

# Protection against glutamate-induced cytotoxicity in C6 glial cells by thiol antioxidants

DERICK HAN,<sup>1</sup> CHANDAN K. SEN,<sup>1,2</sup> SASHWATI ROY,<sup>1,2</sup>

MICHAEL S. KOBAYASHI,<sup>1</sup> HANS J. TRITSCHLER,<sup>3</sup> AND LESTER PACKER<sup>1</sup>

<sup>1</sup>Department of Molecular and Cell Biology, University of California, Berkeley,

California 94720-3200; <sup>2</sup>Department of Physiology, Faculty of Medicine, University of Kuopio,

FIN 70211, Finland; <sup>3</sup>ASTA Medica, D-60314 Frankfurt Am Main, Germany

**Han, Derick, Chandan K. Sen, Sashwati Roy, Michael S. Kobayashi, Hans J. Tritschler, and Lester Packer.**

Protection against glutamate-induced cytotoxicity in C6 glial cells by thiol antioxidants. *Am. J. Physiol.* 273 (Regulatory Integrative Comp. Physiol. 42): R1771–R1778, 1997.—In many cell lines, glutamate cytotoxicity is known to be mediated by an inhibition of cystine transport. Because glutamate and cystine share the same transporter, elevated levels of extracellular glutamate competitively inhibit cystine transport leading to depletion of intracellular glutathione. A glutathione-depleted state impairs cellular antioxidant defenses resulting in oxidative stress. It was therefore of interest to investigate whether proglutathione agents, e.g., *N*-acetylcysteine and lipoic acid, are able to protect against glutamate cytotoxicity. Both lipoic acid (100  $\mu$ M–1 mM) and *N*-acetylcysteine (100  $\mu$ M–1 mM) completely protected C6 cells from the glutamate-induced cell death. Both agents facilitate extracellular supply of cysteine, the reduced form of cystine, that is transported into the cell by a glutamate-insensitive transport mechanism. Protection by lipoic acid and *N*-acetylcysteine corresponded with a sparing effect on cellular glutathione, which is usually depleted after glutamate treatment. In the presence of L-buthionine-(*S,R*)-sulfoximine, a  $\gamma$ -glutamylcysteine synthetase inhibitor, low doses (<100  $\mu$ M) of lipoic acid and *N*-acetylcysteine did not protect cells against glutamate-induced cytotoxicity. At higher concentrations (>500  $\mu$ M), however, both lipoic acid and *N*-acetylcysteine provided partial protection against glutamate cytotoxicity even in glutathione synthesis-arrested cells. These results indicate that at low concentrations the primary mechanism of protection by the thiol antioxidants was mediated by their proglutathione property rather than direct scavenging of reactive oxygen. At higher concentrations (>500  $\mu$ M), a GSH-independent direct antioxidant effect of lipoic acid and *N*-acetylcysteine was observed. Dichlorofluorescein fluorescence, a measure of intracellular peroxides, increased sixfold after glutamate treatment of C6 cells. Lipoic acid and *N*-acetylcysteine treatment significantly lowered glutamate-induced dichlorofluorescein fluorescence compared with that of controls. Interestingly,  $\alpha$ -tocopherol (50  $\mu$ M) also suppressed glutamate-induced dichlorofluorescein fluorescence, indicating the peroxides detected by dichlorofluorescein were likely lipid hydroperoxides. Both thiol antioxidants, particularly lipoic acid, appear to have remarkable therapeutic potential in protecting against neurological injuries involving glutamate and oxidative stress.

lipoic acid; dihydrolipoic acid; *N*-acetylcysteine; glutathione; oxidative stress

icity and cystine transport inhibition-related oxidative stress. Excitotoxicity in mature neurons is mediated through *N*-methyl-D-aspartate and other glutamate receptors (9). Excess glutamate exposure to neurons leads to membrane depolarization that, if prolonged, leads to cell death. The oxidative stress pathway of glutamate cytotoxicity is the end result of simultaneous inhibition of cystine transport and enhanced generation of reactive oxygen species (29). Cysteine is the rate-limiting amino acid substrate for intracellular glutathione (GSH) synthesis. Because of its redox instability, almost all of the extracellular cysteine is present in the oxidized cystine state (11). Thus extracellular cysteine is the primary source of intracellular cysteine necessary for GSH synthesis. Glutamate and cystine share the same amino acid transporter, the  $x_c^-$  system (3), and therefore compete for transport into cells. Under conditions of elevated extracellular glutamate levels, cystine transport is inhibited, resulting in depletion of cellular GSH. Depletion of GSH, the major cellular antioxidant, results in increased vulnerability of the cell to oxidative stress (29). Peroxides generated by lipoxygenase (29) and monoamine oxidase (25) activity may contribute to oxidative stress that ultimately leads to the death of GSH-depleted cells.

The induction of oxidative stress by glutamate has been demonstrated to be the primary cytotoxic mechanism in cell lines, including C6 and PC-12 cells (14, 23), immature cortical neurons (30), and oligodendroglia cells (32). Antioxidants such as tocopherol and probucol (20) are highly effective in protecting these cells from glutamate cytotoxicity (23, 29, 31). There is growing evidence that a disruption of intracellular redox homeostasis by glutamate is a major contributing mechanism of cellular damage in vivo. Cerebral ischemia has been associated with an 800% increase in extracellular glutamate (5) and a decrease in brain GSH levels (44). In addition cerebral ischemia is known to be associated with increased generation of reactive oxygen species, and antioxidants such as vitamin E have been demonstrated to have protective effects (6). Elevated levels of extracellular glutamate are known to be implicated in neurodegeneration associated with Parkinson's disease. Consistently, substantia nigra of patients with Parkinson's disease have depleted glutathione levels (15). Although there is a strong association between elevated levels of extracellular glutamate and tissue GSH depletion in vivo, it is not known whether this GSH depletion is the result of glutamate inhibition of cystine uptake or the result of enhanced GSH consumption during oxidative stress. Regardless of the cause,

ELEVATED LEVELS of extracellular glutamate are responsible for neuronal damage and degeneration in brain disorders, including stroke, epilepsy, and Parkinson's disease (10). In vitro studies have characterized two major mechanisms of glutamate cytotoxicity: excitotox-

GSH depletion increases the vulnerability of the cell to damage in response to both excitotoxicity and oxidative stress components of glutamate cytotoxicity. Antioxidants, especially pro-GSH agents, may therefore be expected to be of marked therapeutic value in many neurological disorders having a glutamate-dependent pathophysiology (6).

Clinically safe thiol antioxidants such as lipoic acid (LA) and *N*-acetylcysteine (NAC) are receiving considerable attention primarily because of their efficacy in enhancing cellular GSH levels and also because of their direct reactive oxygen-scavenging properties (33, 41). LA, a disulfide, is enzymatically converted to the corresponding dithiol dihydrolipoate (DHLA) by cells (17). LA has been clinically used for the therapeutic treatment of diabetic polyneuropathies (49). NAC has been therapeutically used for the treatment of acquired immunodeficiency syndrome (AIDS; 19), which is associated with increased plasma glutamate levels and low T cell GSH levels (11). Although structurally very different, both LA and NAC treatments have been shown to overcome glutamate inhibition of cystine transport and increase cellular GSH levels in lymphocytes (16, 43). Using the C6 glial cells that are known to be susceptible to glutamate cytotoxicity by the cystine inhibition-oxidative stress pathway (23), we investigated the protective mechanisms of LA and NAC against glutamate-induced cytotoxicity.

## MATERIAL AND METHODS

**Reagents.** (*R,S*)-racemic lipoic acid and racemic dihydrolipoic acid were provided by ASTA Medica (Frankfurt, Germany). Glutathione,  $\alpha$ -tocopherol, *N*-acetylcysteine, L-glutamic acid, and L-buthionine-(*S,R*)-sulfoximine (BSO), were purchased from Sigma Chemical (St. Louis, MO). 2',7'-Dichlorodihydrofluorescein diacetate (DCFH) was purchased from Molecular Probes (Eugene, OR). Dulbecco's modified Eagle's medium (DMEM), trypsin-EDTA, and Dulbecco's phosphate-buffered saline (PBS) were obtained from Life Technologies (Gaithersburg, MD). Fetal bovine serum was obtained from Gemini (Calabasas, CA). It should be noted that a change in serum batch from the same manufacturer changed the concentration of glutamate required to induce cytotoxicity from 10 to 20 mM. Therefore, all experiments reported here were carried out with the same batch of serum. Sodium pyruvate and penicillin-streptomycin were obtained from the Cell Culture Facility at the University of California (San Francisco, CA). High-performance liquid chromatography (HPLC)-grade solvents were purchased from Fisher Scientific (Fair Lawn, NJ).

**Cell culture.** C6 glial cells (rat glial tumor cell line) were purchased from American Type Culture Collection (Rockville, MD). Three different batches of C6 cells were purchased from ATCC on three different occasions. At 10 mM, glutamate was cytotoxic to one batch (F-12612) of cells. All experiments reported here were carried out with this batch of cells. A higher (20 mM) concentration of glutamate was required to achieve comparable cytotoxicity response in another batch (F-13978) of cells that was obtained later.

C6 cells were routinely grown in DMEM supplemented with 10% serum and 100 U/ml penicillin-streptomycin at 37°C in a humidified atmosphere containing 95% air-5% CO<sub>2</sub>. All experiments were performed under the following protocol: cells grown to 85–95% confluency were trypsinized and

subcultivated in culture dishes at a concentration of  $0.25 \times 10^6$  cells per milliliter. The cells were continually cultured at 37°C in a humidified atmosphere containing 95% air-5% CO<sub>2</sub> until the conclusion of the experiments.

**Cell treatment.** In all the experiments, glutamate, LA, NAC, and BSO were dissolved in PBS for treatment of cells. All the solutions were sterilized through a 0.2- $\mu$ m Millipore (Bedford, MA) filter. Control samples were treated with the corresponding amount of PBS.  $\alpha$ -Tocopherol in ethanol (0.1% vol/vol final concn) was added directly to the cell culture medium. The corresponding control was treated with the same volume of ethanol alone. Glutamate, BSO, LA, NAC, or  $\alpha$ -tocopherol was added to the culture medium during cell seeding at final concentrations as indicated in the respective Figs. 1 and 3–8.

**Determination of cell viability.** Cell viability was assayed using lactate dehydrogenase (LDH) leakage as previously described by Murphy et al. (29). After the experiments were completed, culture medium was removed from plates and centrifuged (500  $g \times 5$  min). The cells that detached from the monolayer following glutamate treatment were separated by centrifugation of the growth medium. The supernatant medium was mixed with an equal volume of bovine serum albumin (BSA) solution (5% in PBS) to help stabilize LDH activity in the solution for storage at 4°C (12). The pelleted detached cells were washed once with PBS and treated with a lysis buffer (Triton X-100 0.5% vol/vol in PBS). The resulting lysate was mixed with an equal volume of the BSA solution for storage at 4°C. Attached cells were washed with PBS and treated with the lysis buffer and BSA solution as described above for detached cells. LDH activity was measured from the samples spectrophotometrically (40) within 2 days of storage. Cell viability was determined using the following formula: viability = attached cell LDH activity/total LDH activity (medium LDH activity + detached cell LDH activity + attached cell LDH activity) (29).

**HPLC-electrochemical detection of GSH.** At the end of the each experiment, C6 cells were washed with ice-cold PBS, treated with 2% monochloroacetic acid, and scraped. All the samples were immediately frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  until HPLC analysis. Immediately before the assay, samples were thawed, vortexed, and then centrifuged at 15,000  $g$  for 2 min. 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 (1) or ESA (Chelmsford, MA) coulometric detector (18). An Alltech (Deerfield, IL) Altima C-18 (150 mm  $\times$  4.6 mm, 5  $\mu$ m pore size) column was used for GSH separation in both methods. GSH values were expressed in milligrams of protein. Protein was determined using the Bio-Rad DC assay kit (Hercules, CA).

**Flow cytometric assay of intracellular peroxides.** Intracellular peroxides were detected using DCFH as described by Cathcart et al. (8). Following treatment and incubation with glutamate and various antioxidants, cells were washed three times with PBS. Cells were then detached from monolayer using trypsin and centrifuged. Cells were again washed with PBS and centrifuged. The cells were resuspended in PBS and incubated with DCFH (50  $\mu$ M) for 30 min at 37°C. Cells were then excited with a 488-nm ultraviolet line argon ion laser in a flow cytometer (XL, Coulter, Miami, FL), and the 530-nm emission was recorded in fluorescence channel 1 (FL1). Data were collected from 10,000 viable cells.

**Data presentation.** Results are expressed as means  $\pm$  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. Histograms illustrate typical flow cytometry data of three experiments.

**RESULTS**

*Effect of LA and NAC on C6 GSH levels.* LA treatment ( $>100 \mu\text{M}$ ) significantly ( $P < 0.01$ ) increased C6 cell GSH levels. At 1 mM concentration and 24-h treatment time, LA increased C6 cell GSH level to almost fourfold of the level found in LA nontreated control cells. NAC, on the other hand, did not influence cellular GSH levels in either the micromolar or millimolar range when administered to C6 cells for 24 h (Fig. 1).

*Time course of GSH depletion and cytotoxicity in response to glutamate treatment.* Treatment of C6 cells with 10 mM glutamate resulted in a time-dependent decrease in cell GSH (Fig. 2A) and viability (Fig. 2B). Excess glutamate was cytotoxic to C6 cells, when added to cells at or within several hours of passage of confluent cells. It has been shown (23) that glutamate has a dose-dependent effect on cell viability with maximum effect occurring at 10 mM for C6 cells. A similar cytotoxic dose response of glutamate at 10 mM was observed in this study (data not shown).

*DCF in response to glutamate treatment.* Deesterified DCFH is known to be converted to fluorescent dichlorofluorescein (DCF) following reaction with organic and inorganic hydroperoxides (8). Our flow cytometry data show that C6 cells incubated with 10 mM glutamate for 18 h had accumulated intracellular peroxides (Fig. 3). Following 18 h of exposure to elevated levels of glutamate, peroxide build-up was observed in the entire population, but a subpopulation of cells as shown by the shoulder on the right (Fig. 3B) had particularly high DCF fluorescence. Glutamate-induced accumulation of

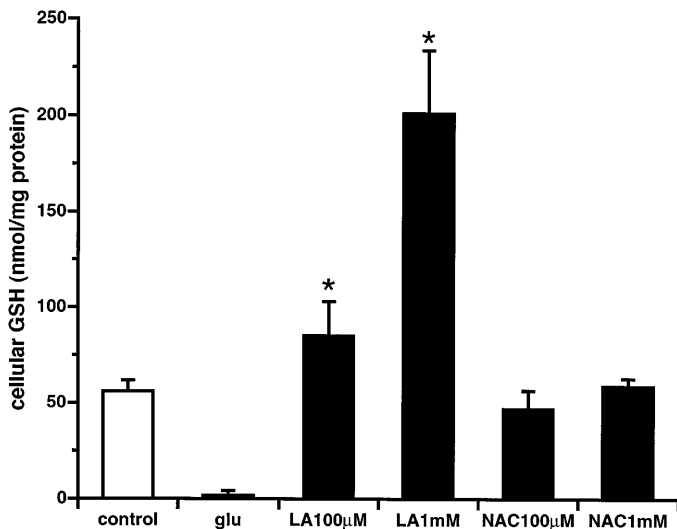


Fig. 1. Effect of lipoic acid (LA) and *N*-acetyl cysteine (NAC) treatment on cellular glutathione (GSH) levels. C6 cells were incubated with LA and NAC for 28 h. After incubation, cells were treated with 2% monochloroacetic acid and scraped, and GSH levels were analyzed by high-performance liquid chromatography (HPLC)-electrochemical detection. Glu, 10 mM glutamate. Values are means  $\pm$  SD;  $n = 3-5$  experiments. \*  $P < 0.01$  compared with nontreated control.

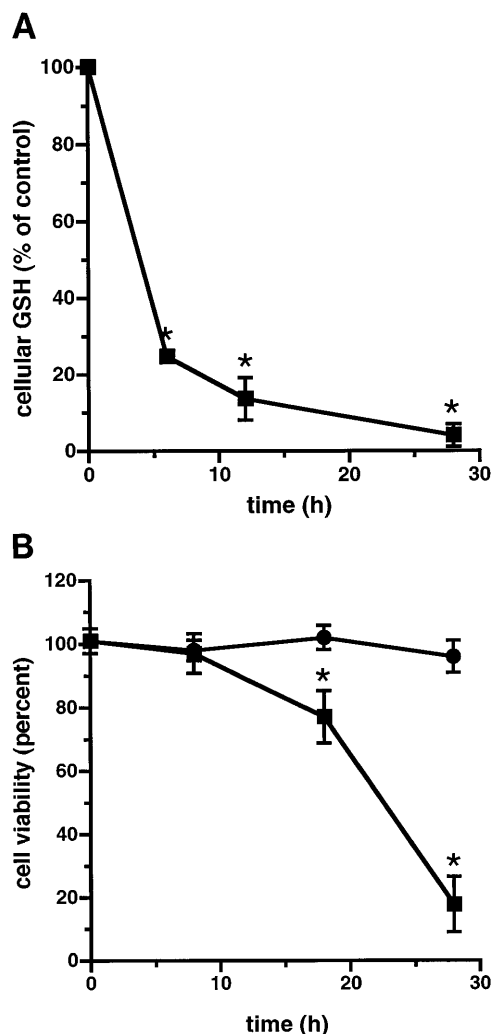


Fig. 2. Time course of cellular GSH depletion and cell cytotoxicity by Glu. C6 cells were incubated with or without 10 mM Glu for up to 28 h. GSH was measured from acidified cell extract by HPLC-electrochemical detection. Cellular GSH changes are expressed as % of nontreated control (A). Cell viability was measured by lactate dehydrogenase (LDH) release (B). Nontreated controls (●); cells treated with 10 mM Glu (■).  $n = 3$  experiments. \*  $P < 0.05$  compared with 0-h value (A) or Glu-nontreated control (B).

intracellular peroxides was markedly increased in BSO-treated GSH deficient cells (Fig. 3, B vs. C).

*Protection of C6 cells against glutamate cytotoxicity by thiol antioxidants.* Treatment of cells with either LA or NAC significantly protected cells from glutamate-induced cytotoxicity in a dose-dependent manner with a maximum effect being reached around 100  $\mu\text{M}$  for both thiol antioxidants (Fig. 4, A and B). Similarly, protection against glutamate cytotoxicity was also evident in cells treated with  $\alpha$ -tocopherol (50  $\mu\text{M}$ ). Both LA and NAC treatment of cells prevented the marked decline of cellular GSH levels that normally occurs after glutamate treatment (Fig. 5). Even when challenged with 10 mM glutamate, 100  $\mu\text{M}$  LA-treated cells maintained 80% of the resting cellular GSH levels. NAC treatment (100  $\mu\text{M}$ ) was not as effective and could maintain only 50% of the GSH level found in glutamate-nontreated control cells. Even in the presence of

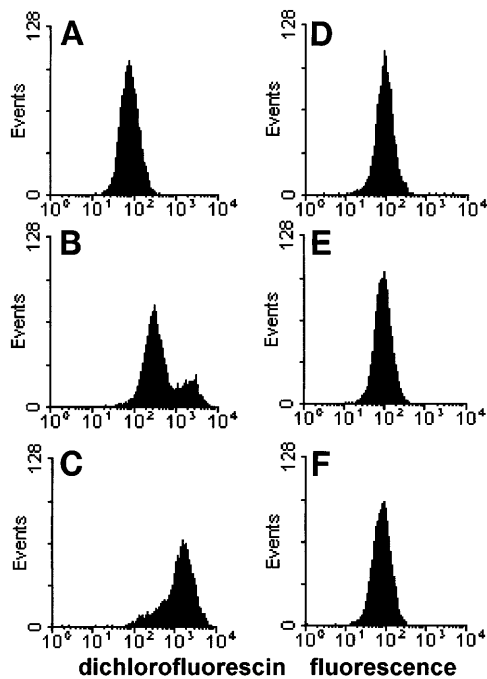


Fig. 3. Glutamate (Glu)-induced intracellular peroxide formation. Shown is dichlorofluorescein (DCF) fluorescence (in arbitrary fluorescence units) in C6 cells either not treated (A) or treated (B) with 10 mM Glu for 18 h. C: DCF fluorescence in GSH synthesis-arrested C6 cells that were exposed to 10 mM Glu. After incubation with the agents indicated, cells were trypsinized, suspended in phosphate-buffered saline (PBS), and incubated with (DCFH) (50  $\mu$ M) for 30 min at 37°C. Fluorescence was read using a Coulter XL flow cytometer. The protective effect of lipate (100  $\mu$ M; D), NAC (100  $\mu$ M; E), and vitamin E (50  $\mu$ M; F) against Glu-induced accumulation of intracellular peroxides is shown. The *ordinate* represents relative number of cellular events collected during measurement shown. Data were collected from 10,000 gated viable cells.

10 mM glutamate, 1 mM LA increased cell GSH levels to fourfold of that found in untreated controls cells.

**Thiol antioxidant protection against glutamate-induced cytotoxicity: role of cell glutathione.** In the presence of 200  $\mu$ M BSO, 100  $\mu$ M of LA or NAC failed to protect cells from glutamate cytotoxicity (Fig. 6). However, at 500  $\mu$ M NAC could completely protect cells from glutamate-induced cell death even in the presence of BSO. Higher concentration of LA (500  $\mu$ M-1 mM) protected only 50% of cells from glutamate cytotoxicity in GSH synthesis-arrested cells. BSO, by itself, had no effect on cell viability, suggesting that GSH deficiency alone was not fatal at least for a short time.

The simultaneous treatment of glutamate and BSO further depleted cell GSH levels to almost undetectable levels (Fig. 7). In GSH synthesis-arrested cells, LA treatment did not increase cell GSH. Both LA (500  $\mu$ M-1 mM) and  $\alpha$ -tocopherol (50  $\mu$ M) protected cells against glutamate-induced cytotoxicity even when cellular GSH level was 99% depleted. NAC (500  $\mu$ M-1 mM) treatment spared 10% of the cells' GSH levels even in the presence of BSO.

**Protection by thiol antioxidants against intracellular peroxide accumulation.** Following 18 h of glutamate treatment, the time point when cell viability begins to decline, we examined DCF fluorescence to get an

indication of cellular oxidant status at a time just preceding cell death. Glutamate treatment markedly increased DCF fluorescence compared with corresponding control cells (Figs. 3 and 8). The thiol antioxidants completely protected cells against glutamate treatment-dependent oxidant build-up (Fig. 3, D and E). Cells treated with 1 mM LA, glutamate, and BSO were only partly (50%) viable, and the viable cells had peroxides levels intermediate between that of control and glutamate-treated cells.  $\alpha$ -Tocopherol treatment also completely protected against peroxide build-up in glutamate-treated cells (Fig. 3, B vs. F). BSO, which depleted GSH levels, did not increase cellular DCF fluorescence, although GSH levels were markedly low. However, cells treated with both glutamate and BSO had the highest DCF fluorescence (Fig. 8 and Fig. 3C) and almost undetectable levels of GSH (Figs. 7 and 8).

## DISCUSSION

*Mechanism by which thiol antioxidants overcome glutamate inhibition of cystine transport.* The intracellular levels of GSH in most cells are determined by the

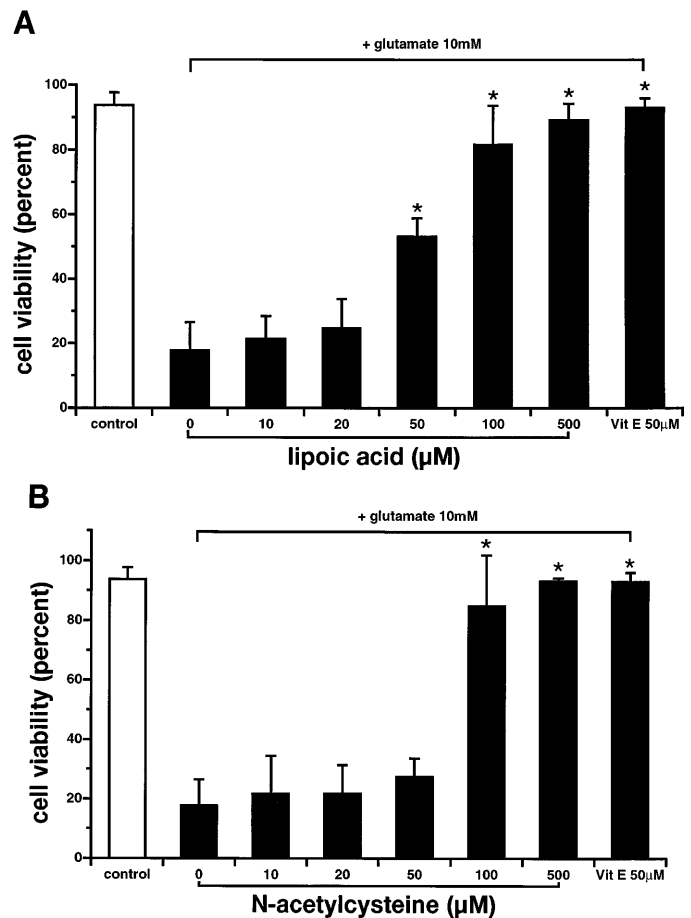


Fig. 4. Protective effects of LA and NAC treatment against Glu cytotoxicity. C6 cells were treated or not (control) with 10 mM Glu with or without antioxidants for 28 h. A: effect of LA. B: effect of NAC. Vit E, 50  $\mu$ M  $\alpha$ -tocopherol. Viability was determined using LDH analysis. Values are presented as % of total cells and show means  $\pm$  SD;  $n = 3-7$  experiments. \*  $P < 0.05$  compared with Glu treated in absence of antioxidant.

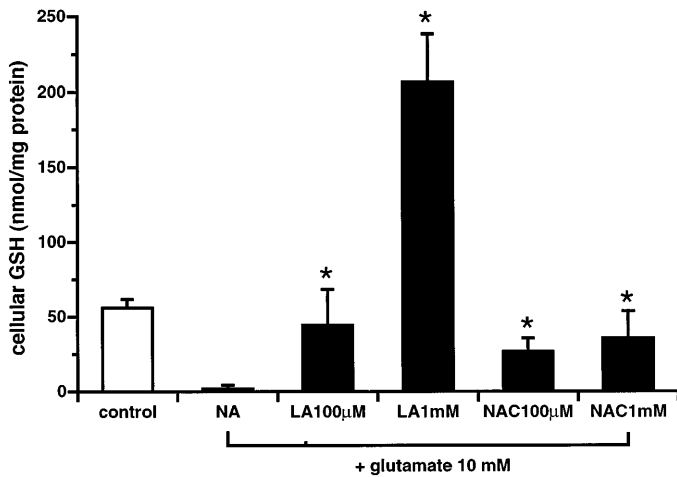


Fig. 5. Protective effect of LA and NAC on cellular GSH levels of Glu-treated cells. C6 cells were treated or not treated (control) with 10 mM Glu with or without antioxidants for 28 h. GSH levels were determined using HPLC-electrochemical detection. NA, no antioxidant treatment. Values are means  $\pm$  SD;  $n = 3-7$  experiments. \*  $P < 0.05$  compared with Glu treated in the absence of antioxidants.

$x_c^-$  system, which mediates cellular import of cystine, and the ASC system, a neutral amino acid transporter that mediates the cellular import of cysteine (4). Because of rapid autooxidation of cysteine to cystine, almost all of the cyst(e)ine present in biological systems is oxidized cystine. For example, plasma cysteine-to-cystine ratio has been estimated to be 1:10 in favor of the oxidized form (24, 39) while cell culture medium consists of 100% cystine. The nonavailability of cysteine in culture media leaves cultured cells dependent on cystine transport for GSH synthesis. Because cystine and glutamate share the same transport system, elevation of glutamate level in the plasma or extracellular fluids will impair cystine transport. This will restrict cysteine availability within the cell for GSH synthesis resulting in lower cell GSH and thus increased vulnerability of the cell to oxidative stress. Glutamate inhibition of cystine transport has been proposed to be involved in disorders such as cerebral ischemia (29) and AIDS (11).

One strategy to overcome the adverse effect of high glutamate on cell GSH metabolism is to improve the bioavailability of cysteine. Cysteine uptake by cells is not glutamate sensitive, and therefore this strategy should help normalize GSH biosynthesis even in the presence of elevated levels of extracellular glutamate. LA administered to cells is known to be rapidly reduced intracellularly to DHLA at the expense of cellular-reducing equivalents, and DHLA is released into the medium (17, 38). DHLA is a strong reducing agent with a standard reduction potential of  $-320$  mV and can chemically reduce extracellular cystine to cysteine. As a result, even in the presence of elevated levels of extracellular glutamate, cysteine supply for GSH synthesis inside the cell is restored (16, 43). NAC treatment, on the other hand, is believed to be transported into cells and deacetylated to form cysteine (45). There is also evidence that NAC may form mixed disulfides with

cysteine that are transported into cells by several amino acid transporters (22, 37). Both LA and NAC treatment can thus bypass glutamate inhibition of cystine transport by improving cysteine bioavailability within the cell and therefore have remarkable therapeutic potential (41).

*Thiol antioxidant protection against glutamate-induced cytotoxicity.* Both LA and NAC treatment enhanced cellular GSH levels and clearly protected C6 cells from glutamate-induced cytotoxicity. Treatment of 100  $\mu$ M of LA or NAC to cells could not completely restore cellular GSH levels, with LA maintaining 80%

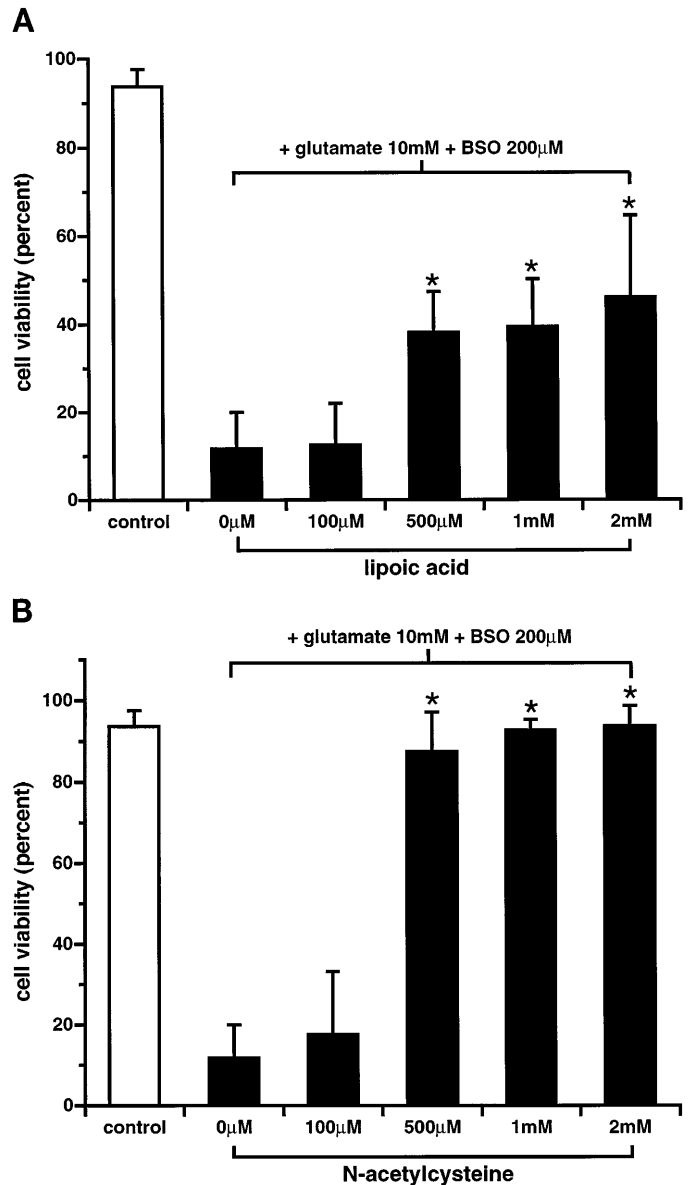


Fig. 6. Protective effects of LA and NAC against Glu cytotoxicity in the presence of L-buthionine-(S,R)-sulfoximine (BSO). C6 cells were treated with 10 mM Glu and 200  $\mu$ M BSO with or without antioxidants for 28 h. The control data are from cells that were treated with neither Glu nor BSO. Cell viability was determined by LDH analysis. Values are means  $\pm$  SD;  $n = 3-7$  experiments. \*  $P < 0.05$  compared with Glu- and BSO-treated cells in the absence of antioxidants. A: effect of LA; B: effect of NAC.

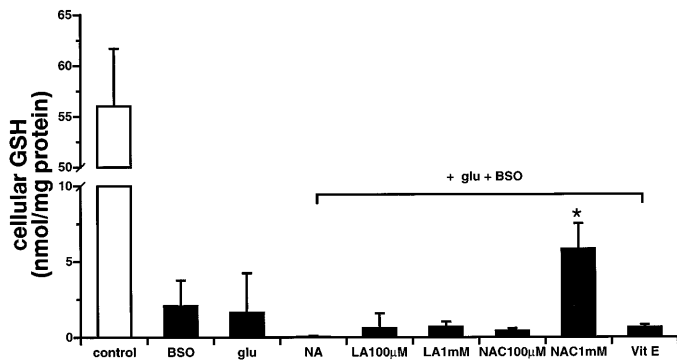


Fig. 7. Effect of LA and NAC on cellular GSH levels following Glu (10 mM) and BSO (200  $\mu$ M) treatment. C6 cells were treated or not (control) as indicated with respective agents for 28 h. GSH levels of cell extracts were determined using HPLC-electrochemical detection. Values are means  $\pm$  SD;  $n = 3-7$  experiments. \* $P < 0.01$  compared with cells treated with 10 mM Glu. Vit E, 50  $\mu$ M  $\alpha$ -tocopherol.

and NAC maintaining only 50% of the level of GSH found in glutamate nontreated control cells. Uhlig and Wendel (47) have suggested that a drop in cellular GSH to  $\sim 10\%$  of normal levels should not affect cellular function and integrity significantly. So although LA or NAC pretreatment did not restore GSH levels completely, both thiol agents helped sustain high enough levels of GSH to maintain antioxidant defense even in the presence of glutamate. The observation that a low micromolar dose (100  $\mu$ M) of LA or NAC could not protect cells from glutamate cytotoxicity in the presence of BSO indicates that at low concentrations the primary mechanism for the protection provided by LA and NAC is dependent on the ability of these thiol antioxidants to favorably influence cellular GSH metabolism. At higher doses ( $\geq 500$   $\mu$ M), both LA and NAC protected against glutamate cytotoxicity even in GSH synthesis-arrested cells, suggesting that the effects at these concentrations were independent of the pro-GSH properties of LA and NAC. Previously, transcription regulatory effects of both LA (42) and NAC (48) have been observed to be independent of the pro-GSH proper-

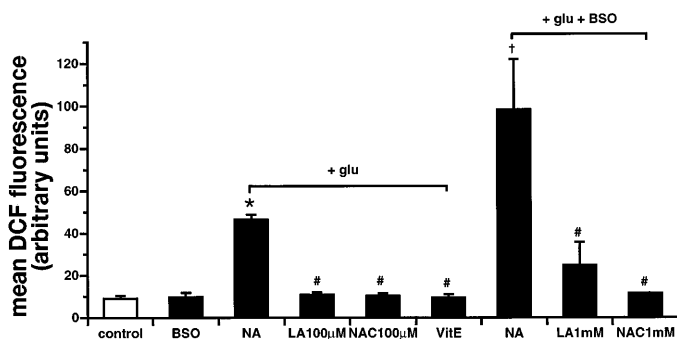


Fig. 8. Protective effect of antioxidants on Glu-induced intracellular peroxide accumulation. C6 cells were treated with compounds as indicated for 18 h. After treatment, cells were trypsinized, dissolved in PBS, and incubated with DCFH (50  $\mu$ M) for 30 min at 37°C. Fluorescence was read using a Coulter flow cytometer. Values are means  $\pm$  SD;  $n = 3-5$  experiments. \* $P < 0.01$  higher compared with Glu nontreated control (open bar); † $P < 0.01$  higher compared with Glu; # $P < 0.01$  lower compared with Glu. BSO, 200  $\mu$ M; Vit E, 50  $\mu$ M  $\alpha$ -tocopherol.

ties of these compounds. The protective effects of high concentrations of LA and NAC against glutamate cytotoxicity even in BSO-treated cells is of mechanistic interest but may have little physiological relevance because such high concentrations are unlikely to be achieved in tissue after supplementation of these agents. Pharmaceutical studies have shown that LA given orally to rats may reach up to 70  $\mu$ M in plasma (36), although a human oral study reported less (46). NAC oral studies reveal that up to 25  $\mu$ M can be seen in human plasma after oral intake (21).

**Glutamate-induced peroxide accumulation.** Several researchers have used DCF fluorescence to determine intracellular peroxide levels after glutamate treatment (29). In this work, a similar DCF increase was observed in cells treated with glutamate. The build-up of intracellular peroxides after glutamate treatment may be associated with the simultaneous GSH depletion within the cell. Under normal conditions, GSH peroxidase, using GSH as substrate, effectively decomposes both organic and inorganic peroxides in cells (13). However, severe depletion of cellular GSH stores, as observed following glutamate treatment, may impair GSH peroxidase function and result in peroxide build-up. Thus glutamate-induced peroxide accumulation inside the cell was markedly increased in GSH synthesis-arrested cells. The peroxides detected by DCF fluorescence in glutamate-treated cells were likely lipid peroxides because  $\alpha$ -tocopherol treatment was able to alleviate glutamate-enhanced DCF fluorescence. There is little evidence and possibility that a lipophilic antioxidant such as  $\alpha$ -tocopherol would scavenge high levels of aqueous hydrogen peroxide, whereas there is strong evidence that  $\alpha$ -tocopherol is highly effective in terminating lipid peroxidation reactions. Our hypothesis is supported by a recent observation (31) that glutamate treatment of PC-12 cells induced lipid peroxidation. Given the catalytic nature of lipid hydroperoxides, if GSH peroxidase cannot function to remove a small amount of peroxides, the potential generation of further lipid peroxidation is greatly increased especially in the presence of iron. The impairment of GSH peroxidase-dependent metabolism of hydroperoxides due to lack of GSH may be a key aspect of glutamate cytotoxicity. The thiol antioxidants thus counteracted and alleviated the build-up of intracellular peroxides in response to glutamate treatment by ensuring adequate substrate supply for glutathione peroxidase function. This protective effect of thiol antioxidants may be a sum of their effects in preventing glutamate-induced loss of cellular GSH and direct reactive oxygen-scavenging properties. High levels of thiol antioxidants were able to suppress glutamate-induced increase in DCF fluorescence even in BSO-treated GSH-deficient cells. This may be the result of intracellular NAC and DHLA decomposing peroxides both enzymatically (26) and nonenzymatically (2, 34).

### Perspectives

Elevated levels of glutamate have been implicated in a wide range of neurological diseases, including epi-

lepsy, cerebral ischemia, Huntington's disease, and Parkinson's disease. Receptor-mediated glutamate excitotoxicity is believed to be a major mechanism of damage in these pathologies (10). Consequently, treatment for many glutamate-associated neurological disorders has focused on glutamate receptor biology. Although the cystine inhibition-oxidative stress pathway requires 100 times more glutamate than the receptor-mediated excitotoxicity pathway of glutamate-induced cell death, GSH depletion should not be ruled out as a contributing mechanism underlying glutamate-related neurological damage. The observations that GSH is depleted in certain regions of the brain after cerebral ischemia (35) and in Parkinson's disease (15) lend support to this idea. Enhancement of tissue GSH by proglutathione drugs may therefore be expected to be of therapeutic value. However, GSH modulation in the brain is complicated by the fact that the blood-brain barrier can obstruct the passage of a number of drugs. A NAC tracer study detected radiolabeled NAC in all areas of the body except the central nervous system (27). LA, on the other hand, has not only been reported to cross the blood-brain barrier (35), but also has been shown to increase GSH in certain areas of rat brains after intravenous injection. In addition, LA treatment has been shown in two cerebral ischemia models to protect against reperfusion injury and GSH depletion in the brain (7, 35). Another intriguing aspect of LA has been reported by Muller and Kriegstein (28), who showed that pretreatment of 1  $\mu$ M LA can protect chick neurons from excitotoxicity induced by glutamate treatment. Our work and the work by Muller and Kriegstein demonstrate that LA can protect cells from both excitotoxicity and cystine-inhibition oxidative stress aspects of glutamate. The complete protection of LA against glutamate cytotoxicity observed in this study may explain the remarkable efficacy of LA in protecting rats against cerebral ischemia injury as reported before. In view of the above-mentioned properties of LA, it may be concluded that LA has remarkable therapeutic potential in protecting against neurological injuries involving glutamate and oxidative stress.

This research was supported by the National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-50430.

Address for reprint requests: C. K. Sen, 251 Life Science Addition, Dept. of Molecular and Cell Biology, Univ. of California, Berkeley, CA 94720-3200.

Received 16 May 1997; accepted in final form 14 August 1997.

## REFERENCES

- Allison, L. A., and R. E. Shoup. Dual-electrode liquid chromatography detector for thiols and disulfides. *Anal. Chem.* 55: 8–12, 1983.
- Aruoma, O. I., B. Halliwell, B. M. Hoey, and J. Butler. The antioxidant action of *N*-acetylcysteine: its reaction with hydrogen peroxide, hydroxyl radical, superoxide, and hypochlorous acid. *Free Radic. Biol. Med.* 6: 593–597, 1989.
- Bannai, S., and E. Kitamura. Transport interaction of L-cystine and L-glutamate in human diploid fibroblasts in culture. *J. Biol. Chem.* 255: 2372–2376, 1980.
- Bannai, S., and N. Tateishi. Role of membrane transport in metabolism and function of glutathione in mammals. *J. Membr. Biol.* 89: 1–8, 1986.
- Benveniste, H., J. Drejer, A. Schousboe, and N. H. Diemer. Elevation of the extracellular concentrations of glutamate and aspartate in rat hippocampus during transient cerebral ischemia monitored by intracerebral microdialysis. *J. Neurochem.* 43: 1369–1374, 1984.
- Bondy, S. C., and C. P. LeBel. The relationship between excitotoxicity and oxidative stress in the central nervous system. *Free Radic. Biol. Med.* 14: 633–642, 1993.
- Cao, X., and J. W. Phillis. The free radical scavenger,  $\alpha$ -lipoic acid, protects against cerebral ischemia-reperfusion injury in gerbils. *Free Radic. Res.* 23: 365–370, 1995.
- Cathcart, R., E. Schwieters, and B. N. Ames. Detection of picomole levels of lipid hydroperoxides using a dichlorofluorescein fluorescent assay. *Methods Enzymol.* 105: 352–358, 1984.
- Choi, D. W., M. Maulucci-Gedde, and A. R. Kriegstein. Glutamate neurotoxicity in cortical cell culture. *J. Neurosci.* 7: 357–368, 1987.
- Coyle, J. T., and P. Puttfarcken. Oxidative stress, glutamate, and neurodegenerative disorders. *Science* 262: 689–695, 1993.
- Droge, W., H. P. Eck, and S. Mihm. HIV-induced cysteine deficiency and T-cell dysfunction—a rationale for treatment with *N*-acetylcysteine. *Immunol. Today* 13: 211–214, 1992.
- Ely, D., G. Dunphy, H. Dollwet, H. Richter, F. Sellke, and M. Azodi. Maintenance of left ventricular function (90%) after twenty-four-hour heart preservation with deferoxamine. *Free Radic. Biol. Med.* 12: 479–485, 1992.
- Flohe, L. The selenoprotein glutathione peroxidase. In: *Glutathione: Chemical, Biochemical and Medical Aspects*, edited by D. D. R. Poulson and A. O. John. New York: Wiley, 1989, p. 643–731.
- Froissard, P., and D. Duval. Cytotoxic effects of glutamic acid on PC12 cells. *Neurochem. Int.* 24: 485–493, 1994.
- Gotz, M. E., A. Freyberger, and P. Riederer. Oxidative stress: a role in the pathogenesis of Parkinson's disease. *J. Neural Transm. Suppl.* 29: 241–249, 1990.
- Han, D., G. Handelman, L. Marcocci, C. K. Sen, S. Roy, H. Kobuchi, H. J. Tritschler, L. Flohe, and L. Packer. Lipoic acid increases de novo synthesis of cellular glutathione by improving cystine utilization. *Biofactors.* 6: 321–338, 1997.
- Handelman, G. J., D. Han, H. Tritschler, and L. Packer. Alpha-lipoic acid reduction by mammalian cells to the dithiol form, and release into the culture medium. *Biochem. Pharmacol.* 47: 1725–1730, 1994.
- Harvey, P. R., R. G. Ilson, and S. M. Strasberg. The simultaneous determination of oxidized and reduced glutathiones in liver tissue by ion pairing reverse phase high performance liquid chromatography with a coulometric electrochemical detector. *Clin. Chim. Acta* 180: 203–212, 1989.
- Herzenberg, L. A., S. C. De Rosa, J. G. Dubs, M. Roederer, M. T. Anderson, S. W. Ela, S. C. Deresinski, and L. A. Herzenberg. Glutathione deficiency is associated with impaired survival in HIV disease. *Proc. Natl. Acad. Sci. USA* 94: 1967–1972, 1997.
- Hiramatsu, M., J. Liu, R. Edamatsu, S. Ohba, D. Kadowaki, and A. Mori. Probulcol scavenged 1,1-diphenyl-2-picrylhydrazyl radicals and inhibited formation of thiobarbituric acid reactive substances. *Free Radic. Biol. Med.* 16: 201–206, 1994.
- Holdiness, M. R. Clinical pharmacokinetics of *N*-acetylcysteine. *Clin. Pharmacokinet.* 20: 123–134, 1991.
- Issels, R. D., A. Nagele, K. G. Eckert, and W. Wilmanns. Promotion of cystine uptake and its utilization for glutathione biosynthesis induced by cysteamine and *N*-acetyl cysteine. *Biochem. Pharmacol.* 37: 881–888, 1988.
- Kato, S., K. Negishi, K. Mawatari, and C. H. Kuo. A mechanism for glutamate toxicity in the C6 glioma cells involving inhibition of cystine uptake leading to glutathione depletion. *Neuroscience* 48: 903–914, 1992.
- Lash, L. H., and D. P. Jones. Distribution of oxidized and reduced forms of glutathione and cysteine in rat plasma. *Arch. Biochem. Biophys.* 240: 583–592, 1985.
- Maher, P., and J. B. Davis. The role of monoamine metabolism in oxidative glutamate toxicity. *J. Neurosci.* 16: 6394–6401, 1996.
- Maiorino, M. Effect of  $\alpha$ -lipoic acid on Se-dependent glutathione peroxidases. In: *Biological Oxidants and Antioxidants*, edited by

- L. Packer and E. Cadenas. Stuttgart: Hippokrates verlag Stuttgart, 1994, p. 69–75.
27. **McLellan, L. I., A. D. Lewis, D. J. Hall, J. D. Ansell, and C. R. Wolf.** Uptake and distribution of *N*-acetylcysteine in mice: tissue-specific effects on glutathione concentrations. *Carcinogenesis* 16: 2099–2106, 1995.
  28. **Muller, U., and J. Krieglstein.** Prolonged pretreatment with alpha-lipoic acid protects cultured neurons against hypoxic, glutamate-, or iron-induced injury. *J. Cereb. Blood Flow Metab.* 15: 624–630, 1995.
  29. **Murphy, T. H., M. Miyamoto, A. Sastre, R. L. Schnaar, and J. T. Coyle.** Glutamate toxicity in a neuronal cell line involves inhibition of cystine transport leading to oxidative stress. *Neuron* 2: 1547–1558, 1989.
  30. **Murphy, T. H., R. L. Schnaar, and J. T. Coyle.** Immature cortical neurons are uniquely sensitive to glutamate toxicity by inhibition of cystine uptake. *FASEB J.* 4: 1624–1633, 1990.
  31. **Naito, M., H. Umegaki, and A. Iguchi.** Protective effects of probucol against glutamate-induced cytotoxicity in neuronal cell line PC12. *Neurosci. Lett.* 186: 211–213, 1995.
  32. **Oka, A., M. J. Belliveau, P. A. Rosenberg, and J. J. Volpe.** Vulnerability of oligodendroglia to glutamate: pharmacology, mechanisms, and prevention. *J. Neurosci.* 13: 1441–1453, 1993.
  33. **Packer, L., S. Roy, and C. K. Sen.**  $\alpha$ -Lipoic acid: a metabolic antioxidant and potential redox modulator of transcription. *Adv. Pharmacol.* 38: 79–101, 1996.
  34. **Packer, L., E. H. Witt, and H. J. Tritschler.**  $\alpha$ -Lipoic acid as a biological antioxidant. *Free Radic. Biol. Med.* 19: 227–250, 1995.
  35. **Panigrahi, M., Y. Sadguna, B. R. Shivakumar, S. V. Kolluri, S. Roy, L. Packer, and V. Ravindranath.**  $\alpha$ -Lipoic acid protects against reperfusion injury following cerebral ischemia in rats. *Brain Res.* 717: 184–188, 1996.
  36. **Peter, G., and H. O. Borbe.** Absorption of [7,8-<sup>14</sup>C]rac-a-lipoic acid from in situ ligated segments of the gastrointestinal tract of the rat. *Arzneimittelforschung* 45: 293–299, 1995.
  37. **Phelps, D. T., S. M. Deneke, D. L. Daley, and B. L. Fanburg.** Elevation of glutathione levels in bovine pulmonary artery endothelial cells by *N*-acetylcysteine. *Am. J. Respir. Cell Mol. Biol.* 7: 293–299, 1992.
  38. **Roy, S., C. K. Sen, H. J. Tritschler, and L. Packer.** Modulation of cellular reducing equivalent homeostasis by  $\alpha$ -lipoic acid. *Biochem. Pharmacol.* 53: 393–399, 1997.
  39. **Sastre, R., and D. L. Rabenstein.** Determination of cysteine in plasma and urine and homocysteine in plasma by high-pressure liquid chromatography. *Anal. Biochem.* 90: 684–692, 1978.
  40. **Schnaar, R. L., P. H. Weigel, M. S. Kuhlenschmidt, Y. C. Lee, and S. Roseman.** Adhesion of chicken hepatocytes to polyacrylamide gels derivatized with *N*-acetylglucosamine. *J. Biol. Chem.* 253: 7940–7951, 1978.
  41. **Sen, C. K.** Nutritional biochemistry of cellular glutathione. *J. Nutr. Biochem.* In press.
  42. **Sen, C. K., and L. Packer.** Antioxidant and redox regulation of gene transcription. *FASEB J.* 10: 709–720, 1996.
  43. **Sen, C. K., S. Roy, D. Han, and L. Packer.** Regulation of cellular thiols in human lymphocytes by  $\alpha$ -lipoic acid: a flow cytometric analysis. *Free Radic. Biol. Med.* 22: 1241–1257, 1997.
  44. **Shivakumar, B. R., S. V. Kolluri, and V. Ravindranath.** Glutathione and protein thiol homeostasis in brain during reperfusion after cerebral ischemia. *J. Pharmacol. Exp. Ther.* 274: 1167–1173, 1995.
  45. **Sjodin, K., E. Nilsson, A. Hallberg, and A. Tunek.** Metabolism of *N*-acetyl-L-cysteine. Some structural requirements for the deacetylation and consequences for the oral bioavailability. *Biochem. Pharmacol.* 38: 3981–3985, 1989.
  46. **Teichert, J., and R. Preuss.** HPLC-methods for determination of lipoic acid and its reduced form in human plasma. *Int. J. Clin. Pharmacol. Ther. Toxicol.* 30: 511–512, 1992.
  47. **Uhlig, S., and A. Wendel.** The physiological consequences of glutathione variation. *Life Sci.* 51: 1083–1094, 1992.
  48. **Yan, C. Y., G. Ferrari, and L. A. Greene.** *N*-acetylcysteine-promoted survival of PC12 cells is glutathione-independent but transcription-dependent. *J. Biol. Chem.* 270: 26827–26832, 1995.
  49. **Ziegler, D., M. Hanefeld, K. J. Ruhnau, H. P. Meissner, M. Lobisch, K. Schutte, and F. A. Gries.** Treatment of symptomatic diabetic peripheral neuropathy with the anti-oxidant alpha-lipoic acid. A 3-week multicentre randomized controlled trial (ALADIN Study). *Diabetologia* 38: 1425–1433, 1995.