

Effects of Citicoline on Phospholipid and Glutathione Levels in Transient Cerebral Ischemia

Rao Muralikrishna Adibhatla, PhD; J.F. Hatcher, BS; R.J. Dempsey, MD

Background and Purpose—Cytidine-5'-diphosphocholine (citicoline or CDP-choline) is an essential intermediate in the biosynthesis of phosphatidylcholine, an important component of the neural cell membrane. Citicoline provided significant neuroprotection after transient forebrain ischemia in gerbils. This study was undertaken to examine changes and effects of citicoline on phospholipids and glutathione synthesis after transient cerebral ischemia and reperfusion.

Methods—Ten-minute transient forebrain ischemia was induced by bilateral carotid artery occlusion in male Mongolian gerbils with reperfusion up to 6 days. Citicoline (500 mg/kg IP in saline) was given to gerbils just after the end of ischemia, at 3-hour reperfusion, and daily thereafter until 1 day before euthanasia. Hippocampal lipids were extracted and analyzed by thin-layer and gas chromatography. Glutathione was measured by using an enzymatic recycling assay. Glutathione reductase activity was determined by measuring NADPH oxidation.

Results—Significant decreases in phospholipids occurred at 1-day reperfusion. Citicoline significantly restored the phosphatidylcholine, sphingomyelin, and cardiolipin levels but did not affect phosphatidylinositol and phosphatidylserine at 1 day. The phospholipids returned to sham levels over days 2 to 6 and were unaffected by citicoline. Ceramide levels significantly increased by 3 and 6 days of reperfusion and were unaltered by citicoline. Ischemia resulted in significant decreases in glutathione and glutathione reductase activity over 3 days of reperfusion. Citicoline significantly increased total glutathione and glutathione reductase activity and decreased the glutathione oxidation ratio, an indicator of glutathione redox status.

Conclusions—Our data indicated that the effects of citicoline on phospholipids occurred primarily during the first day of reperfusion, with effects on glutathione being important over the 3-day reperfusion period. (*Stroke*. 2001;32:2376-2381.)

Key Words: cytidine diphosphate choline ■ glutathione reductase ■ mitochondria ■ neuronal death
■ phosphatidylcholines ■ phospholipases ■ gerbils

Transient forebrain ischemia results in delayed hippocampal CA₁ neuronal death beginning 3 days after the incident and culminating by day 6.^{1,2} Activation of phospholipases, hydrolysis of phospholipids, arachidonic acid release (Figure 1), and lipid peroxidation are important promoters of neuronal death after transient cerebral ischemia.³⁻⁵ Arachidonic acid metabolism generates reactive oxygen species,^{4,6} which form lipid peroxides, and, subsequently, 4-hydroxynonenal, which initiates apoptosis.⁷ Cytidine 5'-diphosphocholine (citicoline or CDP-choline), an endogenous intermediate in the biosynthesis of phosphatidylcholine (PtdCho)⁸ (Figure 2), has shown beneficial effects in cerebral ischemia⁹ and other models of central nervous system injury.¹⁰⁻¹⁴

Exogenous citicoline is hydrolyzed, absorbed as cytidine and choline, and resynthesized in the brain from its phosphorylated components (Figure 2) by cytidine triphosphate-phosphocholine cytidyltransferase.¹⁵ As the intermediate in PtdCho synthesis, it is generally believed that citicoline

neuroprotection is associated with increased PtdCho synthesis. Daily treatment for 90 days with citicoline increased brain PtdCho levels by 25% in normal rats.¹⁶ Pretreatment with citicoline attenuated the loss of PtdCho during ischemia (no reperfusion).¹⁷

We have previously shown that citicoline attenuated arachidonic acid release and partially restored phospholipid loss after ischemia/1-day reperfusion.^{9,18} Citicoline can increase the synthesis of PtdCho⁸ and sphingomyelin^{19,20} and has been shown to prevent the activation of phospholipase A₂.²¹ Therefore, citicoline may affect the levels of many lipids after ischemia and reperfusion. In our previous studies, citicoline administered at 0 and 3 hours after transient ischemia showed significant neuroprotection, but greater protection was obtained if citicoline treatment was given daily after the initial 2 doses.^{9,22} These data indicate that citicoline is acting on events beyond the first day of reperfusion that are important for its neuroprotective effects.

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From the Department of Neurological Surgery (R.M.A., J.F.H., R.J.D.), the Cardiovascular Research Center (R.M.A.), University of Wisconsin, Madison, and the Veterans Administration Hospital (R.M.A., R.J.D.), Madison, Wis.

Reprint requests to Dr Rao Muralikrishna Adibhatla, Department of Neurological Surgery, H4-330, Clinical Science Center, 600 Highland Ave, University of Wisconsin-Madison, Madison, WI 53792-3232. E-mail adibhatl@neurosurg.wisc.edu

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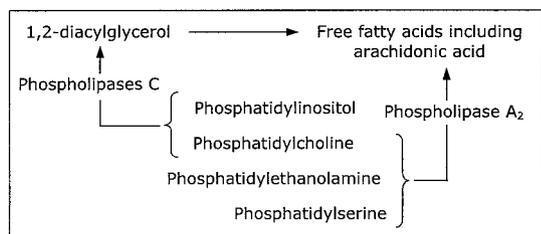


Figure 1. Actions of phospholipases.

Choline liberated from citicoline can be metabolized to methionine,²³ which may be further converted to glutathione through *S*-adenosyl-L-methionine (Figure 2). Glutathione is one of the primary endogenous antioxidant defense systems in the brain. Significant decreases in glutathione and glutathione reductase activity have been reported after transient cerebral ischemia.²⁴ Citicoline may provide neuroprotection by increasing this defense system, because oxidative damage contributes to neuronal death.³⁻⁵ The aim of the present study was to determine whether daily administration of citicoline prevents lipid alteration over 6 days of reperfusion and also increases glutathione levels in transient forebrain ischemia in the gerbil.

Materials and Methods

The following materials were obtained from the indicated suppliers: chemicals, lipid standards, and glutathione reductase (Sigma Chemical Co); citicoline (BioMol); high-performance liquid chromatographic grade solvents and E Merck silica gel 60 thin-layer chromatographic (TLC) plates (Fisher Scientific); and silica gel G, H, and GHL TLC plates (Analtech).

Transient Forebrain Ischemia

All surgical procedures were conducted according to the animal welfare guidelines set forth in the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health, US Department of Health and Human Services, publication No. 85-23, 1985) and were approved by the animal care committee of the University of Wisconsin-Madison. Male Mongolian gerbils (50 to 80 g) were anesthetized with 1% halothane in 70:30 N₂O:O₂. Both carotid arteries were exposed by a neck incision, occluded for 10 minutes, and reperfused for up to 6 days.^{3,9,18,22} Citicoline (500 mg/kg IP) was given to gerbils just after the end of ischemia, at 3 hours of reperfusion, and daily thereafter until 1 day before euthanasia.⁹ For a 60-g gerbil, each citicoline dose (200 mg/mL saline) was given in a volume of 150 μ L. Treatment with citicoline did not affect the brain temperature, mean arterial blood pressure, or arterial PO₂ and PCO₂ for the sham-operated and ischemic groups during 3-hour postischemic reperfusion.^{9,11,18,25} A total of 84 gerbils were used for lipid analyses in the present study and divided into the following groups: sham-operated group (shams)+vehicle (saline), n=12; shams+citicoline, n=8; ischemia+vehicle (saline), n=8 for each time point (1, 2, 3, and 6 days); and ischemia+citicoline, n=8 for

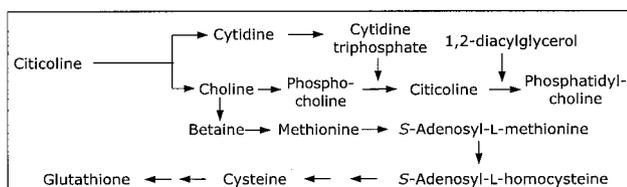


Figure 2. Citicoline metabolism and PtdCho synthesis.

each time point (1, 2, 3, and 6 days). For glutathione studies, 96 gerbils were assigned to the following 12 groups (n=8 each): shams+vehicle (saline), shams+citicoline, ischemia+vehicle (saline) (30 minutes, 2 hours, 6 hours, 1 day, and 3 days), and ischemia+citicoline (30 minutes, 2 hours, 6 hours, 1 day, and 3 days). Sham-operated gerbils received either saline or citicoline at 0 hours, at 3 hours, and daily on days 1 and 2 or on days 1 to 5 and were euthanized on day 3 (glutathione) or day 6 (lipids). Brains of the anesthetized gerbils were frozen in situ, and hippocampi were dissected at 0°C for analysis.^{3,9}

Lipid Analysis

All solvents and extracts were purged with nitrogen during the extraction, TLC, and methylation of lipids. Lipids from hippocampi were extracted into chloroform:methanol (1:2 [vol/vol]) containing 0.01% butylated hydroxytoluene. The TLC conditions and solvent systems for separation of lipids have been described previously.^{18,22} The lipids were identified by using authentic standards, converted to fatty acid methyl esters, and analyzed by gas chromatography with the use of 17:0 as an internal standard.¹⁸ Blank TLC regions did not show any gas chromatographic peaks corresponding to palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2), arachidonic (20:4), and docosahexaenoic (22:6) acids.

Glutathione Reductase Activity, Total Glutathione (GSH+GSSG) and GSSG

Hippocampi were homogenized in 0.1 mol/L sodium phosphate/5 mmol/L EDTA, pH 7.5. Immediately after homogenization, several aliquots of each sample were frozen in liquid nitrogen and stored at -70°C. The remaining homogenate was diluted with 2 vol phosphate/EDTA buffer. After centrifugation (18 000g for 10 minutes), the supernatant was assayed for glutathione reductase activity by using a 96-well microtiter plate reader (Spectra Max 190, Molecular Devices). The activity was determined by measuring the rate of decrease in absorbance of NADPH at 340 nm in the presence of oxidized glutathione (GSSG)²⁶ compared with a standard curve of glutathione reductase.

For total glutathione, 1 aliquot of each sample was thawed and diluted with 9 vol of 5.55% 5-sulfosalicylic acid to remove proteins. After centrifugation, the supernatants (5 and 10 μ L) were analyzed for total glutathione in a 96-well microtiter plate by use of the 5,5'-dithiobis-(nitrobenzoic acid) enzymatic recycling assay.²⁷ Reduced glutathione (GSH) dissolved in 5% 5-sulfosalicylic acid was used for the standard curve. 5-Sulfosalicylic acid was added to samples and standards so that all wells contained 10 μ L of 5% 5-sulfosalicylic acid in a final reaction volume of 175 μ L.

For measurement of GSSG, a second aliquot was thawed and diluted with an equal volume of 10% 5-sulfosalicylic acid. After centrifugation as described above, 100 μ L of supernatant was derivatized with 2 μ L of 2-vinylpyridine and 6 μ L triethanolamine to remove GSH. The reaction was conducted in the dark for 1 hour at room temperature and then assayed as described above for total glutathione, except GSSG was used for the standard curve.

Statistical Analysis

Data are presented as mean \pm SD and were analyzed by using ANOVA, followed by the Bonferroni multigroup comparison post hoc test. A value of $P < 0.05$ was considered significant.

Results

In the Table, the total represents the sum of each of the individual fatty acids: palmitic, stearic, oleic, linoleic, arachidonic, and docosahexaenoic in the respective lipids. Significant differences are reported for the ischemia/saline groups compared with the sham/saline group and for the ischemia/citicoline groups compared with the corresponding ischemia/saline group, and probability values are given in the Table.

Time Course of Lipid Changes and Citicoline Effect After 10-min Forebrain Ischemia/Reperfusion

Reperfusion	Total Fatty Acids, $\mu\text{mol/g}$ Wet Wt					
	Phospholipids Altered by Citicoline					
	Phosphatidylcholine		Sphingomyelin		Cardiolipin	
	Saline	Citicoline	Saline	Citicoline	Saline	Citicoline
Sham	27.9 \pm 1.49	27.7 \pm 1.55	2.09 \pm 0.16	2.13 \pm 0.17	1.60 \pm 0.29	1.58 \pm 0.32
1-day	22.7 \pm 1.46*	24.8 \pm 0.70†§	1.81 \pm 0.12†	2.11 \pm 0.28§	1.25 \pm 0.17†	1.63 \pm 0.20§
2-day	28.0 \pm 0.57	26.4 \pm 2.34	2.28 \pm 0.14	2.20 \pm 0.28	1.81 \pm 0.01	1.99 \pm 0.22
3-day	26.2 \pm 1.21	26.2 \pm 1.14	2.13 \pm 0.15	2.26 \pm 0.25	1.46 \pm 0.52	1.82 \pm 0.37
6-day	28.0 \pm 1.54	26.2 \pm 0.56	2.26 \pm 0.02	2.11 \pm 0.19	1.60 \pm 0.10	1.40 \pm 0.26
Reperfusion	Phospholipids Unaltered by Citicoline					
	Phosphatidylethanolamine		Phosphatidylinositol		Phosphatidylserine	
	Saline	Citicoline	Saline	Citicoline	Saline	Citicoline
	Saline	Citicoline	Saline	Citicoline	Saline	Citicoline
Sham	21.3 \pm 1.60	21.3 \pm 1.7	2.84 \pm 0.24	2.81 \pm 0.19	9.24 \pm 1.13	9.32 \pm 1.04
1-day	22.0 \pm 1.42	20.3 \pm 2.15	1.86 \pm 0.18*	1.94 \pm 0.25*	6.99 \pm 0.37*	6.33 \pm 1.02*
2-day	21.2 \pm 0.61	19.8 \pm 1.44	2.61 \pm 0.47	2.43 \pm 0.08	9.32 \pm 0.44	8.77 \pm 0.31
3-day	19.5 \pm 1.80	20.4 \pm 0.82	2.46 \pm 0.13	2.51 \pm 0.01	8.94 \pm 0.41	8.61 \pm 0.41
6-day	20.9 \pm 0.54	19.5 \pm 1.14	2.74 \pm 0.05	2.46 \pm 0.07	9.40 \pm 0.50	8.70 \pm 0.52
Reperfusion	Phospholipase/Sphingomyelinase Products Unaltered by Citicoline					
	Free Fatty Acids		1,2-Diacylglycerol		Ceramide	
	Saline	Citicoline	Saline	Citicoline	Saline	Citicoline
	Saline	Citicoline	Saline	Citicoline	Saline	Citicoline
Sham	0.38 \pm 0.09	0.37 \pm 0.08	0.31 \pm 0.05	0.32 \pm 0.04	0.16 \pm 0.03	0.16 \pm 0.03
1-day	0.58 \pm 0.24*	0.24 \pm 0.12‡	0.33 \pm 0.12	0.25 \pm 0.02	0.13 \pm 0.02	0.13 \pm 0.02
2-day	0.48 \pm 0.07	0.41 \pm 0.01	0.43 \pm 0.04	0.43 \pm 0.14	0.19 \pm 0.02	0.19 \pm 0.03
3-day	0.49 \pm 0.16	0.48 \pm 0.03	0.37 \pm 0.07	0.40 \pm 0.05	0.22 \pm 0.01†	0.22 \pm 0.04
6-day	0.43 \pm 0.05	0.49 \pm 0.03	0.38 \pm 0.02	0.45 \pm 0.05	0.26 \pm 0.05*	0.27 \pm 0.11

Total fatty acids (palmitic 16:0, stearic 18:0, oleic 18:1, linoleic 18:2, arachidonic 20:4 and docosahexaenoic 22:6) in $\mu\text{mol/g}$ wet wt. of hippocampi are given for the respective lipids.

* $P < 0.01$ and † $P < 0.05$ vs shams; ‡ $P < 0.01$ and § $P < 0.05$ vs respective ischemia/saline group.

Our studies showed that PtdCho, phosphatidylinositol, phosphatidylserine, cardiolipin, and sphingomyelin levels were decreased after 10-minute ischemia/1-day reperfusion (Table). Citicoline administration significantly restored Ptd-Cho, sphingomyelin, and cardiolipin levels at 1 day of reperfusion but had no effect on phosphatidylinositol or phosphatidylserine.¹⁸ Phospholipids returned to sham levels over 2 to 6 days of reperfusion, and citicoline had no significant effect on these levels. Sham groups treated with citicoline did not show any significant changes in lipids compared with saline-treated sham groups (Table).

Phosphatidylethanolamine and 1,2-diacylglycerol levels were unaltered over 6 days after ischemia, with or without citicoline treatment (Table). Free fatty acids were significantly elevated only at 1 day of reperfusion, which returned to sham levels with citicoline treatment (Table). In contrast, ceramide levels were significantly increased at 3 and 6 days of reperfusion and were not significantly altered by citicoline.

Glutathione and Glutathione Reductase

Total glutathione (GSH+GSSG) (Figure 3A) and GSSG (Figure 3B) levels and glutathione reductase activity (Figure 3C) were determined in the gerbil hippocampus over a 3-day reperfusion period after 10 minutes of transient forebrain ischemia and treatment with either saline or citicoline.

After ischemia, total glutathione levels remained unaltered during 6-hour reperfusion but showed statistically significant decreases at 1 and 3 days ($P < 0.01$ compared with shams). When citicoline was administered at the onset of reperfusion, total glutathione was significantly increased at 30 minutes ($P < 0.01$ compared with shams and $P < 0.05$ compared with ischemia/saline at 30-minute reperfusion). By 2 hours, total glutathione had returned to sham levels. After 2 doses of citicoline at 0 and 3 hours of reperfusion, total glutathione showed a second increase at 6 hours ($P < 0.01$ compared with shams and compared with ischemia/saline at 6-hour reperfusion), which declined to sham levels by 1 day. Further treatment with citicoline on days 1 and 2 showed no significant effect on total glutathione on day 3.

GSSG levels (Figure 3B) remained low throughout 3 days of reperfusion and accounted for only 2% to 4% of total glutathione (taking GSH equivalent as $2 \times \mu\text{mol}$ GSSG). There were no significant changes in GSSG levels over 3 days in the ischemia/saline groups. After citicoline treatment, GSSG levels showed significant decreases at 1 and 3 days ($P < 0.01$ compared with shams and compared with corresponding ischemia/saline groups at 1 and 3 days).

Glutathione reductase activity (Figure 3C) showed a steady decline during the early reperfusion period, which was statistically significant at 2 hours ($P < 0.01$ compared with

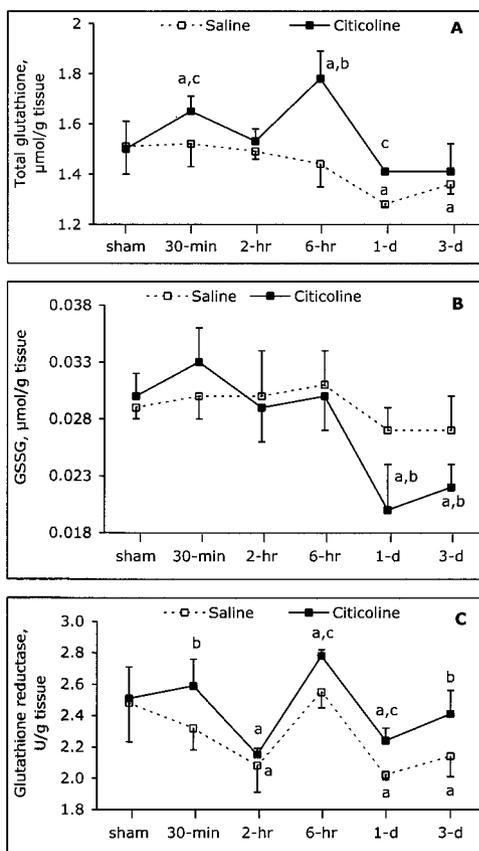


Figure 3. Total glutathione (GSH+GSSG) levels (A), GSSG levels (B), and glutathione reductase activity (C) over 3-day reperfusion after 10-minute transient forebrain ischemia in gerbils. ^a $P < 0.01$ compared with sham; ^b $P < 0.01$ and ^c $P < 0.05$ compared with corresponding ischemia/saline group.

shams). Activity returned to sham levels by 6 hours and showed a second decline at 1 and 3 days ($P < 0.01$ compared with shams). Administration of citicoline at the onset of reperfusion resulted in a significant increase ($P < 0.01$) in reductase activity compared with the corresponding ischemia/saline group at 30 minutes. Similar to ischemia/saline groups, glutathione reductase activity also declined by 2 hours ($P < 0.01$ compared with shams) after citicoline treatment. When citicoline was administered at 0 and 3 hours, the reductase activity was significantly elevated at 6 hours ($P < 0.01$ compared with shams and $P < 0.05$ compared with corresponding ischemia/saline group). The reductase activity also showed a second decline at 1 day ($P < 0.01$ compared with shams), which was elevated compared with reductase activity in the ischemia/saline group at 1 day ($P < 0.05$). Further administration of citicoline on days 1 and 2 restored the reductase activity to sham levels ($P < 0.01$ compared with ischemia/saline group at 3 days).

Discussion

Phospholipases C and A_2 are activated during ischemia by release of glutamate, stimulation of neuronal receptors, and elevation of intracellular Ca^{2+} ,²⁸ resulting in hydrolysis of phospholipids and release of free fatty acids.^{3,6} The actions of these phospholipases are outlined in Figure 1.^{29–31} Sphingo-

myelinase is a C-type phospholipase that hydrolyzes sphingomyelin to release ceramide.³² Arachidonic acid (20:4) metabolism in ischemia/reperfusion generates oxygen radicals that may deplete oxidative defenses.^{4,6}

The loss of phospholipids and increase in free fatty acids suggest activation of phospholipases during 1 day of reperfusion. Citicoline significantly restored the levels of PtdCho, sphingomyelin, and cardiolipin. The partial restoration of PtdCho after 1 day of reperfusion may be due to (1) increasing synthesis and (2) preventing the activation of phospholipase A_2 ,²² and thus may have a dual role in restoring PtdCho levels. Citicoline may have a similar dual function in restoring sphingomyelin levels, because it can serve as a phosphocholine donor to ceramide and may prevent the activation of neutral sphingomyelinase.¹⁸ Data from several studies, together with our results, suggest that citicoline may have prevented cardiolipin hydrolysis by preventing the activation of a Ca^{2+} -dependent 14-kDa phospholipase A_2 .²² Even though phospholipid levels declined, the phospholipase products 1,2-diacylglycerol and ceramide did not show corresponding accumulation (Table). Both 1,2-diacylglycerol and ceramide levels appear to be tightly regulated,³³ suggesting further conversion of these lipids. More extensive discussions of these potential interactions have been presented elsewhere.^{18,22}

Ceramide levels were significantly elevated at 3 and 6 days of reperfusion compared with levels in shams (Table). Although a number of studies have implicated ceramide in the induction of apoptosis,^{34,35} its role in neuronal death remains controversial.^{36,37} In the present study, the increase in ceramide at 3 days corresponds with the onset of neuronal death.² It is conceivable that this increase is a result of neuronal death, inasmuch as ceramide levels showed further increases at 6 days of reperfusion, when neuronal death is culminated. However, citicoline treatment (0 and 3 hours and daily on days 1 to 5) did not alter ceramide levels at 6 days of reperfusion, even though these doses provided significant neuroprotection.⁹ Thus, the increase in ceramide levels did not correlate with the extent of neuronal death.

All major phospholipids returned to sham levels over 2 to 6 days of reperfusion, suggesting that phospholipases are not significantly activated over this time. This is in agreement with previous studies indicating that phospholipases are down-regulated during this period.³⁸ This endogenous restoration of phospholipids was insufficient to prevent neuronal death. In our previous studies, daily administration of citicoline provided greater neuroprotection than did 2 doses at 0 and 3 hours of reperfusion.^{9,22} These studies were undertaken to gain insight into the need for daily administration of citicoline. Although providing neuroprotection, the citicoline doses on days 1 to 5 did not result in significant changes in any of the lipids examined (Table), suggesting that other mechanisms are involved in the neuroprotection.

Choline liberated from citicoline can be metabolized to glutathione through an *S*-adenosyl-L-methionine pathway. Exogenous *S*-adenosyl-L-methionine increased glutathione levels³⁹ and provided significant neuroprotection.²⁵ Increased glutathione may contribute to neuroprotection by attenuating lipid peroxidation.¹⁸ To further investigate the neuroprotec-

tive mechanisms of citicoline, we examined the effects of citicoline on glutathione levels after ischemia/reperfusion.

In the present study, GSSG levels accounted for only 2% to 4% of total glutathione (Figure 3A and 3B); thus, the reduced form of GSH must represent 96% to 98% of the total glutathione. These findings are consistent with those in other ischemic models showing very low levels of GSSG, which were not significantly increased during reperfusion.^{40,41} Compared with total glutathione, GSSG levels showed very small changes; therefore, changes in the total represent alterations in the levels of GSH. Hippocampal extracts were also analyzed by using the glutathione-S-transferase/monochlorobimane method as described, which detects only the reduced form GSH.⁴² The results confirmed that the major fraction ($\approx 98\%$) of glutathione was present as GSH (data not shown).

In saline-treated groups after ischemia, there was a significant decline in total glutathione at 1 day and 3 days of reperfusion (Figure 3A). GSSG levels showed no significant changes over 3 days (Figure 3B). Several factors could contribute to the decrease in total glutathione without an increase in GSSG, such as cleavage of GSH to cysteine,⁴³ formation of mixed disulfides,²⁴ or decreased synthesis of GSH.²⁴ There was significant loss of glutathione reductase activity over 3 days of reperfusion, which may be due to inactivation of the enzyme by oxygen radicals generated during reperfusion.⁴⁴ The decrease in glutathione reductase activity was consistent with other reports.²⁴ Changes in glutathione reductase activity were not accompanied by changes in GSSG levels, suggesting that either the reductase activity did not become limiting or that excess GSSG was excreted or reacted with protein thiols to form mixed disulfides.⁴⁵ There was a significant increase in the glutathione oxidation ratio (calculated as $2 \times \text{GSSG}/\text{total glutathione}$, an indicator of the redox status of glutathione) at 6 hours (0.038 ± 0.003 for sham versus 0.043 ± 0.003 for 6-hour reperfusion, $P < 0.05$) and 1 day (0.043 ± 0.002 , $P < 0.05$).

In citicoline-treated ischemic groups, total glutathione increased from $1.53 \mu\text{mol/g}$ tissue at 2 hours to 1.78 at 6 hours. Two considerations suggest that this represents an increase in GSH through synthesis from cysteine⁴⁵: the levels of GSSG could not account for the increase and were not altered during this time; the levels at 6 hours exceeded sham levels, suggesting that citicoline did not simply prevent oxidation of GSH.

Citicoline treatment decreased the glutathione oxidation ratio at 6 hours (0.043 ± 0.003 for saline versus 0.034 ± 0.003 for citicoline, $P < 0.01$), at 1 day (0.043 ± 0.002 for saline versus 0.028 ± 0.004 for citicoline, $P < 0.01$), and 3 days (0.040 ± 0.003 for saline versus 0.031 ± 0.002 for citicoline, $P < 0.01$). The decreased oxidation ratio at 6 hours was mainly due to an increase in GSH, inasmuch as GSSG levels did not change. At 1 and 3 days, total glutathione levels returned to sham levels, and the decrease in oxidation ratio was due to the decrease in GSSG (Figure 3B). This indicates that citicoline attenuated the oxidative stress over 3 days of reperfusion.

Compared with ischemia/saline treatment, citicoline treatment after ischemia resulted in significant increases in glutathione reductase activity over 3 days of reperfusion (Figure

3C). If the reductase is inactivated by oxygen radicals after ischemia/reperfusion, it is conceivable that citicoline prevented this inactivation by attenuating oxygen radical formation. Citicoline decreased lipid peroxidation after transient cerebral ischemia⁴⁶; this could involve 2 mechanisms. Citicoline prevented activation of phospholipase A_2 ,^{21,22} thus attenuating oxygen radicals formed by arachidonic acid metabolism.^{4,6} The present study has demonstrated that citicoline increases glutathione levels after transient forebrain ischemia, which could increase the scavenging of oxygen radicals by GSH.

The classic viewpoint regarding citicoline is that it increases PtdCho synthesis; however, our data suggest that citicoline may be affecting the activation of phospholipase A_2 ²² and sphingomyelinase, thus preventing the hydrolysis (rather than repairing the damage) of cardiolipin, sphingomyelin, and PtdCho during 1-day reperfusion. Citicoline may prevent membrane phospholipid degradation, which could be independent of choline metabolism. Our data also suggest that citicoline did not alter the activation of other phospholipases, such as phospholipase C or D.²² Citicoline neuroprotection may involve multiple pathways, including the attenuation of phospholipase A_2 activation, stimulation of phospholipid synthesis, and increases in glutathione.¹⁸ The relative importance of these mechanisms may vary with reperfusion time. Our data suggest that the effects of citicoline on phospholipids occur primarily during the first day of reperfusion, with effects on glutathione being important over the 3-day reperfusion period.

Acknowledgments

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