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Lipid Alterations in Transient Forebrain Ischemia: Possible New Mechanisms of CDP-Choline Neuroprotection

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Abstract: We have previously demonstrated that cytidine 5′-diphosphocholine (CDP-choline or citicoline) attenuated arachidonic acid (ArAc) release and provided significant protection for the vulnerable hippocampal CA_1 neurons of the cornu ammonis after transient forebrain ischemia of gerbil. ArAc is released by the activation of phospholipases and the alteration of phosphatidylcholine (PtdCho) synthesis. Released ArAc is metabolized by cyclooxygenases/lipoxygenases to form eicosanoids and reactive oxygen species (ROS). ROS contribute to neurotoxicity through generation of lipid peroxides and the cytotoxic byproducts 4-hydroxynonenal and acrolein. ArAc can also stimulate sphingomyelinase to produce ceramide, a potent pro-apoptotic agent. In the present study, we examined the changes and effect of CDP-choline on ceramide and phospholipids including PtdCho, phosphatidylethanolamine (PtdEtn), phosphatidylinositol (PtdIns), phosphatidylserine (PtdSer), sphingomyelin, and cardiolipin (an exclusive inner mitochondrial membrane lipid essential for electron transport) following ischemia/1-day reperfusion. Our studies indicated significant decreases in total PtdCho, PtdIns, PtdSer, sphingomyelin, and cardiolipin and loss of ArAc from PtdEtn in gerbil hippocampus after 10-min forebrain ischemia/1-day reperfusion. CDP-choline (500 mg/kg i.p. immediately after ischemia and at 3-h reperfusion) significantly restored the PtdCho, sphingomyelin, and cardiolipin levels as well as the ArAc content of PtdCho and PtdEtn but did not affect PtdIns and PtdSer. These data suggest multiple beneficial effects of CDP-choline: (1) stabilizing the cell membrane by restoring PtdCho and sphingomyelin (prominent components of outer cell membrane), (2) attenuating the release of ArAc and limiting its oxidative metabolism, and (3) restoring cardiolipin levels. **Key Words:** *S*-Adenosyl-L-methionine—Arachidonic acid—CA_1 neuronal death—Cardiolipin—Ceramide—Citicoline—Glutathione—Mitochondria—Phosphatidylcholine—Phosphatidylethanolamine—Sphingomyelin.
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Alterations in lipid metabolism including activation of phospholipases and release of arachidonic acid (ArAc) are key events that contribute to neuronal death in cerebral ischemia (Lipton, 1999; Rao et al., 1999*b*,*c*; Sastry and Rao, 2000). Glutamate released during ischemia stimulates neuronal receptors, resulting in elevated intracellular Ca^{2+} and activation of phospholipases C (PLC) and A_2 (PLA_2) (Choi, 1990). PLC hydrolyzes phosphatidylinositol (PtdIns) lipids to release 1,2-diacylglycerol, which is further hydrolyzed to free fatty acids (Bazan, 1970) including ArAc. ArAc enhances glutamate release from hippocampal neurons as well as depolarization-evoked Ca^{2+} accumulation (Freeman et al., 1990; Ruehr et al., 1997). PLA_2 hydrolyzes ArAc at the *sn*-2 position of phosphatidylcholine (PtdCho), phosphatidylethanolamine (PtdEtn), and phosphatidylserine (PtdSer) (Farooqui et al., 1997). Released ArAc is either reincorporated into membranes or metabolized by cyclooxygenases/lipoxygenases to form prostaglandins, leukotrienes, and reactive oxygen species (ROS) (Katsuki and Okuda, 1995). ROS formed by ArAc metabolism generate lipid peroxides and the cytotoxic byproducts 4-hydroxynonenal (Kruman et al., 1997) and acrolein (Uchida et al., 1998; Calingasan et al., 1999), which covalently bind to cellular proteins and alter their function. ArAc can also stimulate sphingomyelinase to produce ceramide (Jayadev et al., 1994), a potent pro-apoptotic agent (Hannun and Obeid, 1995; Green and Reed, 1998; Kinloch et al., 1999; Goswami and Dawson, 2000; Sastry and Rao, 2000). Ceramide and oxygen radicals have been implicated as causative agents in mitochondrial dysfunction and release of cytochrome *c*, which initiates the apoptotic cellular death cascade by activation of caspase-3 (Garcia-Ruiz et al., 1997; Cai and Jones, 1998; Cai et al., 1998; Ghafourifar et al., 1999). Another factor implicated in the initiation of apoptosis is translocation of PtdSer from the inner to the outer leaflet of the mem-

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Abbreviations used: AdoMet, *S*-adenosyl-L-methionine; ArAc, arachidonic acid; CDP-choline, cytidine 5′-diphosphocholine; PLA_2, phospholipase A_2; PLC, phospholipase C; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine; ROS, reactive oxygen species.

brane lipid bilayer (Rimon et al., 1997; Suzuki et al., 1999).

Cytidine 5′-diphosphocholine (CDP-choline or citicoline) is an endogenous intermediate in the biosynthesis of neuronal membrane phospholipids and serves as a choline donor (Alvarez et al., 1999; Rao et al., 1999*a*,*b*). The ability of CDP-choline to alter phospholipid metabolism may be an important function in the treatment of cerebral ischemia (Dorman et al., 1983). Experimental studies have demonstrated that CDP-choline reduces neurodegeneration induced by ischemia, hypoxia, traumatic brain injury, and β-amyloid deposition in animals (D'Orlando and Sandage, 1995; Secades and Frontera, 1995; Weiss, 1995; Dixon et al., 1997; Alvarez et al., 1999; Rao et al., 1999*a*,*b*; Baskaya et al., 2000; Shuaib et al., 2000).

Exogenous CDP-choline stimulates PtdCho synthesis and attenuates the release of ArAc by restoring the phosphotransferase reaction (Trovarelli et al., 1981; Gimenez et al., 1999). Choline liberated from CDP-choline can be converted to *S*-adenosyl-L-methionine (AdoMet) via metabolism to methionine (Trovarelli et al., 1983; Rao et al., 1997, 1999*a*). AdoMet can serve as the methyl donor in the biosynthesis of PtdCho from PtdEtn in cell membranes. Previously, we suggested that CDP-choline neuroprotection may also involve increased sphingomyelin synthesis (Rao et al., 1999*a*) from pro-apoptotic ceramide (Kolesnick and Fuks, 1995; Mathias et al., 1998; Kolesnick and Hannun, 1999; Liu et al., 1999; Goswami and Dawson, 2000). This may have clinical relevance as accumulation of ceramide in human brain after acute internal carotid artery occlusion has been reported (Kubota et al., 1989).

We have previously shown that CDP-choline treatment attenuated ArAc release after ischemia/1-day reperfusion (Rao et al., 1999*a*). As CDP-choline neuroprotection in various CNS injuries may involve more than simply an increase in PtdCho synthesis, we have extended our studies to investigate the effect of CDP-choline on major phospholipids (PtdCho, PtdEtn, PtdIns, PtdSer, sphingomyelin) plus cardiolipin (an exclusive inner mitochondrial lipid essential for electron transport) and ceramide. To the best of our knowledge, this is the first report examining sphingomyelin and ceramide metabolism in ischemia/reperfusion. Our studies showed that PtdCho, PtdIns, PtdSer, cardiolipin, and sphingomyelin levels were decreased after ischemia/1-day reperfusion. Administration of CDP-choline significantly restored PtdCho, sphingomyelin, and cardiolipin levels as well as the ArAc content of PtdCho and PtdEtn but did not affect PtdIns or PtdSer following transient forebrain ischemia of gerbil.

MATERIALS AND METHODS

The following materials were obtained from the indicated suppliers: chemicals and lipid standards (Sigma Chemicals, St. Louis, MO, U.S.A.), CDP-choline (BioMol, Plymouth Meeting, PA, U.S.A.), HPLC-grade solvents, E Merck silica gel 60 TLC plates (Fisher Scientific, Pittsburgh, PA, U.S.A.), and silica gel G, H, and GHL TLC plates (Analtech, Newark, DE, U.S.A.).

Transient forebrain ischemia

All surgical procedures were conducted according to the animal welfare guidelines set forth in the NIH *Guide for the Care and Use of Laboratory Animals* (U.S. Department of Health and Human Services publication 85-23, 1985) and were approved by the Animal Care Committee of the University of Wisconsin–Madison. Male Mongolian gerbils (50–80 g) were anesthetized with 1% halothane in 70:30 N_2O/O_2. Both carotid arteries were exposed (with the aid of a surgical microscope) by a horizontal neck incision, occluded with aneurysm clips for 10 min, and reperfused for 1 day (Rao et al., 1997, 1999*a–c*, 2000). Brain temperature was measured by means of a thermocouple probe placed in the temporalis muscle (Busto et al., 1987). Body and cranial temperatures were maintained at 37–38 and 36–37°C, respectively, using a thermostatically controlled water blanket and heating lamp. CDP-choline (500 mg/kg i.p.) was given to gerbils just after the end of ischemia and at 3-h reperfusion (Rao et al., 1999*a*). Mean arterial blood pressure and blood gases P_{O_2} and P_{CO_2} were monitored. Arterial blood gases were maintained within normal limits (P_{O_2} 75–100 mm Hg; P_{CO_2} 25–40 mm Hg) (Rao et al., 1997) for the sham and ischemic groups during the ischemia and for 3-h postischemia reperfusion. Treatment with CDP-choline did not affect the physiological variables (Rao et al., 1999*a*). The groups and numbers of gerbils used in this study were the following: shams (n = 12); shams treated with CDP-choline (n = 8); ischemia/0 reperfusion (n = 8); ischemia/1-day reperfusion + vehicle (0.9% saline) (n = 8); and ischemia/1-day reperfusion + CDP-choline (n = 8). Brains of the anesthetized gerbils were frozen in situ, and hippocampi were dissected at 0°C for lipid analysis (Rao et al., 1999*a–c*, 2000).

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 following TLC plates and solvent systems (by volume) were used to separate various lipids: (1) cardiolipin: silica gel GHL, chloroform/methanol/acetone/ammonium hydroxide (60:28:20:2.5); (2) PtdCho, PtdEtn, PtdIns, PtdSer: Merck silica gel 60, chloroform/methanol/acetic acid/formic acid/water (70:30:14:4:2); (3) sphingomyelin: silica gel H, chloroform/methanol/acetic acid/formic acid/water (70:30:14:4:2); and (4) ceramide: silica gel H, chloroform/methanol/acetic acid (94:2:5). The lipids were identified using authentic standards and were converted to methyl esters by heating at 70°C for 30 min (100°C for 2 h for sphingomyelin and ceramide) in 1 ml of methanol containing 20 μl of concentrated sulfuric acid, 0.01% butylated hydroxytoluene, and 10 nmol of heptadecanoic acid (17:0) as internal standard (Rao et al., 1999*a*,*c*). The methyl esters were extracted into hexane and analyzed with a Hewlett Packard 6890 gas chromatograph using a capillary column (HP cross-linked FFAP) and equipped with an autosampler. Quantification was based on external standard calibration with 17:0 as internal standard. Blank TLC regions did not show any GC peaks corresponding to palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2), ArAc (20:4), or docosahexaenoic (22:6) acids.

Statistical analysis

Data were presented as means ± SD and assessed using ANOVA followed by Dunnett's multigroup-comparison post-

TABLE 1. *Cardiolipin, sphingomyelin, and ceramide in gerbil hippocampus following ischemia (I) and 0- or 1-day reperfusion*

	Total fatty acids (μmol/g of tissue)		
	Cardiolipin	Sphingomyelin	Ceramide
Sham (n = 12)	1.60 ± 0.29	2.09 ± 0.16	0.161 ± 0.032
Sham + CDP-choline (n = 8)	1.58 ± 0.32	2.13 ± 0.17	0.156 ± 0.030
I/0 reperfusion (n = 8)	1.50 ± 0.09	1.62 ± 0.20[a]	0.153 ± 0.038
I/1-day reperfusion (n = 8)	1.25 ± 0.17[a]	1.81 ± 0.12[a]	0.133 ± 0.020
I/1-day reperfusion + CDP-choline (n = 8)	1.63 ± 0.20[b]	2.11 ± 0.28[c]	0.128 ± 0.024

For individual fatty acids, sham levels were as follows (μmol/g of tissue): Cardiolipin: palmitic (16:0), 0.32 ± 0.08; stearic (18:0), 0.34 ± 0.08; oleic (18:1), 0.34 ± 0.08; linoleic (18:2), 0.10 ± 0.07; ArAc (20:4), 0.22 ± 0.07; docosahexaenoic (22:6), 0.28 ± 0.08. Sphingomyelin: palmitic (16:0), 0.30 ± 0.06; stearic (18:0), 1.79 ± 0.14. Ceramide: palmitic (16:0), 0.037 ± 0.019; stearic (18:0), 0.124 ± 0.014.

[a] $p < 0.01$ compared with shams; [b] $p < 0.01$ and [c] $p < 0.05$ compared with vehicle-treated ischemia/1-day reperfusion.

test (GraphPad Prism Software, San Diego, CA, U.S.A.). A value of $p < 0.05$ was considered significant.

RESULTS

Shams treated with CDP-choline did not show any significant changes in phospholipids compared with shams without CDP-choline (Tables 1 and 2).

CDP-choline restored cardiolipin levels after ischemia/reperfusion

Cardiolipin composition in gerbil (see legend for Table 1) was comparable with that in rat (Nakahara et al., 1991, 1992). In Table 1 and subsequent tables, the total represents the sum of each of the individual fatty acids. After ischemia/0 reperfusion, cardiolipin levels were not

TABLE 2. *Fatty acid composition of PtdCho, PtdEtn, PtdIns, and PtdSer in gerbil hippocampus following ischemia (I) and 0- or 1-day reperfusion*

	Fatty acids (μmol/g of tissue)					
Lipid	Palmitic (16:0)	Stearic (18:0)	Oleic (18:1)	Arachidonic (20:4)	Docosahexaenoic (22:6)	Total
PtdCho						
Sham	15.34 ± 0.90	4.38 ± 0.23	5.40 ± 0.27	1.70 ± 0.15	1.10 ± 0.07	27.9 ± 1.49
Sham + CDP-choline	15.18 ± 0.65	4.42 ± 0.21	5.35 ± 0.25	1.73 ± 0.18	1.05 ± 0.08	27.7 ± 1.55
I/0 reperfusion	14.63 ± 0.88	3.73 ± 0.06[a]	4.33 ± 0.20[a]	1.50 ± 0.18[b]	1.08 ± 0.10	25.3 ± 1.13[a]
I/1-day reperfusion	13.01 ± 0.85[a]	3.37 ± 0.17[a]	4.19 ± 0.29[a]	1.14 ± 0.15[a]	1.03 ± 0.09	22.7 ± 1.46[a]
I/1-day reperfusion + CDP-choline	14.10 ± 1.4[b,e]	3.61 ± 0.46[a,e]	4.47 ± 0.62[a,e]	1.48 ± 0.09[b,c]	1.15 ± 0.17	24.8 ± 0.70[a,c]
PtdEtn						
Sham	2.06 ± 0.30	5.90 ± 0.49	2.18 ± 0.27	3.36 ± 0.29	7.82 ± 0.61	21.32 ± 1.60
Sham + CDP-choline	2.04 ± 0.23	5.82 ± 0.42	2.20 ± 0.25	3.39 ± 0.24	7.80 ± 0.57	21.25 ± 1.73
I/0 reperfusion	1.84 ± 0.17	5.69 ± 0.11	1.63 ± 0.19[a]	3.12 ± 0.25	7.43 ± 0.65	19.71 ± 0.63
I/1-day reperfusion	1.71 ± 0.08[a]	6.84 ± 0.52[a]	1.89 ± 0.27	2.84 ± 0.20[a]	8.70 ± 0.64[b]	21.98 ± 1.42
I/1-day reperfusion + CDP-choline	1.66 ± 0.26[a,e]	5.83 ± 0.50[c]	1.64 ± 0.35[a,e]	3.03 ± 0.25[b,e]	8.09 ± 0.80[e]	20.25 ± 2.15[e]
PtdIns						
Sham	0.64 ± 0.12	1.17 ± 0.16	0.19 ± 0.06	0.84 ± 0.08	—	2.84 ± 0.24
Sham + CDP-choline	0.62 ± 0.11	1.18 ± 0.14	0.20 ± 0.09	0.81 ± 0.09	—	2.81 ± 0.19
I/0 reperfusion	0.55 ± 0.10	1.01 ± 0.07[b]	0.17 ± 0.01	0.71 ± 0.03[a]	—	2.44 ± 0.19[a]
I/1-day reperfusion	0.37 ± 0.09[a]	0.79 ± 0.04[a]	0.12 ± 0.02[b]	0.58 ± 0.07[a]	—	1.86 ± 0.18[a]
I/1-day reperfusion + CDP-choline	0.37 ± 0.08[a,e]	0.82 ± 0.13[a,e]	0.11 ± 0.02[a,e]	0.64 ± 0.06[a,e]	—	1.94 ± 0.25[a,e]
PtdSer						
Sham	0.52 ± 0.11	4.43 ± 0.68	1.13 ± 0.12	0.27 ± 0.06	2.89 ± 0.35	9.24 ± 1.13
Sham + CDP-choline	0.54 ± 0.12	4.46 ± 0.66	1.15 ± 0.10	0.25 ± 0.04	2.92 ± 0.38	9.32 ± 1.04
I/0 reperfusion	0.47 ± 0.05	3.34 ± 0.09[a]	0.58 ± 0.09[a]	0.13 ± 0.01[a]	2.06 ± 0.16[a]	6.57 ± 0.18[a]
I/1-day reperfusion	0.30 ± 0.08[a]	3.47 ± 0.18[a]	0.73 ± 0.14[a]	0.16 ± 0.02[a]	2.32 ± 0.16[a]	6.99 ± 0.37[a]
I/1-day reperfusion + CDP-choline	0.36 ± 0.18[b,e]	3.11 ± 0.49[a,e]	0.65 ± 0.15[a,e]	0.14 ± 0.03[a,e]	2.07 ± 0.38[a,e]	6.33 ± 1.02[a,e]

[a] $p < 0.01$ and [b] $p < 0.05$ compared with sham; [c] $p < 0.01$ and [d] $p < 0.05$ compared with vehicle-treated ischemia/1-day reperfusion; [e] No significant difference compared with ischemia/1-day reperfusion + vehicle.

TABLE 3. *Composition of ArAc (20:4) and docosahexaenoic acid (22:6) as percentage of total fatty acids in PtdEtn and PtdCho after ischemia (I) and 0- or 1-day reperfusion in gerbil hippocampus*

	PtdEtn		PtdCho	
	% arachidonic (20:4)	% docosahexaenoic (22:6)	% arachidonic (20:4)	% docosahexaenoic (22:6)
Sham	15.8 ± 0.97	36.7 ± 1.07	6.1 ± 0.45	3.9 ± 0.26
Sham + CDP-choline	15.9 ± 0.83	36.7 ± 0.96	6.3 ± 0.51	3.8 ± 0.31
I/0 reperfusion	15.8 ± 0.82	37.7 ± 2.13	5.9 ± 0.47	4.3 ± 0.27[a]
I/1-day reperfusion	12.9 ± 0.49[a]	39.6 ± 0.52[a]	5.0 ± 0.39[a]	4.5 ± 0.33[a]
I/1-day reperfusion + CDP-choline	15.0 ± 0.46[b]	39.9 ± 1.05[a,c]	6.0 ± 0.37[b]	4.6 ± 0.20[a,c]

[a] $p < 0.01$ compared with sham; [b] $p < 0.01$ compared with vehicle-treated ischemia/1-day reperfusion; [c] No significant difference compared with ischemia/1-day reperfusion + vehicle.

significantly altered. However, there was a significant decrease in cardiolipin after ischemia/1-day reperfusion ($p < 0.01$ compared with shams), which was significantly restored by CDP-choline treatment ($p < 0.01$ compared with vehicle-treated ischemia/1-day reperfusion).

CDP-choline restored sphingomyelin without altering ceramide levels after ischemia/reperfusion

Sphingomyelin and ceramide from gerbil hippocampi contained primarily stearic acid (18:0) with lesser amounts of palmitic acid (16:0) (see legend for Table 1). Sphingomyelin levels decreased significantly following ischemia with 0- or 1-day reperfusion ($p < 0.01$ compared with shams) (Table 1). CDP-choline significantly restored the sphingomyelin levels after ischemia/1-day reperfusion ($p < 0.05$ compared with vehicle-treated 1-day reperfusion). Ceramide levels showed no significant changes after ischemia with 0- and 1-day reperfusion or following CDP-choline treatment (Table 1).

CDP-choline restored total PtdCho levels and ArAc content of PtdCho and PtdEtn after ischemia/1-day reperfusion

After ischemia/0 reperfusion, there were significant decreases (compared with sham) in total PtdCho ($p < 0.01$), PtdIns ($p < 0.01$), and PtdSer ($p < 0.01$) (Table 2). ArAc levels also significantly decreased in PtdCho ($p < 0.05$), PtdIns, and PtdSer ($p < 0.01$) after ischemia/0 reperfusion but not in PtdEtn. Following ischemia/1-day reperfusion, there were significant decreases ($p < 0.01$) in total PtdCho, PtdIns, and PtdSer but not PtdEtn (Table 2). ArAc levels in PtdCho, PtdEtn, PtdIns, and PtdSer significantly decreased at 1-day reperfusion ($p < 0.01$). CDP-choline significantly restored both the ArAc levels and the total fatty acids of PtdCho ($p < 0.01$ compared with vehicle-treated 1-day reperfusion). There was no significant increase in absolute levels of ArAc or total fatty acids in PtdEtn, PtdIns, or PtdSer following CDP-choline treatment (Table 2).

There were also significant alterations in the composition of PtdCho and PtdEtn (Table 3). Thus, whereas total PtdEtn fatty acids were unchanged following ischemia/1-day reperfusion, ArAc content as a percentage of total fatty acids showed a significant decline after ischemia/1-day reperfusion ($p < 0.01$ compared with shams). This change in composition was observed also in PtdCho, even though total fatty acids also decreased ($p < 0.01$ compared with shams). Treatment with CDP-choline significantly restored the ratio of ArAc to total fatty acids in both PtdCho and PtdEtn (Table 3; $p < 0.01$ compared with vehicle-treated 1-day reperfusion). In contrast, the percentage of docosahexaenoic acid (22:6) in total fatty acids of both PtdCho and PtdEtn showed significant increases at 1-day reperfusion ($p < 0.01$ compared with shams), which were not affected by CDP-choline administration (Table 3).

DISCUSSION

In our previous studies, treatment with CDP-choline prior to ischemia did not significantly alter the release of ArAc following 10-min ischemia/0 reperfusion (Rao et al., 1999*a*). Therefore, subsequent studies have focused on the effect of CDP-choline on metabolic events during reperfusion. In the present studies, we measured changes in lipids following ischemia/0 reperfusion, where phospholipases are known to be activated (Choi, 1990), and at 1-day reperfusion, where we have demonstrated an increase in ArAc that was attenuated by administration of CDP-choline (Rao et al., 1999*a*). We also determined the effect of CDP-choline on alterations in lipids at 1-day reperfusion. Our data showing that CDP-choline did not significantly alter the phospholipid levels in shams (Tables 1 and 2) are consistent with other reports (Agut et al., 1993; López-Coviella et al., 1995).

Ischemia causes a decrease in the cellular energy levels that is followed by various biochemical changes in the cell, including dysfunctional mitochondrial respiratory activity (Ankarcrona, 1998; Green and Kroemer, 1998; Green and Reed, 1998; Fiskum et al., 1999). It has been shown that various factors influence ischemic mitochondrial dysfunction, such as generation of ROS, intracellular pH, Ca^{2+} homeostasis, and free fatty acids. Cardiolipin is an exclusive inner mitochondrial phospho-

lipid enriched with unsaturated fatty acids and is essential for mitochondrial electron transport (Hoch, 1992). Studies have indicated that degradation of mitochondrial phospholipids also occurs and could affect the cellular energy metabolism via mitochondrial functions during ischemia/reperfusion (Nakahara et al., 1991, 1992). Loss of cardiolipin has been implicated as a causative factor in mitochondrial dysfunction and release of cytochrome c, which activates caspase-3 and initiates the apoptotic cell death cascade (Hoch, 1992; Cai et al., 1998; Green and Reed, 1998).

Our studies demonstrated a significant loss of cardiolipin in the hippocampus following ischemia/1-day reperfusion but not after ischemia/0 reperfusion (Table 1). These data are consistent with previous reports showing that cardiolipin levels did not change during ischemia but declined following reperfusion (Nakahara et al., 1991, 1992). In our studies, CDP-choline prevented the loss of cardiolipin at 1-day reperfusion. The mechanism of cardiolipin degradation is not known at this time, although the involvement of PLA_2 has been suggested (Nakahara et al., 1992). CDP-choline was shown to inhibit the activation of mitochondrial PLA_2 (Arrigoni et al., 1987; Gimenez and Aguilar, 1998; Knapp and Wurtman, 1999), and this mechanism needs further investigation.

Another factor that may contribute to mitochondrial dysfunction is release of ceramide by sphingomyelinase (Levade and Jaffrezou, 1999; Liu et al., 1999). Ceramide has been implicated as a potent inducer of apoptosis (Hannun and Obeid, 1995; Green and Reed, 1998; Perry and Hannun, 1998; Kinloch et al., 1999; Goswami and Dawson, 2000) by inhibiting the mitochondrial electron transport (Garcia-Ruiz et al., 1997) and releasing cytochrome c (Ghafourifar et al., 1999).

In our studies, significant loss of sphingomyelin was observed after ischemia/0- or 1-day reperfusion (Table 1). Even though sphingomyelin levels decreased, ceramide levels did not increase; instead, a small but nonsignificant decrease occurred after ischemia/1-day reperfusion. Other studies have also shown loss of sphingomyelin during ischemia without a corresponding increase in ceramide, and a threefold increase in ceramide was observed only after prolonged (4 days) ischemia with no reperfusion (Kubota et al., 1996). To the best of our knowledge, this is the first report examining sphingomyelin and ceramide metabolism in ischemia/reperfusion.

Once generated, ceramide may transiently accumulate or be converted into various metabolites (Mathias et al., 1998). Phosphorylation by ceramide kinase generates ceramide 1-phosphate, whereas deacylation by various ceramidases yields sphingosine, which may then be phosphorylated to sphingosine 1-phosphate (Liu et al., 1999). Ceramide can be converted back to sphingomyelin by transfer of phosphocholine from PtdCho by sphingomyelin synthase (Vos et al., 1997; Mathias et al., 1998). However, ceramide levels appear to be tightly regulated in most cells (Kolesnick and Fuks, 1995). Our data suggest that ceramide released from sphingomyelin was further metabolized, thus preventing its accumulation.

Administration of CDP-choline restored the sphingomyelin levels after 1-day reperfusion (Table 1), which may have involved increased synthesis of sphingomyelin. PtdCho, derived primarily from de novo synthesis (Eq. 1), and CDP-choline (Eq. 2) may each serve as the phosphocholine donor to form sphingomyelin from ceramide (Stoffel and Melzner, 1980; Vos et al., 1997; Goswami and Dawson, 2000):

$$\text{CDP-choline} + \text{1,2-diacylglycerol} \rightarrow \text{PtdCho} + \text{cytidine 5}'\text{-monophosphate}$$

$$\text{PtdCho} + \text{ceramide} \rightarrow \text{sphingomyelin} + \text{1,2-diacylglycerol} \quad (1)$$

$$\text{CDP-choline} + \text{ceramide} \rightarrow \text{sphingomyelin} + \text{cytidine 5}'\text{-monophosphate} \quad (2)$$

The current study provided evidence for enhanced sphingomyelin synthesis by administration of CDP-choline. It is conceivable that a distinct novel pathway of neuroprotection by CDP-choline involves stimulation of sphingomyelin synthesis (Aronowski et al., 1996). Alternatively, sphingomyelinase is stimulated by tumor necrosis factor-α (Levade and Jaffrezou, 1999; Liu et al., 1999), which is induced over 1–6 h following transient forebrain ischemia in gerbil (Saito et al., 1996). The stimulation of sphingomyelinase may be mediated through activation of PLA_2 and release of ArAc (Jayadev et al., 1994). If CDP-choline prevents PLA_2 activation (Arrigoni et al., 1987), this could interrupt the signaling from tumor necrosis factor-α to sphingomyelinase.

Activation of phospholipases is harmful to neurons in several ways: (1) membrane destabilization through phospholipid degradation (Trovarelli et al., 1983; Rao et al., 1999*a*,*c*), (2) increased calcium influx, (3) formation of lyso-PtdCho that can be metabolized to platelet-activating factor (Bazan, 1998; Jean et al., 1998), and (4) ArAc release and metabolism by cyclooxygenases/lipoxygenases. Oxidative metabolism of ArAc is considered to be a major source of ROS in ischemia/reperfusion (Katsuki and Okuda, 1995; Rao et al., 1999*b*), which may be directly related to its neurotoxicity.

Significant loss of PtdCho, PtdIns, and PtdSer occurred after ischemia/1-day reperfusion (Table 2). Although the total amount of PtdEtn was not significantly altered following 1-day reperfusion, there was a significant decrease in the levels of ArAc and the proportion of ArAc in the total fatty acids present in PtdEtn. This was also observed for the ArAc proportion of fatty acids present in PtdCho and may be due to activation of PLA_2, which selectively hydrolyzes ArAc at the *sn*-2 position of PtdCho and PtdEtn. The loss of PtdIns suggests activation of a PtdIns-specific PLC (Rhee and Bae, 1997). Both total PtdCho and the ArAc content of PtdCho and

PtdEtn, but not PtdIns, were restored after ischemia/1-day reperfusion by administration of CDP-choline (Tables 2 and 3).

CDP-choline may increase PtdCho via two pathways (Rao et al., 1999*a*): (1) transfer of phosphocholine to 1,2-diacylglycerol to form PtdCho and (2) choline liberated from CDP-choline that can be converted to AdoMet via metabolism to methionine. AdoMet serves as a methyl donor in the conversion of PtdEtn to PtdCho and provided neuroprotection in transient forebrain ischemia of gerbil (Rao et al., 1997). To determine whether CDP-choline stimulated the conversion of PtdEtn to PtdCho, we examined the docosahexaenoic acid (22:6) proportion of PtdCho. As PtdEtn contains a high level of 22:6 (~37% of total fatty acids) whereas PtdCho contains a much lower proportion (4%) (Table 3), an increase in PtdEtn conversion to PtdCho following CDP-choline treatment might have been reflected in an increase in the docosahexaenoic (22:6) content of PtdCho. Somewhat surprisingly, the proportion of 22:6 in both PtdCho and PtdEtn increased following ischemia/reperfusion, and CDP-choline treatment had no effect on this increase.

CDP-choline could conceivably restore PtdCho levels, in addition to increasing PtdCho synthesis, by preventing the activation of PLA_2 (Arrigoni et al., 1987), which may account for the effect of CDP-choline on restoring the ArAc content of PtdEtn. The observation that CDP-choline did not restore PtdIns suggests it did not increase its synthesis or had no effect on activation of PtdIns-PLC. Thus, the action of CDP-choline may be twofold: repairing the ischemic damage (increased PtdCho synthesis) and preventing hydrolysis of phospholipids (by inhibiting the activation of PLA_2). Which of these two pathways is predominant requires further study.

Ischemic injury to brain is multidimensional

Our data suggest multiple beneficial effects of CDP-choline: (1) stabilizing the cell membrane by restoring PtdCho and sphingomyelin (prominent components of outer cell membrane), (2) attenuating the release of ArAc and limiting its oxidative metabolism, and (3) restoring cardiolipin levels. The pharmacological action of CDP-choline may extend beyond its effect on phospholipid synthesis, because it provides choline for acetylcholine synthesis and cytidine for cytidilic nucleotides. In addition, its metabolites betaine, methionine, and AdoMet are involved in numerous metabolic pathways (Galletti et al., 1991; Rao et al., 1997; Shuaib et al., 2000). However, due to the multiple pathways involved in ischemic injury (e.g., excitatory amino acids and their receptors, calcium channels, phospholipases, ArAc release and its oxidative metabolism, sphingomyelin and cardiolipin hydrolysis, formation of ROS and lipid peroxidation products 4-hydroxynonenal and acrolein), no single agent is likely to provide complete neuroprotection following transient ischemia. Combining agents with different mechanisms of action will probably be necessary for full recovery (Moore and Traystman, 1994; De Keyser et al., 1999). CDP-choline (itself a multifunctional agent) together with other neuroprotective agents was effective in ischemia models (Onal et al., 1997; Andersen et al., 1999; Schabitz et al., 1999; Shuaib et al., 2000).

Note added in proof: Recent studies showed attenuation of glutathione levels after a 10-min ischemia/reperfusion in gerbil hippocampus (Baek et al., 2000). An additional benefit of CDP-choline may be increased glutathione synthesis through AdoMet metabolism, thereby augmenting the cellular defenses against oxidative damage (Fernandez-Checa et al., 1998; Lu, 1999).

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