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Pyruvate Released by Astrocytes Protects Neurons from Copper-Catalyzed Cysteine Neurotoxicity

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We have found previously that astrocytes can provide cysteine to neurons. However, cysteine has been reported to be neurotoxic although it plays a pivotal role in regulating intracellular levels of glutathione, the major cellular antioxidant. Here, we show that cysteine toxicity is a result of hydroxyl radicals generated during cysteine autoxidation. Transition metal ions are candidates to catalyze this process. Copper substantially accelerates the autoxidation rate of cysteine even at submicromolar levels, whereas iron and other transition metal ions, including manganese, chromium, and zinc, are less efficient. The autoxidation rate of cysteine in rat CSF is equal to that observed in the presence of ~0.2 μM copper. In tissue culture tests, we found that cysteine toxicity depends highly on its autoxidation rate and on the total amount of cysteine being oxidized, suggesting that the toxicity can be attributed to the free radicals produced from cysteine autoxidation, but not to cysteine itself.

We have also explored the *in vivo* mechanisms that protect against cysteine toxicity. Catalase and pyruvate were each found to inhibit the production of hydroxyl radicals generated by cysteine autoxidation. In tissue culture, they both protected primary neurons against cysteine toxicity catalyzed by copper. This protection is attributed to their ability to react with hydrogen peroxide, preventing the formation of hydroxyl radicals. Pyruvate, but not catalase or glutathione peroxidase, was detected in astrocyte-conditioned medium and CSF. Our data therefore suggest that astrocytes can prevent cysteine toxicity by releasing pyruvate.

Key words: glia; glutathione; toxicity; oxidative stress; transition metal; autoxidation; conditioned medium

Glutathione is the major cellular antioxidant and thiol compound, and it plays a central role in cellular antioxidative defense. It is synthesized from glutamate, cysteine, and glycine. Cysteine is the rate-limiting precursor of glutathione synthesis (Beutler, 1989). Neurons prefer to take up cysteine, rather than cystine, to synthesize glutathione (Kranich et al., 1996). Our recent data have shown that astrocytes can provide cysteine to neurons indirectly by releasing glutathione, and that cysteine is maintained at a stable level in CSF and astrocyte-conditioned medium (ACM) (Wang and Cynader, 2000). However, cytotoxic effects of cysteine have also been noted, particularly in neurons. Extensive degenerative changes in the CNS are induced after subcutaneous injection of cysteine in newborn mice (Olney et al., 1972) and rats (Karlsen et al., 1981). Cysteine is also toxic to cultured neurons (Puka-Sundvall et al., 1995), hepatocytes (Saez et al., 1982), and kidney cell lines (Nath and Salahudeen, 1993). The underlying mechanisms of cysteine toxicity have been studied, and there are several theories. (1) Olney et al. (1990) found that NMDA antagonists can prevent cysteine toxicity and that cysteine is a bicarbonate-sensitive excitotoxin. They suggest a direct toxicity of cysteine. (2) Some researchers reported that glutathione potentiates glutamate toxicity by modulating the redox site of the NMDA receptor-channel complex (Sucher and Lipton, 1991; Janaky et al., 1993; Regan and Guo, 1999). Like glutathione, cysteine can also regulate redox status by participating in the thiol/disulfide exchange reaction, thus exerting an indirect toxic effect. (3) Cysteine autoxidation can generate free radicals, which are cytotoxic (Saez et al., 1982; Nath and Salahudeen, 1993). This last mechanism has not been studied in neurons. Because neurons are vulnerable to oxidative stress, attributable to the high oxygen consumption of the brain, their high proportion of membrane polyunsaturated fatty acids, and the weak activities of their antioxidative enzymes (Makar et al., 1994), free radicals may play an important role in cysteine neurotoxicity in brain. In the experiments reported here, we explore the free radical mechanisms of cysteine toxicity. We investigate the influence of cysteine autoxidation, the generation of free radicals from cysteine autoxidation, and its neurotoxicity to cultured neurons.

Another aim of our experiments is to identify possible protective mechanisms against cysteine toxicity. The data to be reported indicate that cysteine in the extracellular fluid of the CNS will constantly generate reactive oxygen intermediates via an autoxidation process. The organism must have developed certain mechanisms to remove the free radicals produced. In this paper, we have explored the possible protective effects of catalase and pyruvate on cysteine toxicity. Pyruvate, as well as other α-ketoacids, can react with hydrogen peroxide (H_2O_2) nonenzymatically, being converted to carbon dioxide and the carboxylic acid with one less carbon: $R\text{-}COCOOH + H_2O_2 \rightarrow R\text{-}COOH + H_2O + CO_2$ (Holleman, 1904; Bunton, 1949). Removing H_2O_2 will prevent the formation of the hydroxyl radical (·OH), which is the major damaging radical. The protective effects of pyruvate against oxidative stress in biological systems have been reported (O'Donnell-Tormey et al., 1987; Desagher et al., 1997; Giando-

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menico et al., 1997). The enzymatic activities of glutathione peroxidase (GPx), catalase, and pyruvate were examined in ACM and the CSF.

MATERIALS AND METHODS

Materials. Long–Evans pregnant rats were obtained from Charles River (Laval, Quebec, Canada). Coumarin-3-carboxylic acid (CCA) was obtained from Aldrich (Milwaukee, WI). 7-Hydroxycoumarin-3-carboxylic acid (7-OHCCA) was obtained from Molecular Probes (Eugene, OR). Fetal bovine serum (FBS), trypsin, and 10 mM Dulbecco's PBS were obtained from Life Technologies (Grand Island, NY), and DNase I was obtained from Boehringer Mannheim (Mannheim, Germany). Cysteine, glutathione, sodium pyruvate, sodium lactate, cupric sulfate, cuprous chloride, ferric chloride, ferrous sulfate, manganese sulfate, chromium chloride, zinc chloride, hemin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), iodoacetic acid, 1-fluoro-2,4-dinitrobenzene (FDNB), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), isopropanol, dithiothreitol, 2-mercaptoethanol, *tert*-butyl hydroperoxide (t-BuOOH), H_2O_2, catalase, glutathione peroxidase, glutathione reductase, lactic dehydrogenase (LDH), β-nicotinamide adenine dinucleotide reduced form (β-NADH), β-nicotinamide adenine dinucleotide phosphate reduced form (β-NADPH), Earle's balanced salt solution (EBSS), Eagle's MEM, cystine-free Eagle's MEM (M-2289), phenol red-free Eagle's MEM (M-4144), insulin, transferrin, selenium, and poly-L-lysine were obtained from Sigma (St. Louis, MO). The Angiocath catheter was obtained from Becton Dickinson (Sandy, UT). Centricon-3 was obtained from Amicon (Beverly, MA). HPLC was performed using the 712 Gilson gradient system from Gilson Medical Electronics (Middleton, WI). The 3-amino propyl ion-exchange column, with a particle size of 5 μm and dimensions of 4.6 × 200 mm, was obtained from CEL Associates (Houston, TX). Fluorescent measurement was accomplished using the luminescence spectrometer model LS 50B from Perkin-Elmer (Buckinghamshire, UK).

Primary cultures of cortical neurons and astrocytes. Primary cultures of cortical neurons were prepared in serum-free medium. Cerebral cortices of 18-d-old rat embryos were taken, and meninges were removed. The tissue was dissected and enzymatically digested with 0.25% trypsin and 0.1 mg/ml DNase. The dissociated cells were suspended in the serum-free medium and plated onto glass coverslips (28 mm in diameter) at a density of 1×10^5 cells/cm^2. The coverslips were coated with poly-L-lysine and dried. Before the dissociated cells were plated, the coverslips were briefly coated with 10% FBS-supplemented MEM for 5 min and rinsed twice with Hank's solution. The latter procedure helps cell attachment in serum-free medium (Wang and Cynader, 1999). The culture medium was serum-free Eagle's MEM, supplemented with glucose (33 mM), glutamine (2 mM), $NaHCO_3$ (26 mM), and a mixture of insulin (10 mg/l), transferrin (5.5 mg/l), and sodium selenite (5 μg/l). Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. After 24 hr in culture, the cells were used for neurotoxicity assays.

To prepare neuron-conditioned medium (NCM), the primary neurons were cocultured with a confluent astrocyte feeder layer in serum-free MEM using a noncontact method, which we have described in detail previously (Wang and Cynader, 1999). At 7 d *in vitro* (DIV), the astrocyte feeder layer was removed. The serum-free medium was added to the neuronal cultures at a concentration of 1.33 ml per 1×10^6 cells. The NCM was collected 24 hr later.

Astrocyte cultures were prepared by a method modified from that described by McCarthy and de Vellis (1980). Cerebral cortices of 2-d-old newborn rats were used. The tissue was enzymatically digested as described above. The dissociated cells were plated in poly-L-lysine-precoated 75 cm^2 plastic flasks at a high density of 2×10^5 cells/cm^2. The culture medium was Eagle's MEM, supplemented with glucose (33 mM), glutamine (2 mM), $NaHCO_3$ (26 mM), and 10% FBS. The medium was changed twice a week. The cultures were grown to confluence in 2 weeks. At 14 DIV, the flasks were tightly sealed and shaken at 260 rpm for 18 hr. Suspended cells in the flasks were discarded after shaking, and the adherent cells were flat, polygonal astrocytes, which were identified by morphology and glial fibrillary acidic protein immunostaining. The density of confluent astrocytes was $\sim 1 \times 10^5$ cells/cm^2. Astrocyte-enriched cultures that were 2- to 6-week-old in flasks were used to make ACM.

To prepare ACM, serum-free MEM was added to the confluent astrocyte cultures at a concentration of 1.33 ml per 1×10^6 cells (10 ml per flask). The ACM was collected 24 hr later.

MTT colorimetric assay of neuronal viability. Neuronal survival was determined by the MTT method, modified from Denizot and Lang (1986). The tetrazolium salt MTT is reduced into a blue formazan by the mitochondrial enzyme succinate-dehydrogenase (Slater et al., 1963), and the amount of formazan produced is proportional to the number of living cells. The cultured neurons were incubated with 0.5 mg/ml of MTT in phenol red-free MEM. After a 3 hr incubation at 37°C in a humidified atmosphere of 5% CO_2, the medium was removed, and 1 ml of propanol was added to each dish to solubilize the formazan. The optical density was measured at 560 nm with 690 nm as a reference ($OD_{560-690}$). The cell viability was normalized as a percentage of control.

HPLC analysis of cysteine, cystine, and related compounds. The HPLC analysis of cysteine, cystine, and related compounds, including glutathione and cysteine-glutathione disulfide, has been described in detail elsewhere (Wang and Cynader, 2000), with modifications from Reed et al. (1980). Briefly, a 100 μl sample was first reacted with 12.5 μl of 100 mM iodoacetic acid in 0.2 mM m-cresol purple and 12.5 μl of $NaHCO_3$ (0.24 M)/NaOH (0.12 M) buffer for 30 min. Then, 112.5 μl of 1% (v/v) FDNB in ethanol was added, and the mixture was stored at 4°C overnight. Finally, 12.5 μl of 1 M lysine was added to eliminate the unreacted FDNB, and the sample was then ready for analysis. HPLC solvent A was 80% methanol in water. Solvent B was 0.8 M sodium acetate in 64% methanol. A 100 μl sample was injected into the HPLC column. The mobile phase was maintained at 80% A/20% B for 5 min, followed by a gradient elution to 1% A/99% B over 10 min, and held for 5 min. The flow rate was 1.5 ml/min. The concentrations of cysteine and related compounds in the samples were calculated from the peak areas. The minimal detectable concentrations in this experiment were 0.1 μM for thiols and 0.05 μM for disulfides.

Spectrophotometric assay of cysteine with Ellman's reagent (DTNB). This is an alternative method for quantitating cysteine. It is based on the thiol/disulfide reaction of thiol and DTNB, a disulfide, liberating the chromophore 5-mercapto-2-nitrobenzoic acid (Ellman, 1959; Hu, 1994). The advantage of this reagent is that its reaction with thiols is faster (in seconds) than iodoacetic acid (in minutes), which is used in our HPLC analysis. Although it cannot detect multiple components of thiols and disulfides, it is suitable for simple systems, such as the study of cysteine autoxidation in pure solutions. DTNB stock solution (10 mM) was prepared in methanol. Hemin stock solution (10 mM) was prepared in 50 mM NaOH. The stock solutions of other transition metal ions (100 μM to 10 mM), including $FeSO_4$, $FeCl_3$, CuCl, $CuSO_4$, $MnSO_4$, $CrCl_3$, and $ZnCl_2$, were prepared in dH_2O. These stock solutions were freshly prepared. The reaction mixture included transition metal ions and cysteine in PBS. After the reaction, 0.1 ml of DTNB was added to 0.9 ml of the reaction mixture. It was measured against a reference of 1 mM of DTNB in PBS. An additional blank control containing all components except cysteine was evaluated to correct the absorption of transition metal ions. Cysteine concentrations were measured from absorbance at 412 nm and calculated on the basis of cysteine standards.

Procedures for obtaining CSF. CSF was obtained from the rat cerebellomedullary cistern. Three-month-old male Long–Evans rats were anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (5 mg/kg). The rat was placed in a stereotaxic frame. The skin was incised along the midline over the occipital crest, and the muscles were separated. A puncture through occipital foramen magnum was made with an Angiocath catheter. Approximately 100 μl of CSF was slowly drawn over 2 min. The CSF was centrifuged at 300 × *g* for 2 min to remove the tiny amount of contaminating blood cells. To examine the cysteine autoxidation rate in CSF, 10 μl of 1 mM cysteine solution was added to 90 μl of CSF, to reach a final concentration of 100 μM. The original cysteine concentration in the CSF (~1.12 μM) (Wang and Cynader, 2000) can be ignored. The CSF was incubated at 37°C in a humidified atmosphere of 5% CO_2.

Fluorimetric assay of hydroxyl radicals. Production of ·OH was estimated by using CCA. Nonfluorescent CCA was converted by ·OH to highly fluorescent 7-OHCCA (Collins et al., 1994). For measuring ·OH produced during autoxidation of 100 μM cysteine in the presence of 0.2 μM Cu^{2+}, the reaction mixture was prepared as follows in sequence: 0.885 ml of PBS, 0.1 ml of 10 mM CCA (final concentration: 1 mM), 10 μl of 10 mM cysteine, and 5 μl of 40 μM Cu^{2+}. The solution was left at 37°C and 100% air to finish the reaction completely. After 4 hr incubation, the samples were measured using a fluorescence spectrometer, with excitation wavelength of 400 nm and emission of 450 nm. CCA (1 mM) in PBS was used as the reference. A standard curve was calculated by measuring the fluorescence intensities of a series of concentrations of 7-OHCCA. The produced ·OH from cysteine autoxidation was represented by the corresponding 7-OHCCA concentrations.

Pyruvate assay. Pyruvate in ACM, NCM, and the CSF was measured using an enzymatic method described by Von Korff (1969). LDH catalyzes the conversion of pyruvate to lactate with NADH: pyruvate + NADH ⇆ lactate + NAD^+. The reaction mixture was prepared as follows: 198 μl of PBS, 100 μl of 1 mM NADH, 100 μl of the sample, and 2 μl of 0.4 U/μl LDH. The decrease of NADH was monitored at 340 nm with a spectrophotometer for 300 sec until a constant value was obtained. Pyruvate concentration was calculated from the oxidized NADH (ϵ_{NADH} = 6290 M^{-1} cm^{-1} at 340 nm).

Determination of enzyme activities. GPx activity was determined using a method modified from Takahashi et al. (1987). GPx activity was assessed from the oxidation of NADPH in the presence of glutathione reductase and oxidized glutathione formed by GPx. The reaction mixture included Tris-HCl (0.1 M, pH 8.0), NADPH (0.2 mM), EDTA (0.5 mM), glutathione (2 mM), glutathione reductase (1 U/ml), sample (100 μl), and t-BuOOH (100 μM). The total volume was 1 ml, and t-BuOOH was added last. The oxidation of NADPH was monitored against a reference mixture without sample and t-BuOOH. A negative control containing all components except the sample was used to correct the oxidation of glutathione and NADPH by t-BuOOH. The samples were adult rat CSF and ACM. The latter was 10× concentrated ACM prepared by filtering ACM in a centricon with a 3 kDa molecular weight cutoff. Calculation of GPx activity was based on the consumption of NADPH at A_{340} (ϵ_{NADPH} = 6220 M^{-1} cm^{-1} at 340 nm).

Catalase activity was measured by monitoring the absorbance change of H_2O_2 (Duffy et al., 1998). The reaction mixture included PBS, EDTA (0.5 mM), H_2O_2 (10 mM), and 100 μl of sample. The total volume was 1 ml. The reference contained all components except the sample. The samples were adult rat CSF and the concentrated ACM, as above. Absorbance was monitored at 240 nm from 300 to 600 sec. The minimal detectable enzyme activity of this test was 5 mU/ml. One unit of enzyme activity was defined as the amount of the enzyme that decomposes 1 μmol of H_2O_2 per minute.

The data were analyzed by using ANOVA. The *post hoc* tests to determine significant differences between means of individual groups were followed when necessary.

RESULTS

Cysteine autoxidation in the presence of transition metal ions and in CSF

To study the factors determining cysteine autoxidation, we first investigated the autoxidation rate of cysteine in the following three solutions: EBSS, cystine-free MEM (MEM-$CSSC^-$), and MEM-$CSSC^-$ with cultured neurons. EBSS contains the basal salts of MEM. The medium of the primary cortical neurons was replaced by MEM-$CSSC^-$ before testing. Only a small amount of cell debris was present in the cell cultures. All of these solutions were incubated at 37°C and pH 7.4 in a humidified atmosphere of 5% CO_2. Cysteine was added to a final concentration of 100 μM. Cysteine and cystine were measured by HPLC at 0, 5, 15, 30, 60, and 120 min after cysteine was added to the three solutions. The results showed that cysteine was gradually oxidized to cystine. Only a small proportion of cysteine (~1–2%) was oxidized to cysteic acid (data not shown). Cysteine concentrations in MEM-$CSSC^-$ and neuronal culture medium did not differ significantly from the corresponding values in EBSS ($p > 0.05$) (Fig. 1*A*). This result demonstrates that the components of culture medium and cultured cells have no obvious influence on cysteine autoxidation.

Transition metal ions, such as Fe^{3+} and Cu^{2+}, are believed to catalyze the oxidation of reducing agents, including thiol-containing compounds (Halliwell and Gutteridge, 1985). The effects of Fe^{3+} and Cu^{2+} on cysteine autoxidation were measured under the same conditions as described above. Fe^{3+}, at concentrations of 0.1, 1.0, and 10 μM, had no obvious effects on the autoxidation rate of cysteine, as assessed by comparing cysteine concentrations with the corresponding value in EBSS ($p > 0.05$) (Fig. 1*B*). There were also no obvious differences among the three different concentrations ($p > 0.05$). Our results showed that Cu^{2+} substantially accelerated the autoxidation rate of cysteine at concentrations between 0.1 and 10 μM (Fig. 1*C*). The efficacy of submicromolar levels of Cu^{2+} in catalyzing cysteine autoxidation has significant physiological meaning because these are well within the physiological range. The concentrations of loosely bound copper in human CSF have been reported to be in the range of 0.13–0.75 μM (Gutteridge, 1984).

We compared the effects of variable oxidation valences of iron (Fe^{2+} and Fe^{3+}) and copper (Cu^+ and Cu^{2+}) on cysteine autoxidation. The effects of hemin, the low molecular weight complex of Fe^{3+}, and some other biologically important transition metal ions, including Mn^{2+}, Cr^{3+}, and Zn^{2+}, on cysteine autoxidation were also studied. As shown in Figure 2, cysteine autoxidation with Fe^{2+} was faster than that with Fe^{3+} at concentrations of 100 and 200 μM but was not significantly different at concentrations of 1 and 10 μM. The effects of Cu^+ and Cu^{2+} on cysteine autoxidation were not significantly different at concentrations of 0.01–1 μM. Hemin was more efficient in catalyzing cysteine autoxidation than Fe^{2+} and Fe^{3+}, but still much less efficient than Cu^+ and Cu^{2+}. The concentration for hemin to catalyze the half oxidation of 100 μM cysteine in 60 min was ~10 μM, whereas the concentration for Cu^+ and Cu^{2+} was between 0.1 and 0.2 μM, a ~50- to 100-fold difference. Mn^{2+} was efficient at concentrations of 100 and 200 μM. No catalyzing effects of Cr^{3+} and Zn^{2+} were observed at concentrations between 1 and 200 μM.

Cysteine autoxidation in CSF was examined. As described in the Materials and Methods, cysteine was added to rat CSF to a concentration of 100 μM. The samples were taken for test at 15, 30, 60, and 120 min after this addition. The results showed that cysteine was oxidized at a moderate rate in the CSF (Fig. 3*A*). Most of the cysteine was converted to cystine (~80%). The other small portion of cysteine participated in the thiol/disulfide exchange reaction and formed mixed disulfides, such as cysteine-glutathione disulfide and cysteine-protein disulfides. We compared cysteine autoxidation in CSF and in Cu^{2+}-supplemented solutions (Fig. 3*B*). The autoxidation rate of cysteine in CSF was roughly equal to that observed in solutions of 0.2–0.3 μM Cu^{2+}. In Discussion, we propose that copper is likely the major catalyst in CSF for cysteine autoxidation. We chose 0.2 μM Cu^{2+} to mimic the cysteine autoxidation in CSF in the following toxicity experiments.

Neurotoxic effects of cysteine

Cysteine neurotoxicity was investigated in our cell culture system. We observed the effects of the autoxidation rate of cysteine and the amount of cysteine being oxidized, as well as the effects of cysteine concentrations, on cysteine neurotoxicity. In Figure 4, primary neurons were cultured in the presence of a series of concentrations of cysteine (0, 10, 20, 50, 100, 200, 500, 1000, and 2000 μM) and in the absence or presence of copper (0, 0.2, and 1.0 μM) in 2 ml culture medium (MEM). Neurotoxicity was estimated 24 hr later by the MTT assay. The role of copper in this experiment was to modulate the autoxidation rate of cysteine. Without addition of cysteine, copper itself had no visible effect on neuronal survival. The neuronal survivals in the presence of copper and absence of cysteine were 99.6 ± 11.3% (mean ± SEM; Cu^{2+} = 0.2 μM) and 105 ± 5% (Cu^{2+} = 1.0 μM); there were no significant differences from the control 100 ± 1.5% (Cu^{2+} = 0) ($p > 0.05$). Without Cu^{2+}, cysteine was toxic only at relatively high concentrations (EC_{50} ~600 μM). With the addition of Cu^{2+}, the toxic concentrations of cysteine decreased (EC_{50}

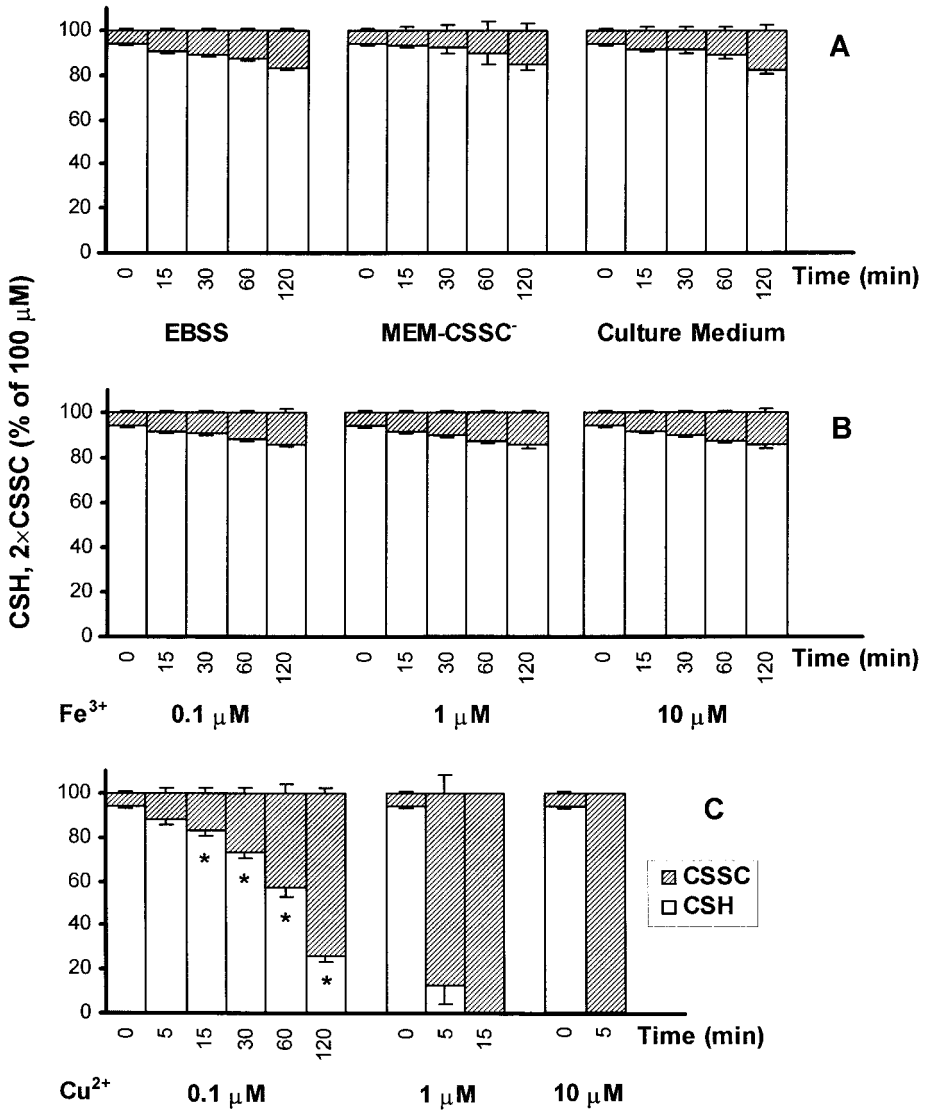

Figure 1. The factors influencing cysteine (*CSH*) autoxidation. The reaction conditions to assess cysteine autoxidation were 37°C and pH 7.4 in a humidified atmosphere of 5% CO_2 and 95% air. The samples were taken at different time points for HPLC assays. *A*, Cysteine (100 μM) autoxidation in Earle's balanced salt solution (*EBSS*), cystine-free MEM (*MEM-CSSC*$^-$), and neuronal culture medium. The latter was made from MEM-CSSC$^-$, which replaced the original cystine-containing MEM in the neuronal cultures before testing. Cysteine was gradually oxidized to cystine. Cysteine concentrations in MEM-CSSC$^-$ and culture medium were not significantly different from the corresponding values in EBSS ($p > 0.05$). *B*, The effects of iron on cysteine autoxidation. Ferric chloride ($FeCl_3$), at concentrations of 0.1, 1, and 10 μM, was incubated with 100 μM cysteine in EBSS. Cysteine concentrations in the three Fe^{3+} solutions were not significantly different from the corresponding values in EBSS ($p > 0.05$). *C*, The effects of copper on cysteine autoxidation. Cupric sulfate ($CuSO_4$), at concentrations of 0.1, 1, and 10 μM, was incubated with 100 μM cysteine in EBSS. Cysteine autoxidation was substantially accelerated in the presence of Cu^{2+} at all three concentrations. Results are normalized as the percentage of 100 μM total cysteine. Each *column* represents the average of three independent experiments performed in duplicate (mean ± SEM). $^*p < 0.01$ versus corresponding values of cysteine autoxidation in EBSS.

~30 μM at 0.2 μM Cu^{2+} and EC_{50} ~12 μM at 1.0 μM Cu^{2+}). This result demonstrates that cysteine toxicity is closely related to its autoxidation rate.

The effect on its toxicity of the total amounts of cysteine being oxidized was investigated further. Cu^{2+} (0.2 μM) was added for the purpose of catalyzing cysteine autoxidation. Cysteine (10 μM) was added to the culture medium either 1, 5, or 10 times at a rate of once every 5 min, respectively. Cysteine (10 μM) was completely oxidized in 5 min (Fig. 5). Although a single dose of cysteine showed no obvious toxicity (91 ± 5.1% of viability), multiple additions of cysteine substantially increased cysteine toxicity. Neuronal survival was 91.5 ± 5.1% (mean ± SEM; $p >$ 0.05 vs control) with the addition of 10 μM cysteine one time and decreased to 28.9 ± 4.4% ($p < 0.01$ vs control) with five additions of 10 μM cysteine and to 12.6 ± 1.5% ($p < 0.01$ vs control) with 10 applications. Note that the repeated addition increased the total amounts of cysteine being oxidized, whereas the maximal concentration of cysteine never exceeded 10 μM at any one time. These results demonstrate that cysteine toxicity is closely related to the total amount of cysteine being oxidized.

Taken together, these data suggest that cysteine autoxidation, rather than cysteine itself, is responsible for cysteine toxicity. Using CCA as a probe, we measured the production of ·OH generated from cysteine autoxidation. The hydroxyl radical reacts with CCA to generate 7-OHCCA. Cysteine in PBS was incubated at 37°C and pH 7.4 for 4 hr. As shown in Figure 6*A*, little ·OH was produced from cysteine autoxidation without the presence of Cu^{2+}. In the presence of 0.2 μM Cu^{2+}, hydroxyl radicals were generated in substantial amounts during cysteine autoxidation, and the amount of ·OH increased with cysteine concentration. We also compared the generation of ·OH with some other thiols. Glutathione, *N*-acetyl-cysteine, homocysteine, dithiothreitol, and 2-mercaptoethanol can all generate ·OH with Cu^{2+} as a catalyst to variable degrees (Fig. 6*B*). Disulfides (cystine and glutathione disulfide) and sulfur-containing amino acid (cysteic acid) did not generate ·OH.

The effects of catalase and pyruvate on the cysteine toxicity and the generation of hydroxyl radicals

When cysteine is oxidized, O_2 accepts electrons one by one, and reactive oxygen intermediates are produced. The hydroxyl radical is the major reactive oxygen species to cause tissue damage and is generated from H_2O_2. Therefore it is reasonable to expect that catalase, which decomposes H_2O_2, could prevent ·OH generation

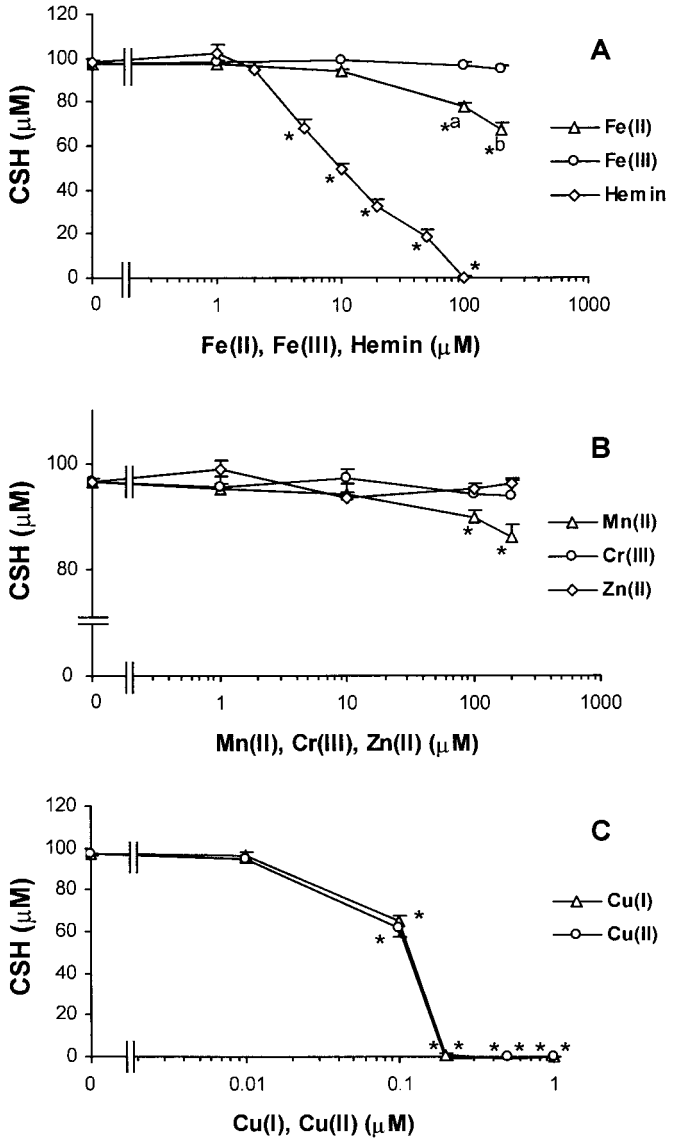

Figure 2. Comparisons of cysteine autoxidation in the presence of several transition metal ions. A series of concentrations of $FeSO_4$, $FeCl_3$, hemin, $MnSO_4$, $CrCl_3$, $ZnCl_2$, CuCl, and $CuSO_4$ were reacted with 100 μM cysteine, respectively. The control was 100 μM cysteine without transition metal ions. The reaction mixtures were incubated in PBS at pH 7.4 and 37°C in a humidified 100% air. Cysteine concentrations were determined with Ellman's reagent after 60 min. *A*, Cysteine autoxidation with Fe^{2+}, Fe^{3+} (1–200 μM), and hemin (1–100 μM). *B*, Cysteine autoxidation with Mn^{2+}, Cr^{3+}, and Zn^{2+} (1–200 μM). *C*, Cysteine autoxidation with Cu^{+} and Cu^{2+} (0.01–1 μM). Data are the mean ± SEM of three independent experiments in duplicate. $^*p < 0.01$, significantly different from the control. $^{a,b}p < 0.01$ versus corresponding values of Fe^{3+} groups.

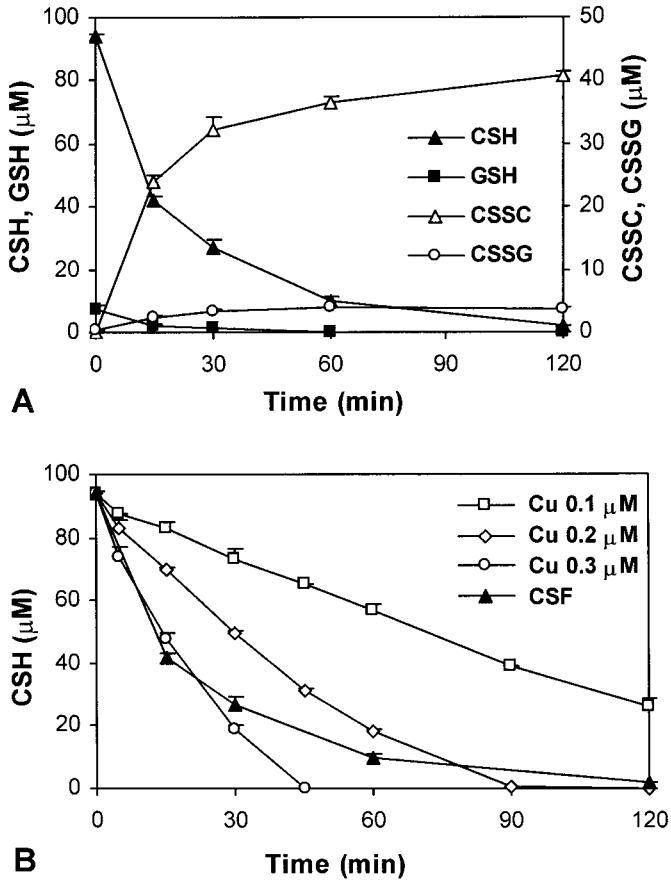

Figure 3. Cysteine autoxidation rates in CSF and comparisons with those in Cu^{2+}-supplemented solutions. CSF was taken from 3-month-old rats as described in Materials and Methods. Cysteine was added to the CSF with a final concentration of 100 μM. The CSF was incubated under conditions of pH 7.4 and 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Samples were taken at several time points for HPLC assays. *A*, Time course of the concentrations of cysteine and related compounds in the CSF during cysteine autoxidation. *B*, Comparison of cysteine autoxidation in CSF and in Cu^{2+}-supplemented solutions. Cu^{2+} was prepared in EBSS at concentrations of 0.1, 0.2, and 0.3 μM and incubated under the same conditions as the CSF. Data are the mean ± SEM of three independent experiments in duplicate. *CSSC*, Cystine; *CSSG*, cysteine-glutathione disulfide; *GSH*, glutathione.

from cysteine autoxidation and thus reduce cysteine toxicity. Likewise, pyruvate would also be expected to have the same effect because of its reactivity with H_2O_2. The effects of catalase and pyruvate on cysteine neurotoxicity were tested in our primary neuronal cultures. In the presence of Cu^{2+} (0.2 μM), cysteine (100 μM) was toxic to neurons (16.8 ± 2.8% of viability) (Fig. 7*A*). Addition of catalase (10 U/ml) or pyruvate (1 mM) completely prevented cysteine toxicity, with 101 ± 4 and 104 ± 5% of viability, respectively. Pyruvate and lactate are both glucose metabolites and important energy suppliers to neurons (Selak et al., 1985; Pellerin and Magistretti, 1994; Tsacopoulos and Magistretti, 1996). To demonstrate that the preventive effect of pyruvate against cysteine toxicity is caused by its specific reactivity with H_2O_2, other than its energy supplying effect, sodium lactate was used as control. The results showed that lactate could not prevent cysteine neurotoxicity (Fig. 7*A*). The protective capacity of pyruvate was dose dependent. In the presence of 0.2 μM Cu^{2+} and 50 or 100 μM cysteine, increasing concentrations of pyruvate (up to 1 mM) produced a progressive enhancement of neuronal protection (Fig. 7*B*).

Because of their ability to remove H_2O_2, catalase and pyruvate are expected to prevent the formation of ·OH generated from cysteine autoxidation. In the presence of 0.2 μM Cu^{2+} and 100 μM cysteine, the generation of ·OH was measured in PBS at 37°C and pH 7.4. Catalase and pyruvate substantially decreased ·OH production, whereas lactate did not (Fig. 8*A*). Lactate even increased the ·OH production, and the reason for this is unknown. The inhibitory effect of pyruvate on ·OH production from cysteine autoxidation was dose dependent (Fig. 8*B*).

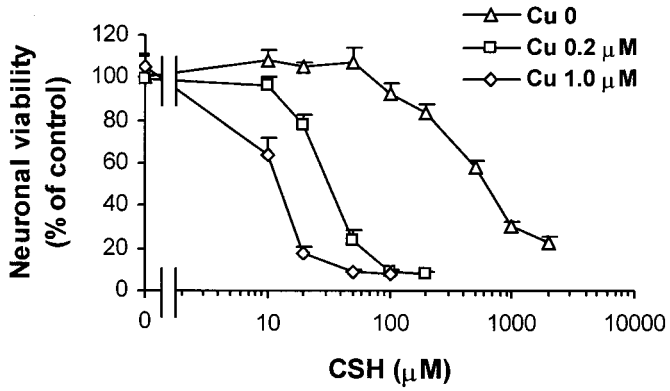

Figure 4. Neurotoxic effects of cysteine in the presence of copper. Primary cortical neurons were cultured in serum-free MEM. Cysteine was added at concentrations as indicated in the presence of 0, 0.2, and 1.0 μM Cu^{2+}. Neuronal viability was estimated 24 hr later using the MTT assay. Results are expressed as the percentage of surviving neurons compared with control cultures (without the addition of cysteine and Cu^{2+}). Data represent the mean ± SEM of three independent experiments in triplicate.

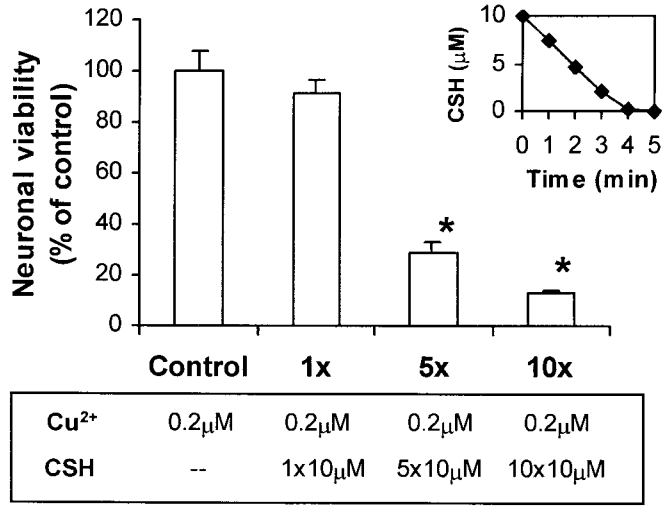

Figure 5. Effect of total amount of cysteine being oxidized on neuronal survival. Primary cortical neurons were cultured in serum-free MEM. The concentration of Cu^{2+} was 0.2 μM. Cysteine (10 μM) was added each time and once or repetitively every 5 min for a total of 5 and 10 times. Cysteine (10 μM) was completely oxidized within 5 min in the presence of 0.2 μM Cu^{2+} (*inset*, *top right*). Neuronal viability was estimated 24 hr later using the MTT assay. Results are expressed as the percentage of surviving neurons compared with control cultures (without addition of cysteine). Data represent the mean ± SEM of three independent experiments in triplicate. $^{*}p < 0.01$, significantly different from the control.

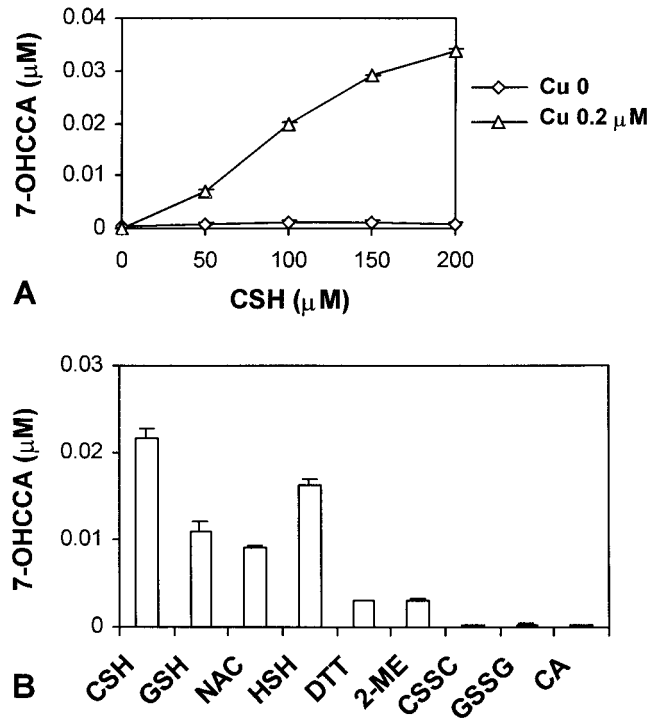

Figure 6. Generation of hydroxyl radical (·OH) by the autoxidation of cysteine and other thiols. Cysteine and related compounds were incubated in PBS under the conditions of pH 7.4 and 37°C in a humidified atmosphere of 100% air. CCA (1 mM) was added to react with the generated ·OH, producing 7-OHCCA. Fluorescence was measured 4 hr after the reaction began. *A*, Cysteine, at concentrations of 0–200 μM, was incubated in the presence or absence of 0.2 μM Cu^{2+}. *B*, Generation of ·OH from the autoxidation of thiols. Thiols, disulfides, and sulfur-containing amino acid (100 μM of each) were incubated with 0.2 μM Cu^{2+}. The thiols include cysteine (*CSH*), glutathione (*GSH*), *N*-acetylcysteine (*NAC*), homocysteine (*HSH*), dithiothreitol (*DTT*), and 2-mercaptoethanol (*2-ME*). The disulfides include cystine (*CSSC*) and glutathione disulfide (*GSSG*). The sulfur-containing amino acid is cysteic acid (*CA*). Data represent the mean ± SEM of three independent experiments in duplicate.

Pyruvate released by astrocytes

We explored two potential extracellular antioxidative mechanisms, which may prevent the toxic effects of cysteine autoxidation: (1) enzymatic mechanisms and (2) small molecule antioxidant mechanisms. For the former, we tested the activities of GPx and catalase in ACM and CSF. The concentrated ACM prepared from primary confluent astrocyte cultures was used as described in Materials and Methods. CSF was taken from 3-month-old rats. The results showed that the activities of the two enzymes were not detected in ACM and CSF. GPx activity of ACM was 1.6 ± 0.2 mU/ml (mean ± SEM, $n = 6$), which was not significantly different from the negative control (1.9 ± 0.4 mU/ml; $n = 6$; $p > 0.05$). GPx activity of CSF was 2.2 ± 0.2 mU/ml ($n = 6$), which was not significantly different from the negative control (1.7 ± 0.2 mU/ml; $n = 6$; $p > 0.05$). Catalase activity was not detected in ACM and CSF (<5 mU/ml, $n = 6$, respectively).

Because pyruvate can scavenge H_2O_2, it may act as an extracellular antioxidant *in vivo*. Pyruvate concentration was therefore assayed in ACM, NCM, and CSF. Relatively high levels of pyruvate were found in ACM (254 ± 15 μM) and in CSF (131 ± 9 μM). Pyruvate was also detected in NCM at relatively low concentrations (45.6 ± 4.4 μM) (Table 1), suggesting that the pyruvate pool of the extracellular space and CSF was contributed mainly by astrocytes. The time course of pyruvate release by astrocytes was also measured (Fig. 9). In the ACM made from pyruvate-free medium, pyruvate concentration increased rapidly within 12 hr and reached a peak at 24 hr.

DISCUSSION

The biological role of cysteine is double edged. It is a very important amino acid for the synthesis of glutathione, which is the major cellular antioxidant. Neurons, in particular, prefer to take up cysteine, rather than cystine, to synthesize glutathione (Kranich et al., 1996). On the other hand, cysteine has been found to be cytotoxic. Subcutaneous injection of cysteine in newborn

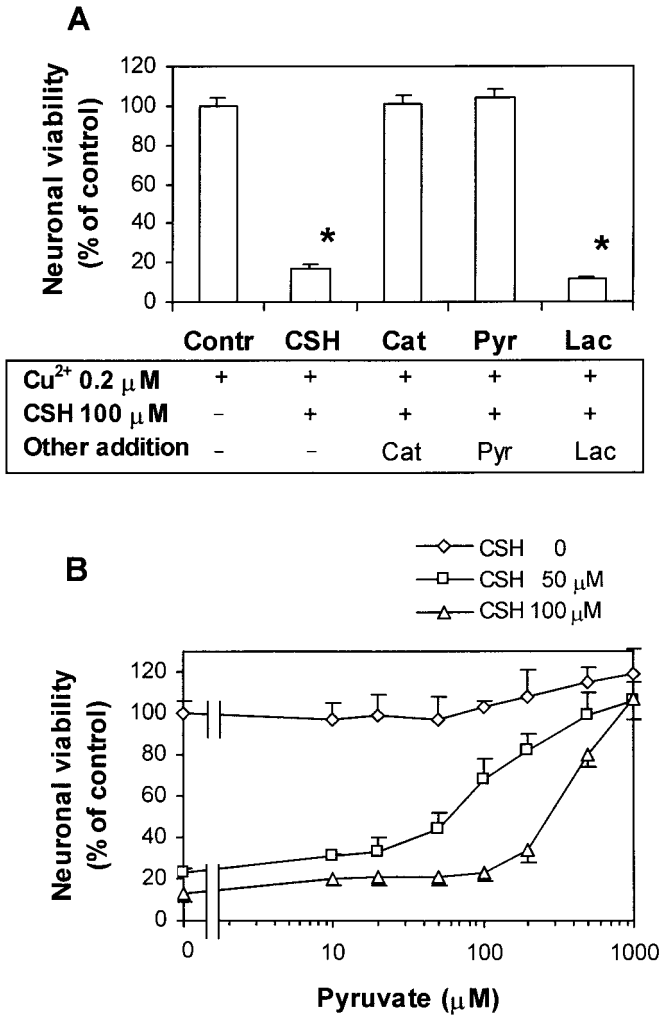

Figure 7. The protective effects of catalase and pyruvate on cysteine neurotoxicity. Primary cortical neurons were cultured in serum-free MEM. Cysteine toxicity was induced by addition of 100 μM cysteine and 0.2 μM Cu^{2+}. Neuronal viability was estimated 24 hr later using the MTT assay. Results are expressed as the percentage of surviving neurons compared with control cultures (without addition of cysteine). *A*, Catalase (10 U/ml), pyruvate (1 mM), and lactate (1 mM) were added immediately before addition of cysteine and Cu^{2+}. Data represent the mean ± SEM of three independent experiments in triplicate. *B*, Dose–response curve illustrating the neuroprotective effect of pyruvate. Cysteine concentrations were 0, 50, and 100 μM, respectively. Data represent the mean ± SEM of three independent experiments in duplicate. $^*p < 0.01$, significantly different from the control.

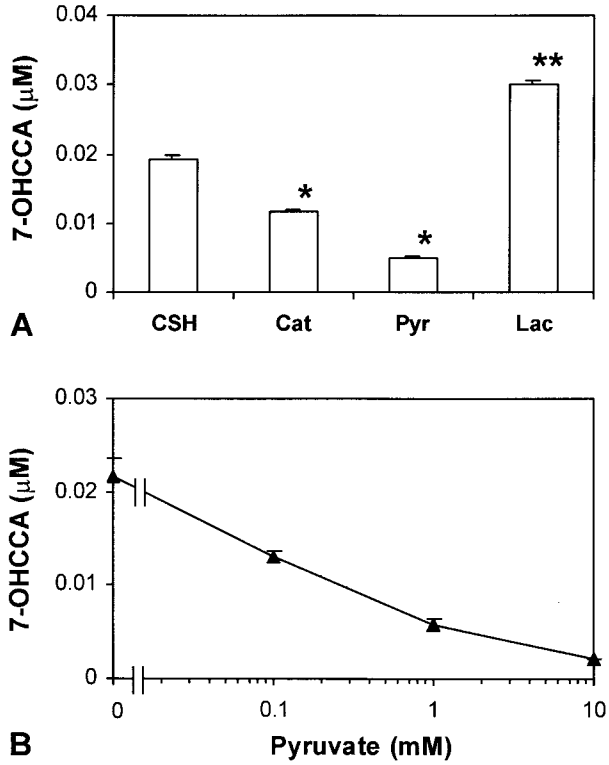

Figure 8. Effects of catalase and pyruvate on the generation of ·OH from cysteine autoxidation. Cysteine (100 μM) and Cu^{2+} (0.2 μM) were incubated in PBS under the conditions of pH 7.4 and 37°C in a humidified atmosphere of 100% air. CCA (1 mM) was added to react with the generated ·OH, producing 7-OHCCA. The fluorescence was measured 4 hr after the reaction. Data represent the mean ± SEM of three independent experiments in duplicate. *A*, Catalase (10 U/ml), pyruvate (1 mM), and lactate (1 mM) were added immediately before addition of cysteine and Cu^{2+}. *B*, Pyruvate, at concentrations of 0, 0.1, 1, and 10 mM, was added immediately before addition of cysteine and Cu^{2+}. *, $^{**}p < 0.01$, significantly different from the control (cysteine only).

Table 1. Pyruvate contents in ACM, NCM, and CSF

	Pyruvate concentrations (μM)	
	Mean ± SEM	Range
ACM ($n = 6$)	254 ± 15	184–297
NCM ($n = 6$)	45.6 ± 4.4	36.9–62.3
CSF ($n = 6$)	131 ± 9	98.9–145

ACM was prepared from confluent astrocyte cultures, and NCM was from 7 DIV neuronal cultures in serum-free MEM. The conditioned media were collected 24 hr later for pyruvate assay. CSF was obtained from 3-month-old male rats.

mice (1 mg/gm weight) (Olney et al., 1972) and rats (1.2 mg/gm weight) (Karlsen et al., 1981) caused extensive neuronal death. Cysteine was toxic to cultured hepatocytes (4 mM) (Saez et al., 1982), kidney cell lines (4 mM) (Nath and Salahudeen, 1993), and primary neurons (1 mM) (Puka-Sundvall et al., 1995). In our neuronal cultures, 1 mM cysteine decreased neuronal survival to 30.5% (EC_{50} ~600 μM) in the absence of copper (Fig. 4), similar to other findings (Puka-Sundvall et al., 1995). Importantly, our results showed that cysteine toxicity was greatly increased in the presence of even submicromolar levels of copper, whereas copper itself was noncytotoxic without cysteine (Fig. 4). Copper appears to function as a catalyst to accelerate cysteine autoxidation (Fig. 10).

Iron is generally considered the major transition metal ion in mediating the production of free radicals. Our experiments show that copper is much more efficient in catalyzing cysteine autoxidation than iron and some other transition metal ions, such as manganese, chromium, and zinc. Variable valences of copper do not influence its catalyzing efficacy. For iron, the soluble low molecular weight Fe^{3+} complex, hemin, is more efficient in catalyzing cysteine autoxidation than Fe^{2+} and Fe^{3+}. Fe^{3+} is insoluble at neutral pH, whereas Fe^{2+} is relatively soluble. Fe^{2+}, however, will be oxidized to Fe^{3+} under aerobic conditions and form deposits. Whether solubility is the cause of these differences is unknown. Even the catalyzing efficacy of hemin is still much lower than that of copper. Our finding that copper is a more potent catalyst than iron is not unique. It has been reported that

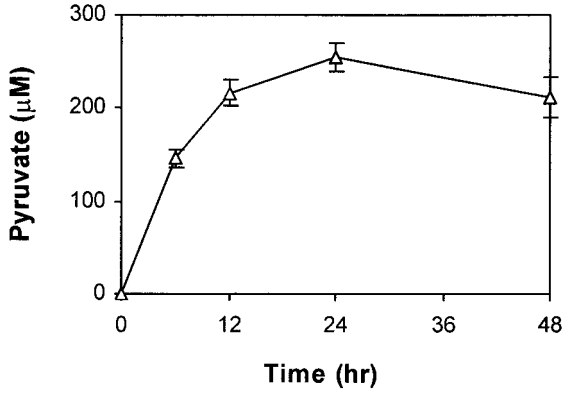

Figure 9. Time course of pyruvate release by astrocytes. Confluent astrocytes in flasks were rinsed with Hank's solution. The serum-free MEM was added at a concentration of 1.33 ml per 1×10^6 cells. Samples of the ACM were taken at 0, 6, 12, 24, and 48 hr and used for pyruvate assays. Data represent the mean ± SEM of three independent experiments in triplicate.

copper is more efficient than iron in mediating paraquat toxicity (Chevion, 1988) and in catalyzing dialuric acid autoxidation (Munday, 1988).

Our data show that cysteine was oxidized at a moderate rate in CSF. The autoxidation rate of 100 μM cysteine in CSF is equivalent to that in the presence of ~0.2 μM Cu^{2+} (Fig. 3). According to different authors, total copper concentrations in human CSF are in the range of 0.22–1.7 μM (14.2–109 μg/l) (Kapaki et al., 1997; Jimenez-Jimenez et al., 1998; Joergstuerenburg et al., 1999; Stuerenburg, 2000). CSF also contains some other physiologically important transition metal ions, such as iron, manganese, chromium, and zinc. Their concentrations in human CSF are 1.1–3.8 μM for Fe (Jimenez-Jimenez et al., 1998; LeVine et al., 1998), 12–60 nM for Mn (D'Amico and Klawans, 1976; Kapaki et al., 1997; Jimenez-Jimenez et al., 1998), 0.28 μM for Cr (Aguilar et al., 1998), and 0.16–2.6 μM for Zn (Palm and Hallmans, 1982; Kapaki et al., 1997; Jimenez-Jimenez et al., 1998). The binding status of copper in CSF is of particular importance with regard to its catalyzing effect, because only protein-unbound or loosely bound copper, which can form a low molecular weight complex with cysteine, can catalyze cysteine autoxidation (Halliwell and Gutteridge, 1985). The concentration of loosely bound copper detected in human CSF ranges from 0.13 to 0.75 μM (Gutteridge, 1984). By comparing the efficient catalyzing concentrations of these transition metal ions and their physiological ranges, we suggest that copper is the major determinant influencing the cysteine autoxidation rate in CSF.

The mechanism of copper-catalyzed cysteine autoxidation has been investigated extensively (Cavallini et al., 1969; Munday, 1989; Kachur et al., 1999). It has been suggested that the intermediate cysteine–Cu complex is initially formed in a 2:1 ratio. Cysteine, as well as other low molecular weight thiol compounds, can donate electrons via catalysts. O_2 generally acts as an oxidant, accepting electrons one by one to generate reactive oxygen species: $\cdot O_2^-$, H_2O_2, and ·OH. In CSF, cysteine (1.12 ± 0.14 μM) and glutathione (5.87 ± 0.29 μM) are the major low molecular weight thiols (Wang and Cynader, 2000). The constant autoxidation of these thiols will place neurons in a situation of oxidative stress if no mechanisms exist to remove the generated oxygen radicals. The hydroxyl radical, which has an extremely short half-life of 10^{-9} sec (Pryor, 1986), is the major damaging radical. Once ·OH is produced, it rapidly attacks polyunsaturated fatty acids to initiate the chain reaction of lipid peroxidation, as well as DNA, proteins, and carbohydrates. H_2O_2 itself is stable and nontoxic and is the one-step precursor of ·OH. Eliminating H_2O_2 can therefore block the formation of ·OH. Although cytoplasmic enzymes, such as GPx and catalase, can eliminate membrane-permeable H_2O_2 intracellularly, H_2O_2 permeating the cell membrane itself is risky for cells. In addition, extracellularly generated H_2O_2 will be reduced to ·OH if it is not scavenged immediately. Therefore, it will be more efficient and beneficial if extracellularly derived H_2O_2 can be eliminated *in situ* in the extracellular space.

We have explored two possible extracellular H_2O_2-eliminating mechanisms, including enzymatic mechanisms and small molecule antioxidant mechanisms. The activities of the two most plausible antioxidative enzymes, GPx and catalase, were not detected in ACM or CSF. The trophic effect of pyruvate on neuronal survival has long been reported (Selak et al., 1985; Katoh-Semba et al., 1988; Izumi et al., 1994; Matsumoto et al., 1994). Recently, pyruvate was found to protect neurons (Desagher et al., 1997) or cell lines (Giandomenico et al., 1997) from H_2O_2-induced toxicity. We show that pyruvate, as well as catalase, can inhibit the production of ·OH generated from cysteine autoxidation by removing H_2O_2. The neuroprotective effect of pyruvate cannot be attributed to its role in energy metabolism,

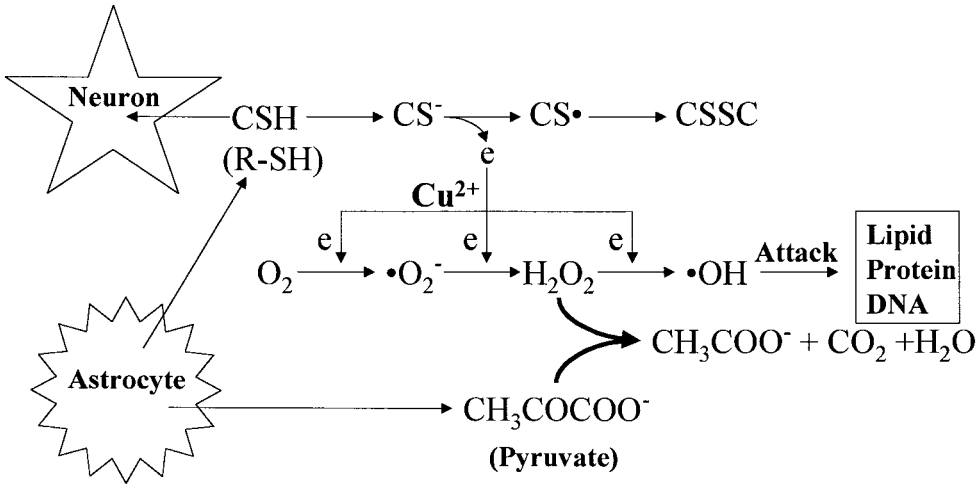

Figure 10. Diagram of the proposed mechanism of protection by astrocytes in preventing cysteine toxicity catalyzed by copper. Astrocytes release glutathione and indirectly produce cysteine in the extracellular fluid of the CNS. Cysteine, as well as glutathione or other thiols, will be oxidized to disulfide under the catalysis of protein-unbound or loosely bound copper. Molecular oxygen, as the oxidant, accepts electrons step by step to produce superoxide radicals, hydrogen peroxide, and hydroxyl radicals. The latter is the major damaging free radical to the cells. In parallel, astrocytes also release pyruvate, which can react with hydrogen peroxide, preventing the formation of hydroxyl radicals.

because lactate, a metabolic substrate, did not prevent the cysteine neurotoxicity and ·OH production from cysteine autoxidation.

The source of pyruvate in the extracellular fluid and CSF has not been studied extensively, as has lactate. The lactate pool is mainly contributed by astrocytes, and much evidence supports an astrocyte-to-neuron lactate shuttle mechanism (Walz and Mukerji, 1988; Dringen et al., 1993; Tsacopoulos and Magistretti, 1996; Schousboe et al., 1997). A specific monocarboxylate transporter is responsible for the transport of pyruvate and lactate across the plasma membrane (Poole and Halestrap, 1993; Garcia et al., 1994). It is a facilitated transport, and the net flux is mainly determined by the concentration gradient of substrates across the membrane. Our studies have examined pyruvate concentrations in different compartments: ACM (254 ± 15 μM) > CSF (131 ± 9 μM) > NCM (45.6 ± 4.4 μM). The related data from other investigators are as follows: rat ACM, 320 μM (Selak et al., 1985); adult rat CSF, ~200 μM (Vannucci and Duffy, 1976); basal intracellular pyruvate concentration of cultured striatal neurons, ~90 μM (Desagher et al., 1997). The concentration gradients among these different compartments suggest a net flux of pyruvate from astrocytes to extracellular pool, then to neurons. In agreement with this, other reports show that astrocytes release pyruvate (Pellerin and Magistretti, 1994) and that neurons can use pyruvate for their function recovery or survival (Selak et al., 1985; Matsumoto et al., 1994; Yoshioka et al., 2000). Given the evidence above, it is suggested that the extracellular pool of pyruvate is contributed mainly by astrocytes rather than neurons. It is still unknown whether other brain cells such as choroidal epithelial cells or ependymocytes contribute to the pyruvate pool.

Another possible source of pyruvate is from blood. Pyruvate can be transported through the blood–brain barrier by the monocarboxylate transporter (Pardridge and Oldendorf, 1977; Miller and Oldendorf, 1986). However, the arteriovenous difference of pyruvate across the brain of adult fed rats was found to be negligible (arterial blood, 141 ± 14 μM; sinus blood, 138 ± 14 μM), whereas the arteriovenous difference for glucose was substantial (510 ± 50 μM) (Hawkins et al., 1971). This suggests that although pyruvate is permeable across the blood–brain barrier, the net transport from blood to brain contributes little to the pyruvate pool of the CNS under normal conditions. Instead, the brain uses glucose and produces pyruvate on its own. Taken together, these findings lead to the set of mechanisms outlined in Figure 10 as those underlying the neurosupportive effect of astrocytes against cysteine toxicity.

Our data clearly indicate the importance of copper in oxidative stress. Wilson's disease is an inherited disorder characterized by hepatic cirrhosis and neuronal degeneration attributable to an impairment of copper excretion (Loudianos and Gitlin, 2000). The pathological changes in the CNS include copper deposition (10- to 15-fold over normal) in virtually all parts of brain with resulting extensive neuronal loss and gliosis in the gray matter (Scheinberg and Sternlieb, 1983). Plasma loosely bound copper and free radical production are markedly increased in Wilson's disease (Ogihara et al., 1995). However, the molecular mechanism of copper-induced oxidative stress and cytotoxicity is still uncertain. Our studies suggest that elevated free copper in the CNS accelerates the autoxidation of cysteine and other reducing agents, resulting in increased production of free radicals and subsequent cytotoxicity in Wilson's disease. Mutations of Cu/Zn superoxide dismutase have been identified in patients with familial amyotrophic lateral sclerosis (FALS) (Rosen et al., 1993). Further studies have found that oxidative stress caused by altered copper coordination is the major pathogenic factor in this FALS model (Estevez et al., 1999; Gabbianelli et al., 1999). Copper has also been found to mediate the deposition of Aβ in Alzheimer's disease (Huang et al., 1999). It is predicted that copper will be found to be involved in the pathogenesis of many neurodegenerative diseases and other oxidative stress-related conditions.

REFERENCES

Aguilar MV, Jimenez-Jimenez FJ, Molina JA, Meseguer I, Mateos-Vega CJ, Gonzalez-Munoz MJ, de Bustos F, Gomez-Escalonilla C, Ort-Pareja M, Zurdo M, Martinez-Para MC (1998) Cerebrospinal fluid selenium and chromium levels in patients with Parkinson's disease. J Neural Transm 105:1245–1251.

Beutler E (1989) Nutritional and metabolic aspects of glutathione. Annu Rev Nutr 9:287–302.

Bunton CA (1949) Oxidation of α-diketones and α-keto-acids by hydrogen peroxide. Nature 163:444.

Cavallini D, De Marco C, Dupre S, Rotilio G (1969) The copper catalyzed oxidation of cysteine to cystine. Arch Biochem Biophys 130:354–361.

Chevion M (1988) A site-specific mechanism for free radical induced biological damage: the essential role of redox-active transition metals. Free Radic Biol Med 5:27–37.

Collins AK, Makrigiorgos GM, Svensson GK (1994) Coumarin chemical dosimeter for radiation therapy. Med Phys 21:1741–1747.

D'Amico DJ, Klawans HL (1976) Direct microdetermination of manganese in normal serum and cerebrospinal fluid by flameless atomic absorption spectrophotometry. Anal Chem 48:1469–1472.

Denizot F, Lang R (1986) Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J Immunol Methods 89:271–277.

Desagher S, Glowinski J, Premont J (1997) Pyruvate protects neurons against hydrogen peroxide-induced toxicity. J Neurosci 17:9060–9067.

Dringen R, Wiesinger H, Hamprecht B (1993) Uptake of L-lactate by cultured rat brain neurons. Neurosci Lett 163:5–7.

Duffy S, So A, Murphy TH (1998) Activation of endogenous antioxidant defenses in neuronal cells prevents free radical-mediated damage. J Neurochem 71:69–77.

Ellman GL (1959) Tissue sulfhydryl groups. Arch Biochem Biophys 82:70–77.

Estevez AG, Crow JP, Sampson JB, Reiter C, Zhuang Y, Richardson GJ, Tarpey MM, Barbeito L, Beckman JS (1999) Induction of nitric oxide-dependent apoptosis in motor neurons by zinc-deficient superoxide dismutase. Science 286:2498–2500.

Gabbianelli R, Ferri A, Rotilio G, Carri MT (1999) Aberrant copper chemistry as a major mediator of oxidative stress in a human cellular model of amyotrophic lateral sclerosis. J Neurochem 73:1175–1180.

Garcia CK, Goldstein JL, Pathak RK, Anderson RG, Brown MS (1994) Molecular characterization of a membrane transporter for lactate, pyruvate, and other monocarboxylates: implications for the Cori cycle. Cell 76:865–873.

Giandomenico AR, Cerniglia GE, Biaglow JE, Stevens CW, Koch CJ (1997) The importance of sodium pyruvate in assessing damage produced by hydrogen peroxide. Free Radic Biol Med 23:426–434.

Gutteridge JMC (1984) Copper-phenanthroline-induced site-specific oxygen-radical damage to DNA. Detection of loosely bound trace copper in biological fluids. Biochem J 218:983–985.

Halliwell B, Gutteridge JMC (1985) Oxygen radicals and the nervous system. Trends Neurosci 8:22–26.

Hawkins RA, Williamson DH, Krebs HA (1971) Ketone-body utilization by adult and suckling rat brain in vivo. Biochem J 122:13–18.

Holleman AF (1904) Notice sur l'action de l'eau oxygenee sur les acides α-cetoniques et sur les dicetones 1.2. Rec Trav Chim Pays-Bas Belg 23:169–172.

Hu ML (1994) Measurement of protein thiol groups and glutathione in plasma. Methods Enzymol 233:380–385.

Huang X, Cuajungco MP, Atwood CS, Hartshorn MA, Tyndall JD, Hanson GR, Stokes KC, Leopold M, Multhaup G, Goldstein LE, Scarpa RC, Saunders AJ, Lim J, Moir RD, Glabe C, Bowden EF, Masters CL, Fairlie DP, Tanzi RE, Bush AI (1999) Cu(II) potentiation of alzheimer abeta neurotoxicity. Correlation with cell-free hydrogen peroxide production and metal reduction. J Biol Chem 274:37111–37116.

Izumi Y, Benz AM, Zorumski CF, Olney JW (1994) Effects of lactate and pyruvate on glucose deprivation in rat hippocampal slices. NeuroReport 5:617–620.

Janaky R, Varga V, Saransaari P, Oja SS (1993) Glutathione modulates the *N*-methyl-D-aspartate receptor-activated calcium influx into cultured rat cerebellar granule cells. Neurosci Lett 156:153–157.

Jimenez-Jimenez FJ, Molina JA, Aguilar MV, Meseguer I, Mateos-Vega

CJ, Gonzalez-Munoz MJ, de Bustos F, Martinez-Salio A, Orti-Pareja M, Zurdo M, Martinez-Para MC (1998) Cerebrospinal fluid levels of transition metals in patients with Parkinson's disease. J Neural Transm 105:497–505.
Joergstuerenburg H, Oechsner M, Schroeder S, Kunze K (1999) Determinants of the copper concentration in cerebrospinal fluid. J Neurol Neurosurg Psychiatry 67:252–253.
Kachur AV, Koch CJ, Biaglow JE (1999) Mechanism of copper-catalyzed autoxidation of cysteine. Free Radic Res 31:23–34.
Kapaki E, Zournas C, Kanias G, Zambelis T, Kakami A, Papageorgiou C (1997) Essential trace element alterations in amyotrophic lateral sclerosis. J Neurol Sci 147:171–175.
Karlsen RL, Grofova I, Malthe-Sorenssen D, Fonnum F (1981) Morphological changes in rat brain induced by L-cysteine injection in newborn animals. Brain Res 208:167–180.
Katoh-Semba R, Keino H, Kashiwamata S (1988) A possible contribution by glial cells to neuronal energy production: enzyme-histochemical studies in the developing rat cerebellum. Cell Tissue Res 252:133–139.
Kranich O, Hamprecht B, Dringen R (1996) Different preferences in the utilization of amino acids for glutathione synthesis in cultured neurons and astroglial cells derived from rat brain. Neurosci Lett 219:211–214.
LeVine SM, Wulser MJ, Lynch SG (1998) Iron quantification in cerebrospinal fluid. Anal Biochem 265:74–78.
Loudianos G, Gitlin JD (2000) Wilson's disease. Semin Liver Dis 20:353–364.
Makar TK, Nedergaard M, Preuss A, Gelbard AS, Perumal AS, Cooper AJ (1994) Vitamin E, ascorbate, glutathione, glutathione disulfide, and enzymes of glutathione metabolism in cultures of chick astrocytes and neurons: evidence that astrocytes play an important role in antioxidative processes in the brain. J Neurochem 62:45–53.
Matsumoto K, Yamada K, Kohmura E, Kinoshita A, Hayakawa T (1994) Role of pyruvate in ischaemia-like conditions on cultured neurons. Neurol Res 16:460–464.
McCarthy KD, de Vellis J (1980) Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. J Cell Biol 85:890–902.
Miller LP, Oldendorf WH (1986) Regional kinetic constants for blood-brain barrier pyruvic acid transport in conscious rats by the monocarboxylic acid carrier. J Neurochem 46:1412–1416.
Munday R (1988) Dialuric acid autoxidation. Effects of transition metals on the reaction rate and on the generation of "active oxygen" species. Biochem Pharmacol 37:409–413.
Munday R (1989) Toxicity of thiols and disulphides: involvement of free-radical species. Free Radic Biol Med 7:659–673.
Nath KA, Salahudeen AK (1993) Autoxidation of cysteine generates hydrogen peroxide: cytotoxicity and attenuation by pyruvate. Am J Physiol 264:F306–314.
O'Donnell-Tormey J, Nathan CF, Lanks K, DeBoer CJ, de la Harpe J (1987) Secretion of pyruvate. An antioxidant defense of mammalian cells. J Exp Med 165:500–514.
Ogihara H, Ogihara T, Miki M, Yasuda H, Mino M (1995) Plasma copper and antioxidant status in Wilson's disease. Pediatr Res 37:219–226.
Olney JW, Ho OL, Rhee V, Schainker B (1972) Cysteine-induced brain damage in infant and fetal rodents. Brain Res 45:309–313.
Olney JW, Zorumski C, Price MT, Labruyere J (1990) L-cysteine, a bicarbonate-sensitive endogenous excitotoxin. Science 248:596–599.
Palm R, Hallmans G (1982) Zinc concentrations in the cerebrospinal fluid of normal adults and patients with neurological diseases. J Neurol Neurosurg Psychiatry 45:685–690.
Pardridge WM, Oldendorf WH (1977) Transport of metabolic substrates through the blood-brain barrier. J Neurochem 28:5–12.
Pellerin L, Magistretti PJ (1994) Glutamate uptake into astrocytes stimulates aerobic glycolysis: a mechanism coupling neuronal activity to glucose utilization. Proc Natl Acad Sci USA 91:10625–10629.
Poole RC, Halestrap AP (1993) Transport of lactate and other monocarboxylates across mammalian plasma membranes. Am J Physiol 264:C761–782.
Pryor WA (1986) Oxy-radicals and related species: their formation, lifetimes, and reactions. Annu Rev Physiol 48:657–667.
Puka-Sundvall M, Eriksson P, Nilsson M, Sandberg M, Lehmann A (1995) Neurotoxicity of cysteine: interaction with glutamate. Brain Res 705:65–70.
Reed DJ, Babson JR, Beatty PW, Brodie AE, Ellis WW, Potter DW (1980) High-performance liquid chromatography analysis of nanomole levels of glutathione, glutathione disulfide, and related thiols and disulfides. Anal Biochem 106:55–62.
Regan RF, Guo Y (1999) Extracellular reduced glutathione increases neuronal vulnerability to combined chemical hypoxia and glucose deprivation. Brain Res 817:145–150.
Rosen DR, Siddique T, Patterson D, Figlewicz DA, Sapp P, Hentati A, Donaldson D, Goto J, O'Regan JP, Deng HX, Rahmani Z, Krizus A, McKenna-Yasek D, Cayabyab A, Gaston SM, Berger R, Tanzi RE, Halperin JJ, Herzfeldt B, Van den Bergh R, Hung WY, Bird T, Deng G, Mulder DW, Smyth C, Laing NG, Soriano E, Pericak-Vance MA, Haines J, Rouleau GA, Gusella JS, Horvitz HR, Brown Jr RH (1993) Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. Nature [Erratum (1993) 364:362] 362:59–62.
Saez G, Thornalley PJ, Hill HA, Hems R, Bannister JV (1982) The production of free radicals during the autoxidation of cysteine and their effect on isolated rat hepatocytes. Biochim Biophys Acta 719:24–31.
Scheinberg IH, Sternlieb I (1983) Wilson's disease. Philadelphia, PA: W. B. Saunders.
Schousboe A, Westergaard N, Waagepetersen HS, Larsson OM, Bakken IJ, Sonnewald U (1997) Trafficking between glia and neurons of TCA cycle intermediates and related metabolites. Glia 21:99–105.
Selak I, Skaper SD, Varon S (1985) Pyruvate participation in the low molecular weight trophic activity for central nervous system neurons in glia-conditioned media. J Neurosci 5:23–28.
Slater TF, Sawyer B, Strauli U (1963) Studies on succinate-tetrazolium reductase systems. III. Points of coupling of four different tetrazolium salts. Biochim Biophys Acta 77:383–393.
Stuerenburg HJ (2000) CSF copper concentrations, blood-brain barrier function, and coeruloplasmin synthesis during the treatment of Wilson's disease. J Neural Transm 107:321–329.
Sucher NJ, Lipton SA (1991) Redox modulatory site of the NMDA receptor-channel complex: regulation by oxidized glutathione. J Neurosci Res 30:582–591.
Takahashi K, Avissar N, Whitin J, Cohen H (1987) Purification and characterization of human plasma glutathione peroxidase: a selenoglycoprotein distinct from the known cellular enzyme. Arch Biochem Biophys 256:677–686.
Tsacopoulos M, Magistretti PJ (1996) Metabolic coupling between glia and neurons. J Neurosci 16:877–885.
Vannucci RC, Duffy TE (1976) Carbohydrate metabolism in fetal and neonatal rat brain during anoxia and recovery. Am J Physiol 230:1269–1275.
Von Korff RW (1969) Purity and stability of pyruvate and α-ketoglutarate. Methods Enzymol 13:519–523.
Walz W, Mukerji S (1988) Lactate production and release in cultured astrocytes. Neurosci Lett 86:296–300.
Wang XF, Cynader MS (1999) Effects of astrocytes on neuronal attachment and survival shown in a serum-free co-culture system. Brain Res Brain Res Protoc 4:209–216.
Wang XF, Cynader MS (2000) Astrocytes provide cysteine to neurons by releasing glutathione. J Neurochem 74:1434–1442.
Yoshioka K, Nisimaru N, Yanai S, Shimoda H, Yamada K (2000) Characteristics of monocarboxylates as energy substrates other than glucose in rat brain slices and the effect of selective glial poisoning: a 31P NMR study. Neurosci Res 36:215–226.