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Short communication

Does CDP-choline modulate phospholipase activities after transient forebrain ischemia?

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Abstract

Ten min forebrain ischemia/1-day reperfusion resulted in significant decreases in total phosphatidylcholine (PtdCho), phosphatidylinositol (PtdIns), and cardiolipin in gerbil hippocampus. CDP-choline restored cardiolipin levels, arachidonic acid content of PtdCho, partially but significantly restored total PtdCho, and had no effect on PtdIns. These data suggest that CDP-choline prevented the activation of phospholipase A_2 (rather than inhibiting phospholipase A_2 activity) but did not affect activities of PtdCho-phospholipases C and/or D, or phosphoinositide-phospholipase C. CDP-choline also provided significant protection for hippocampal CA_1 neurons.

Theme: Disorder of the nervous system

Topic: Ischemia

Keywords: Arachidonic acid; Apoptosis, Cardiolipin; Citicoline; Lysophospholipids, Mitochondria; Neuronal death; Phosphatidylcholine; Phosphatidylinositol; Phospholipase A_2

Alterations in lipid metabolism including activation of phospholipases and release of arachidonic acid (ArAc) are important promoters of neuronal death after transient cerebral ischemia [20,24,29]. Glutamate released during ischemia stimulates neuronal receptors, resulting in elevated intracellular Ca^{++} and activation of phospholipases C (PLC) and A_2 (PLA_2) [4]. PLC enzymes hydrolyze phosphatidylcholine (PtdCho) and phosphatidylinositol (PtdIns) lipids to release 1,2-diacylglycerol (DAG), which is further hydrolyzed to free fatty acids including ArAc. PLA_2 hydrolyzes ArAc at the *sn*-2 position of PtdCho, phosphatidylethanolamine and phosphatidylserine [8,9,11]. Released ArAc is either re-incorporated into membranes or metabolized to form prostaglandins, leukotrienes, and reactive oxygen species (ROS) [16]. ROS generate lipid peroxides, and cytotoxic 4-hydroxynonenal [18,35] and acrolein [34] that covalently bind to cellular proteins and alter their function. ROS have been implicated in mitochondrial dysfunction that initiates apoptotic cellular death [14].

Cytidine 5′-diphosphocholine (CDP-choline or citicoline) is an endogenous intermediate in the biosynthesis of PtdCho [23,24,31]. Exogenous CDP-choline stimulates the PtdCho synthesis and attenuates the release of ArAc [33]. Exogenous CDP-choline is hydrolyzed, absorbed as cytidine and choline, and re-synthesized in the brain from its phosphorylated components by cytidine triphosphate-phosphocholine cytidylyltransferase (PCCT) [5]. Pharmacokinetic studies demonstrated that brain uptake of CDP-choline metabolites occurred within 30-min and may be biologically available for ~3-h after its administration [13]. The ability of CDP-choline to alter phospholipid metabolism may be an important function in preventing neuronal death resulting from CNS injury [2,7,23,31,32,37].

We have previously shown that CDP-choline treatment attenuated ArAc release after ischemia/1-day reperfusion [23]. We have extended our studies to investigate the

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changes and the effect of CDP-choline on PtdCho, PtdIns and cardiolipin (an exclusive inner mitochondrial lipid essential for electron transport) following transient cerebral ischemia. Our studies showed that PtdCho, PtdIns, and cardiolipin levels were decreased after 10-min ischemia/1-day reperfusion in gerbil. CDP-choline (500 mg/kg at 0- and 3-h reperfusion) significantly restored PtdCho and cardiolipin levels, the ArAc content of PtdCho, and had no effect on PtdIns levels at 1-day reperfusion. These doses also provided significant protection to CA_1 hippocampal neurons at 6 days reperfusion.

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

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 (US Department of Health and Human Services Pub 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 neck incision, occluded with aneurysm clips for 10-min and reperfused for 1-day [23,25,27]. Body and cranial temperatures were maintained at 37–38°C 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 [23]. Treatment with CDP-choline did not affect the brain temperature, mean arterial blood pressure or arterial pO_2 and pCO_2 for the sham and ischemic groups during 3-h post-ischemia reperfusion [23,30]. The number of gerbils used in this study were: shams (n=12); shams+CDP-choline (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 in situ frozen and hippocampi were dissected at 0°C for lipid analysis [23,27]. 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, v/v) containing 0.01% butylated hydroxytoluene (BHT). The following TLC plates and solvent systems (by volume) were used to separate various lipids: (1) PtdCho and PtdIns: Merck silica gel 60; chloroform/methanol/acetic acid/formic acid/water (70:30:14:4:2); (2) cardiolipin: silica gel GHL; chloroform/methanol/acetone/ammonium hydroxide (60:28:20:2.5). The lipids were identified using authentic standards and were converted to methyl esters by heating at 70°C for 30-min in 1-ml methanol containing 20-μl concentrated sulfuric acid, 0.01% BHT and 10-nmol of heptadecanoic acid (17:0) as internal standard [23,27]. The methyl esters were extracted into hexane and analyzed with a Hewlett Packard 6890 gas chromatograph. 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) and docosahexaenoic (22:6) acids.

For hippocampal neuronal counts, gerbils were anesthetized 6-days after ischemia and perfused transcardially with buffered paraformaldehyde as described [23]. Brains were sectioned (10-μm thick) coronally and were stained with thionine. The hippocampal CA_1 neurons/mm were counted as described [23,27].

Data were presented as mean±S.D., and analyzed using ANOVA followed by Dunnett's multigroup comparisons post-test (GraphPad Prism Software, San Diego, CA). A value of $P<0.05$ was considered significant.

Significant decreases in hippocampal levels of PtdCho, PtdIns, and cardiolipin occurred after ischemia/1-day reperfusion ($P<0.01$ compared to shams) (Table 1). CDP-choline treatment partially but significantly ($P<0.01$ compared to vehicle-treated ischemia/1-day reperfusion) restored the PtdCho levels, but had no effect on PtdIns (Table 1). ArAc content of PtdCho as a percentage of total fatty acids also declined following ischemia/1-day reperfusion (sham 6.1±0.45% vs. ischemia/1-day reperfusion 5.0±0.39%, $P<0.01$), which was restored by CDP-choline (6.0±0.37%, $P<0.01$ compared to vehicle-treated ischemia/1-day reperfusion). Treatment with CDP-choline also completely prevented cardiolipin loss at 1-day reperfusion ($P<0.01$ compared to vehicle-treated ischemia/1-day reperfusion) (Table 1).

Ischemia resulted in significant neuronal death in the hippocampal CA_1 subfield after 6-day reperfusion (Table 2). CDP-choline (500 mg/kg) given at 0- and 3-h after reperfusion provided significant neuroprotection ($P<0.05$ compared with vehicle treated ischemic) whereas the same two doses delayed by 24-h did not offer any neuroprotection. Since lipid changes were observed in the hippocampus, it is likely that CDP-choline and its metabolites (*S*-adenosyl-L-methionine and glutathione) are uniformly distributed throughout the brain [25].

Two distinct classes of phospholipase C (PLC) exist based on substrate specificity: phosphatidylinositide (PI)-PLC and PtdCho-PLC [26]. These PLCs hydrolyze the respective phospholipids to generate DAG [26], which is further converted to FFA including ArAc [27]. PtdCho can also be hydrolyzed by PtdCho-phospholipase D (PtdCho-PLD). The loss of PtdCho and PtdIns following ischemia/1-day reperfusion suggests that these phospholipases were activated during this time.

Following ischemia/1-day reperfusion, CDP-choline treatment partially restored the PtdCho levels, but had no effect on PtdIns. ArAc composition of PtdCho expressed as percentage of total fatty acids significantly decreased

Table 1
Effect of CDP-choline on fatty acid composition of PtdCho, PtdIns and cardiolipin following forebrain ischemia/1-day reperfusion in gerbil hippocampus

Lipid	Fatty acids, μmol/g tissue						Total
	Palmitic (16:0)	Stearic (18:0)	Oleic (18:1)	Linoleic (18:2)	Arachidonic (20:4)	Docosa-hexaenoic (22:6)	
PtdCho							
Sham (n=12)	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 (n=8)	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/1-d reperfusion (n=8)	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-d R+CDP-choline (n=8)	14.10±1.4[b,d]	3.61±0.46[a,d]	4.47±0.62[a,d]	–	1.48±0.09[b,c]	1.15±0.17	24.8±0.70[a,c]
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/1-d 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-d R+CDP-choline	0.37±0.08[a,d]	0.82±0.13[a,d]	0.11±0.02[a,d]	–	0.64±0.06[a,d]	–	1.94±0.25[a,d]
Cardiolipin							
Sham	0.32±0.08	0.34±0.08	0.34±0.08	0.10±0.07	0.22±0.07	0.28±0.08	1.60±0.29
Sham+CDP-choline	0.30±0.09	0.36±0.07	0.31±0.09	0.09±0.07	0.21±0.07	0.27±0.07	1.54±0.26
I/1-d reperfusion	0.24±0.06	0.26±0.07	0.28±0.03	0.08±0.05	0.16±0.05	0.23±0.07	1.25±0.17[a]
I/1-d R+CDP-choline	0.30±0.13	0.36±0.16	0.38±0.21	0.09±0.04	0.21±0.05	0.29±0.09	1.63±0.20[c]

[a] $P<0.01$ and [b] $P<0.05$ compared to sham; [c] $P<0.01$ and [d] not significant compared to vehicle-treated ischemia/1-day reperfusion.

following ischemia/1-day reperfusion. CDP-choline significantly restored the ArAc content of PtdCho, suggesting that CDP-choline may have prevented activation of PLA_2, which cleaves ArAc at the *sn*-2 position of PtdCho [1]. CDP-choline increased the total PtdCho levels after ischemia/1-day reperfusion, which may be due to increased synthesis, although an effect on PtdCho-PLC and/or PtdCho-PLD activities cannot be excluded. CDP-choline may thus have a dual role in restoring PtdCho levels by preventing PLA_2 activation and increasing its synthesis. The fact that CDP-choline had no effect on the loss of PtdIns suggests that CDP-choline did not alter the PI-PLC activity.

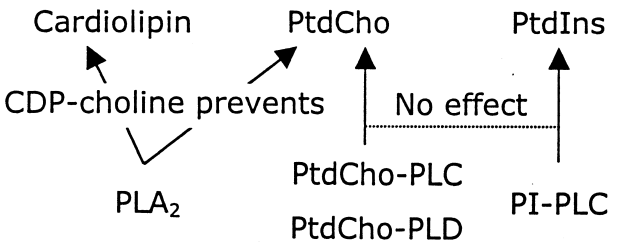
CDP-choline prevented cardiolipin loss at 1-day reperfusion. Activation of mitochondrial PLA_2 following forebrain ischemia/reperfusion in gerbil has been demonstrated, which has been implicated in cardiolipin degradation [22,28]. Significant loss of cardiolipin occurred after ischemia/1-day reperfusion, consistent with the activation of mitochondrial PLA_2. Multiple forms of PLA_2 are present in the brain [10,15,38,39]. CDP-choline prevented activation of mitochondrial PLA_2 [1]. The mitochondrial PLA_2 that is activated following transient ischemia has been characterized as a Ca^{2+}-dependent 14-kDa isoform that acts on both PtdCho and PtdEtn [28]. It is likely that CDP-choline inhibited the activation of this isoform in preserving the cardiolipin levels rather than inhibiting the activity of PLA_2. CDP-choline also restored the ArAc levels of cardiolipin, consistent with an effect on PLA_2 (Table 1). It is conceivable that CDP-choline stimulated cardiolipin synthesis by increasing cytidine diphosphodiacylglycerol, a precursor in the biosynthesis of both PtdIns and cardiolipin [36]. However, as CDP-choline treatment had no effect on PtdIns, it appears unlikely that CDP-choline increased biosynthesis of cytidine diphosphodiacylglycerol. However, as CDP-choline completely recovered cardiolipin levels, one can speculate that CDP-choline prevented cardiolipin and PtdCho hydrolysis by inhibiting the activation of PLA_2.

Table 2
Effect of CDP-choline on hippocampal CA_1 neuronal counts following 10-min ischemia and 6-day reperfusion. CDP-choline (500 mg/kg) was given as two doses either at 0- and 3-h reperfusion or at 24- and 27-h (delayed by 24-h)

Group	CA_1 neuronal counts, cells/mm
Sham	275±10
Sham+CDP-choline	270±11
Ischemia+vehicle	58±8[a]
Ischemia+CDP-choline, 0 and 3-h	123±20[b]
Ischemia+CDP-choline delayed by 24-h	60±10[a]

[a] $P<0.01$ compared to sham.
[b] $P<0.05$ compared to ischemia+vehicle.

CDP-choline administered at 0- and 3-h reperfusion afforded significant but incomplete neuroprotection (Table 2), even though cardiolipin levels were completely restored (Table 1). This may be due in part to the partial restoration of PtdCho at 1-day. Loss of PtdCho may be a significant factor in neuronal death following cerebral ischemia since inactivation of PtdCho synthesis is sufficient in itself to induce cell death [6]. PtdCho hydrolysis by PLA_2 results in formation of lyso-PtdCho, which accumulates only transiently during ischemia and returns to basal levels on reperfusion [17,21]. Lyso-PtdCho is either further hydrolyzed, re-acylated to form PtdCho, or metabolized to platelet activating factor (PAF) [3,8,19]. In cerebral ischemia/reperfusion, the formation of lipid hydroperoxides inhibits lysophospholipid acyltransferase [8], and thus



Scheme 1. Proposed action of CDP-choline on phospholipases.

more of lyso-PtdCho may be converted to PAF. CDP-choline attenuated lipid peroxidation [12,25], and may therefore restore the acyltransferase reaction, allowing lysophospholipids to be re-incorporated into the membrane. Increasing PtdCho synthesis or preventing its hydrolysis by phospholipases may thus be important factors in neuroprotection by CDP-choline. Lack of any significant neuroprotection when treatment with CDP-choline was delayed by 1-day suggests that restoration of cardiolipin and PtdCho during the first 24-h reperfusion is critical for neuronal survival.

Based on these conclusions, a tentative action of CDP-choline on phospholipases is presented in Scheme 1. Currently, future experiments are focused on whether and how CDP-choline modulates activation of phospholipases after transient cerebral ischemia.

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