluble analog trolox (Figure 2). At a concentration of 1 µM, α-tocopherol protected cells almost completely, whereas trolox provided no significant protective effects.

Flavonoids Protect against Glutamate-Induced Cytotoxicity

Flavonoids are important phytonutrients with strong antioxidant activity. Figure 3 demonstrates that the flavonoids tested, rutin and quercetin, protected HT-4 cells from glutamate-induced cytotoxicity. Protection by flavonoids was achieved at concentrations higher than those needed for protection conferred by tocopherol and its analogs. Rutin provided partial protection from

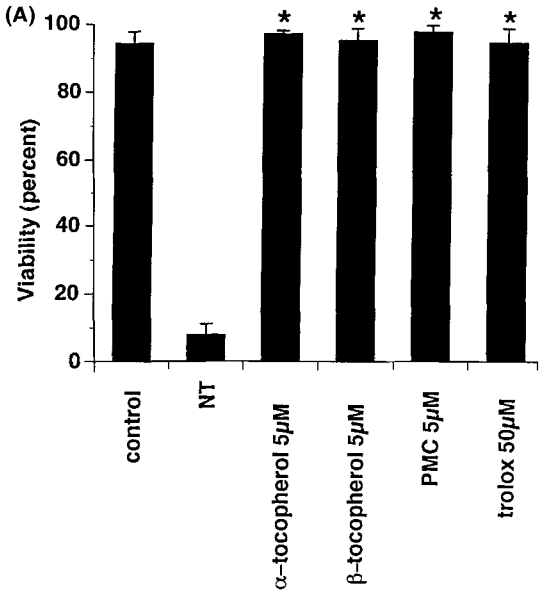

FIGURE 2 α-Tocopherol and tocopherol analogs protect HT-4 cells against glutamate-induced cytotoxicity. HT-4 cells were treated with glutamate (10 mM) with or without antioxidants for 12 h. (A) Minimum doses for tocopherol analogs that conferred complete protection. Control, no glutamate; NT, no antioxidant treatment. (B) Dose comparison of lipophilic α-tocopherol and the hydrophilic analog trolox. Control, no glutamate; NT, no antioxidant treatment; solid bars, trolox; open bars, α-tocopherol. *$P < 0.05$ compared with glutamate-treated cells.

cytotoxicity, and at a concentration of 100 μM, 80% of cells (compared to control cells not treated with glutamate) remained viable. Quercetin and flavone protected cells completely at a concentration of 100 μM (Figure 3).

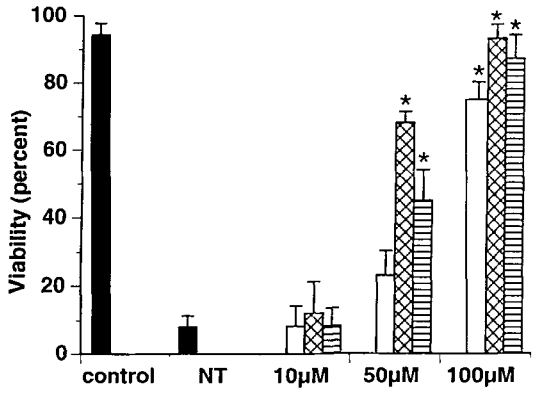

FIGURE 3 Flavonoids protect HT-4 neuronal cells against glutamate-induced cytotoxicity: Conditions were the same as in Figure 2. Control, no glutamate; NT, no antioxidant treatment; open bars, rutin; cross-hatched bars, quercetin; horizontal stripe bars, flavone. Values are shown as mean ± SD. *$P < 0.05$ compared with glutamate-treated cells.

Herbal Extracts Protect against Glutamate-Induced Cytotoxicity

EGb 761 and Pycnogenol® are two common herbal extracts used as nutritional supplements as well as for medical purposes. Since both extracts are known to have antioxidant capacity, their ability to protect glutamate-challenged HT-4 cells was of interest. Both EGb 761 and Pycnogenol® protected cells against glutamate-induced cytotoxicity in a dose-dependent manner (Figure 4). At a concentration of 100 μg/ml, Pycnogenol® provided 91% protection, while EGb 761 provided 83% protection at this concentration, in comparison to control cells.

Protective Actions of Other Free Radical Scavengers

In addition to tocopherol derivatives and bioflavonoids, other common antioxidants were tested for their protective effects against glutamate-induced cytotoxicity in HT-4 cells. Ascorbic acid only provided partial protection to cells, with 42% of cells remaining viable using 200 μM ascorbate. BHT and the iron chelator desferrioxamine mesylate were highly effective in protecting HT-4 cells,

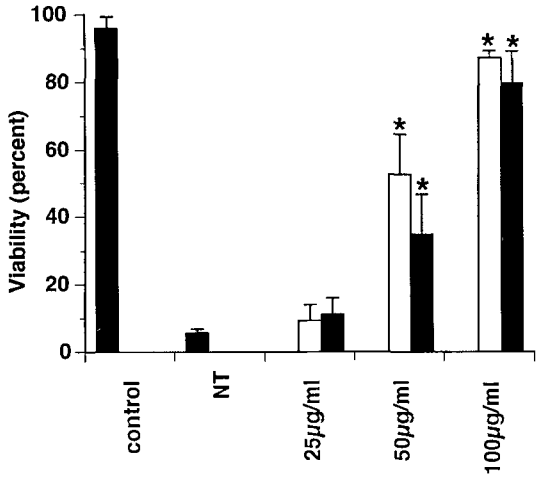

FIGURE 4 Herbal extracts protect against glutamate-induced cytotoxicity. Conditions were the same as in Figure 2. Control, no glutamate; NT, no antioxidant treatment; open bars, Pycnogenol®; solid bars, EGb 761. Values are shown as mean ± SD. $^*P < 0.05$ compared with glutamate-treated cells.

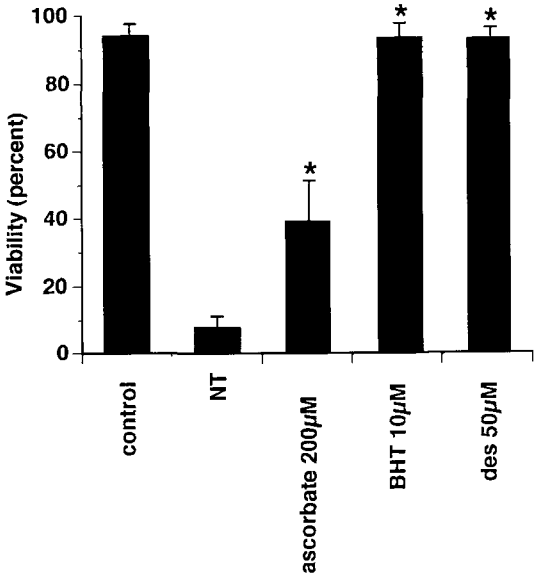

FIGURE 5 Protective effects of butylated hydroxytoluene and desferrioxamine. Conditions were same as in Figure 2. Control, no glutamate; NT, no antioxidant treatment; des, desferrioxamine mesylate; BHT, butylated hydroxytoluene. Values are shown as mean ± SD. $^*P < 0.05$ compared with glutamate-treated cells.

with 10 μM BHT and 50 μM desferrioxamine mesylate conferring complete protection against the glutamate challenge (Figure 5).

Thiol-Based Antioxidants Protect HT-4 Cells against Glutamate-Induced Cytotoxicity

Previously, we demonstrated that thiol-based antioxidants (LA and NAC) protect C6 glial cells from glutamate-induced cytotoxicity. In glutamate-treated HT-4 neuronal cells, we observed a similar cytoprotective effect at concentrations of 100 μM for both LA and NAC (Figure 6). Because the reduced form of α-lipoic acid, dihydrolipoic acid (DHLA), is thought to be involved in protection against glutamate, the effects of a 12 h pretreatment with LA in glutamate-challenged HT-4 cells was tested. Pretreatment with 50 μM LA provided full protection against glutamate-induced cytotoxicity, while pretreatment with 25 μM LA provided protective effects similar to 100 μM LA added simultaneously with glutamate.

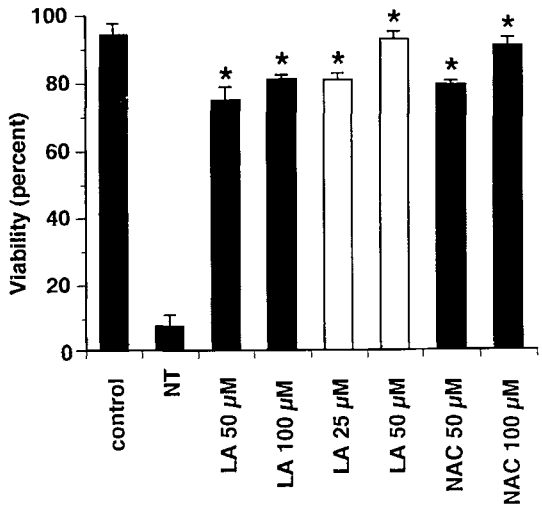

FIGURE 6 Thiol-based antioxidants protect HT-4 cells against glutamate-induced cytotoxicity. LA-pretreated HT-4 cells were treated with LA for 12 h prior to glutamate addition. HT-4 cells were treated with glutamate (10 mM) with or without antioxidants for 12 h. Control, no glutamate; NT, no antioxidant treatment; open bars, 12 h pretreatment with LA prior to glutamate addition. Values are shown as mean ± SD. $^*P < 0.05$ compared with glutamate-treated cells.

Nonthiol-Based Antioxidant Protection is not Mediated by Modulation of Cellular Glutathione

It has been previously demonstrated that thiol-based antioxidants protect cells from glutamate-induced cytotoxicity and prevent the loss of cellular GSH levels. To determine if the antioxidants tested in this investigation also modulated GSH levels, HPLC measurements were performed (Figure 7). At concentrations sufficient to provide protective effects, antioxidants were ineffective in maintaining cellular GSH levels in glutamate-challenged HT-4 cells.

Thiol-Based Antioxidants Enhance Cellular GSH Levels

The thiol-based antioxidants LA and NAC have been previously shown to modulate cellular GSH levels in the C6 glial cell line. To determine if this phenomenon occurs in HT-4 cells, HPLC measurements were performed. LA and NAC added simultaneously with glutamate significantly enhanced cellular GSH levels compared to glutamate-only-treated cells. Pretreatment with 50 μM LA significantly enhanced cellular GSH levels, restoring GSH to levels similar to the 100 μM LA simultaneous treatment, and 25 μM LA pretreatment tended to increase cellular GSH, although not significantly (Figure 7).

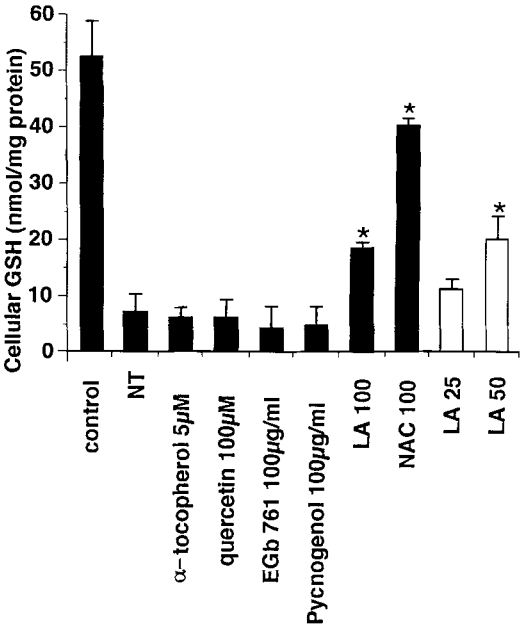

FIGURE 7 Antioxidant protection modulation of cellular GSH levels. HT-4 cells were treated with glutamate (10 mM) with or without antioxidants for 8 h. Control, no glutamate; NT, no antioxidant treatment; open bars, 12 h pretreatment with LA prior to glutamate addition. Values are shown as mean ± SD. $^*P < 0.05$ compared with glutamate-treated cells.

DISCUSSION

There is mounting evidence that free radicals and oxidative damage are involved in a number of neurodegenerative diseases, and that antioxidant therapy is beneficial.[1–6,21] Cell culture models of neurodegeneration have demonstrated that antioxidant administration protects neuronal cells from glutamate challenge, hypoxia, and various other toxins.[1] Glutamate-induced cytotoxicity has been used as a model system to study neurodegeneration due to oxidative stress.[10] Cellular GSH depletion, coupled with oxidative stress, is possibly involved in neurodegeneration of dopaminergic neurons in patients suffering from Parkinson's disease.[27] In this system, cell death is a result of oxidative stress coupled with cellular GSH depletion due to a glutamate-mediated inhibition of cystine transport. Antioxidants such as lipoic acid[15] and vitamin E[10] have been proven to inhibit glutamate-induced cytotoxicity in various cell lines. In the present work we demonstrate that many compounds that have antioxidant activity protect HT-4 cells from glutamate-induced cytotoxicity. α-Tocopherol, its analogues, and flavonoids were all effective in protecting cells from the glutamate challenge.

While a wide range of antioxidants could protect cells from glutamate-induced cytotoxicity, the doses required to protect HT-4 cells against glutamate insult varied for each antioxidant. In this model system concentrations of antioxidants

used may not be optimal or be directly extrapolated to the *in vivo* situation. Lipophilic antioxidants were more effective than the water-soluble antioxidants, consistent with our previous preliminary observations.[22] At low concentrations (5 μM) α-tocopherol had a protective effect while at similar concentrations trolox did not protect cells against glutamate challenge.

The greater protective effects of lipid-soluble antioxidants compared to that of the water-soluble counterparts are probably due mainly to two factors: (i) a greater ability of lipid-soluble antioxidants to permeate membranes, leading to increased uptake by cells, and (ii) the potential of a greater ability to scavenge lipid radicals. MDA levels were observed to be elevated in glutamate-challenged cells, and lipid peroxidation may be crucial in mediating cell death.[23] These two factors are likely to be the most critical determinants for effective concentrations of antioxidants required to protect cells against glutamate-induced cytotoxicity in HT-4 cells.

Herbal extracts such as EGb 761 and Pycnogenol® are rich in polyphenols and have been shown to have potent antioxidant activity.[24,25] EGb 761 has been shown to protect brain neurons from ischemia, and the protective action of this extract has been attributed to its high flavonoid content.[26] Both EGb 761 and Pycnogenol® protected HT-4 cells from glutamate-induced cytotoxicity. Although alterations in signal transduction processes such as calcium modulation[10] may be involved, the fact that both extracts can protect HT-4 cells from glutamate challenge supports the notion that these herbal extracts possess effective antioxidant capacities.

We have previously reported that the thiol-based antioxidants LA and NAC can protect C6 glial cells from glutamate-induced cytotoxicity. A similar protective effect was observed in the HT-4 neuronal cell line. LA administered to cells is known to be rapidly reduced intracellularly to DHLA and released into culture medium. Since the DHLA effluxed from cells may be critical in the protection mechanism against glutamate,[15] pretreatment of cells with LA to increase medium DHLA levels prior to the glutamate challenge was of interest. Here, we report that pretreatment of HT-4 cells with LA provided a marked protective effect using low doses (25–50 μM) of LA. Previously, using the C6 glial cell line, we were unable to achieve complete protection against cytotoxicity with doses of LA lower than 100 μM. By using the pretreatment approach in the HT-4 cell model, the efficacy of LA was markedly improved, and cytoprotective effects were observed at potentially physiologically relevant doses. Pharmaceutical studies have shown that LA given orally to rats may reach up to 70 μM in plasma,[28] although a human oral study reported lower values.[29]

Following glutamate treatment, but prior to cell death (8 h after glutamate treatment), intracellular levels of GSH were observed to be decreased to less than 20% of those of glutamate nontreated controls. The protective effects of the nonthiol antioxidants and herbal extracts tested occurred without affecting the GSH levels. Thus, the antioxidants can apparently substitute for GSH to protect cells from oxidative stress or may reduce glutamate-induced oxidative stress enough so that cells can survive despite lowered GSH levels.

In contrast, the thiol-based antioxidants LA and NAC significantly enhanced GSH levels in glutamate-challenged HT-4 cells. Pretreatment with 50 μM LA restored cellular GSH to levels similar to those of cells treated with 100 μM LA simultaneously with glutamate. Together with the enhanced cytotoxicity protection observed with low dose LA pretreatment, the GSH-sparing effect suggests that modulation of cellular GSH may be responsible for the protective efficacy of LA.

Oxidative stress and peroxides appear to be involved in this nonreceptor-mediated glutamate-induced cytotoxicity model of HT-4 cells. The fact that a wide variety of antioxidants have been found to provide protection supports the above-mentioned hypothesis. Currently, the evaluation of antioxidant activity of pure compounds

or complex mixtures is often performed *in vitro* with chemical methods such as electron spin resonance (ESR) with spin trapping or cyclic voltammetry. The present study using the HT-4 cell glutamate model suggests that it can serve as a reliable and efficient biological screening test for putative antioxidant efficacy.

Acknowledgement

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