CDP-CHOLINE SIGNIFICANTLY RESTORES THE PHOSPHATIDYLCHOLINE LEVELS BY DIFFERENTIALLY AFFECTING PHOSPHOLIPASE A2 AND CTP-PHOSPHOCHOLINE CYTIDYLYLTRANSFERASE AFTER STROKE*
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Running title: CDP-choline and phosphatidylcholine in stroke
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Phosphatidylcholine (PtdCho) is a major membrane phospholipid and its loss is sufficient in itself to induce cell death. PtdCho homeostasis is regulated by the balance between hydrolysis and synthesis. PtdCho is hydrolyzed by phospholipase A2 (PLA2), PtdCho-phospholipase C (PtdCho-PLC) and phospholipase D (PLD). PtdCho synthesis is rate-limited by CTP:phosphocholine cytidylyltransferase (CTT) that makes CDP-choline. The final step of PtdCho synthesis is catalyzed by CDP-choline:1,2-diacylglycerol choline phosphotransferase (CPT). PtdCho synthesis in the brain is predominantly through the CDP-choline pathway. Transient middle cerebral artery occlusion (tMCAO) significantly increased PLA2 activity, secretory PLA2 (sPLA2) IIA mRNA and protein levels, PtdCho-PLC activity and PLD2 protein expression following reperfusion. CDP-choline treatment significantly attenuated PLA2 activity, sPLA2 IIA mRNA and protein levels, and PtdCho-PLC activity, but did not affect PLD2 protein expression. tMCAO also resulted in loss of CCT activity and CCTα protein that were partially restored by CDP-choline. No changes were observed in the cytosolic PLA2 or calcium-independent PLA2 protein levels after tMCAO. The up-regulation of phospholipases (A2, C and D) and down-regulation of CCT collectively resulted in the loss of PtdCho, which was significantly restored by CDP-choline treatment. CDP-choline treatment significantly attenuated the infarction volume by 55% ± 5 after 1 h tMCAO and 1 d reperfusion. Taken together, these results suggest that CDP-choline significantly restores PtdCho levels by differentially affecting sPLA2 IIA, PtdCho-PLC and CCTα after transient focal cerebral ischemia. A hypothetical scheme is proposed integrating results from this study and other reports from the literature.

Focal cerebral ischemia or stroke is characterized by an obstruction of blood flow to the brain, resulting in disruption of glucose and oxygen that supply the brain’s energy needs. Energy failure results in rapid loss of ATP and uncontrolled leakage of ions across the cell membrane, causing membrane depolarization and release of the neurotransmitters such as glutamate and dopamine (1,2). Excess glutamate release and stimulation of its receptors results in activation of phospholipases (3-5), phospholipid hydrolysis and arachidonic acid release (6). Ultimately these processes lead to apoptotic or necrotic cell death (7).

Phosphatidylcholine (PtdCho) (8,9) is the major membrane phospholipid and constitutes ~50% of the total phospholipid content of mammalian cells. In addition to being an essential structural component of cellular membranes, PtdCho is the biosynthetic precursor for other phospholipids such as sphingomyelin and phosphatidylserine, serves as a reservoir for several lipid messengers, and is the source of bioactive lipids such as phosphatidates, 1,2-diacylglycerol (DAG), and arachidonic acid, among others (8). PtdCho homeostasis is regulated by a balance between the opposing actions of hydrolysis and synthesis (10). PtdCho can be hydrolyzed by phospholipase A2 (PLA2), PtdCho-phospholipase C (PtdCho-PLC) and phospholipase D (PLD). PLA2 isozymes occur in multiple forms.
(11-13) in the mammalian cell and are classified as calcium independent (iPLA₂, 84 kDa), and the calcium-dependent cytosolic (cPLA₂, 85-110 kDa) and secretory (sPLA₂, 14-18 kDa) forms. cPLA₂ preferentially hydrolyzes arachidonic acid in the sn-2 position of phospholipids, whereas sPLA₂s and iPLA₂s generally lack specificity for the fatty acid in the sn-2 position (14-17). CTP:phosphocholine cytidylyltransferase (CCT) synthesizes CDP-choline and is the rate-limiting enzyme (18) in PtdCho synthesis. The final step of PtdCho synthesis is catalyzed by CDP-choline:DAG cholinephosphotransferase (CPT) (19). A second route of PtdCho synthesis is phosphatidylethanolamine N-methyltransferase (PEMT), however, this pathway is believed to be of significance only in the liver (20,21). PtdCho synthesis in extra-hepatic tissues including the brain is primarily through the CDP-choline pathway (22). As a PtdCho precursor, CDP-choline (trade names citicoline or Somazina) is virtually without any side effects and has undergone clinical trials for stroke treatment in the USA (2,23,24). CDP-choline has shown benefit in transient cerebral ischemia (25-29), and is in clinical use for stroke treatment in 70 countries including Europe and Japan.

Inhibition of PtdCho synthesis through inactivation of CCT is sufficient to induce cell death (8). In transient global cerebral ischemia (a situation relevant to cardiac arrest), loss of membrane PtdCho resulting from activation of phospholipases (A₂, PtdCho-PLC and D) and loss of CCT activity might have contributed significantly to cerebral ischemic injury (2,30,31). Here we report that transient focal cerebral ischemia (a model for clinical stroke condition) differentially up-regulated sPLA₂IIA, PtdCho-PLC and PLD, and down-regulated CCT, resulting in net loss of PtdCho. Treatment with CDP-choline attenuated sPLA₂IIA mRNA and protein expression, PtdCho-PLC activity, loss of CCT activity and protein levels, significantly restored PtdCho levels and reduced infarction volume after stroke.

EXPERIMENTAL PROCEDURES

Materials

All chemicals and reagents unless stated otherwise were purchased from Sigma (St. Louis, MO). CDP-choline was obtained from BioMol (Plymouth Meeting, PA). The following antibodies were obtained from the indicated suppliers: rabbit polyclonal anti-sPLA₂ (Upstate, Charlottesville, VA), rabbit polyclonal anti-PLD, goat polyclonal anti-CCTα, rabbit polyclonal anti-cPLA₂, and rabbit polyclonal anti-iPLA₂ (Santa Cruz Biotech, Santa Cruz, CA), horseradish peroxidase conjugated goat anti-rabbit IgG (Bio-Rad, Hercules, CA) and donkey anti-goat IgG (Santa Cruz). Detection of Western blots used SuperSignal from Pierce (Rockford, IL).

Focal cerebral 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 Academy Press Washington, D.C. 1996) and were approved by the Animal Care and Use Committee of the University of Wisconsin-Madison. These studies used transient middle cerebral artery occlusion (tMCAO) in spontaneously hypertensive rats (SHR); we (32) and others (33) have shown that SHR provide a consistent infarction volume with a low variability. The coefficient of variation in the injury volumes is much less in SHR compared to Sprague-Dawley rats (32). Male SHR (250-300 g) were purchased from Charles River (Wilmington, MA) and subjected to 1 h (tMCAO) as described earlier (25,32,34). Under halothane anesthesia (1-2%) in an O₂ and N₂O (50:50) mixture, a 3-0 monofilament nylon suture was introduced through the left internal carotid to occlude the middle cerebral artery. Reduction in blood flow was confirmed using a laser Doppler blood perfusion monitor (Vasamedics, LLC, St. Paul, MN) After 1 h occlusion, the suture was withdrawn to restore the blood flow that was confirmed by laser Doppler flowmetry. Mean arterial blood pressure, blood gases PaO₂ and PaCO₂ were monitored via a catheter inserted into the left femoral artery. Body temperature was maintained at 37-38°C by means of a thermostatically controlled water blanket.

Drug treatment

CDP-choline (500 mg/kg i.p.) was dissolved in saline and administered at the onset of reperfusion and at 3 h, and thereafter once daily until one day before euthanasia. This dose and schedule for CDP-choline provided maximum
neuroprotection in previous studies (23,25). Controls received a corresponding volume of saline. Treatment with CDP-choline did not affect the physiological variables and did not cause any hypothermia (35).

**Brain tissue collection**

For PCR and Western blot analyses, rats were terminated by decapitation under deep anesthesia. Brain tissue was rapidly dissected at 0 °C and placed in RNA-Later solution (Qiagen, Valencia, CA) for PCR analyses or flash frozen in liquid N₂ for Western blot analyses. For enzyme assays and lipid analyses, brains of anesthetized rats were in situ frozen, dissected at 0 °C, and stored at -80 °C until analyzed.

**Real-Time PCR**

Expression of sPLA₂ IIA mRNA was quantified using Real-Time PCR as described earlier (36). RNA was extracted from cerebral cortices using mini total RNA purification kit (Qiagen, Valencia, CA). Total RNA (1 μg) was reverse transcribed using Oligo-dT random hexamer primers (Promega Corp., Madison, WI) and M-MLV reverse transcriptase (Promega) in a final volume of 20 μL. 10 ng of cDNA and gene-specific primers were added to SYBR green PCR master mix (Applied Biosystems, Foster City, CA) and subjected to amplification in an ABI Prism 7000 Sequence detection system (Applied Biosystems). The amplified transcripts were quantified with the comparative C₇ method using 18S rRNA as the internal control. The primers were designed based on rat GenBank accession number (NM_031598 for rat sPLA₂ IIA) using primer express software (Applied Biosystems) and purchased from Integrated DNA Technologies (Skokie, IL). Primer sequences were: forward, 5'-ACAAGAAGCCATACCACATCCCA-3'; and reverse, 5'-ACAGTCATGAGTCACACAGCA-CCA-3' which amplified a 252-bp fragment of rat sPLA₂ IIA. Primer sequences for 18S rRNA were: forward 5'-GCAAGAAGCCATACCACATCCCA-3'; and reverse: 5'-CGAACCCTCGACTTTGGTCTC-3' which amplified a 101-bp fragment of rat 18S rRNA.

**PLA₂ and PtdCho-PLC activities**

Brain cortical tissue was homogenized in 10 mM HEPES (pH 7.2, containing 0.5 mM EDTA, 0.5 mM EGTA and protease inhibitor cocktail. PLA₂ and PtdCho-PLC activities were determined in 18,000 x g supernatant. PLA₂ activity was measured as the release of [1-¹⁴C]-arachidonic acid from 1-palmitoyl-2-[¹⁴C]-arachidonyl-sn-glycero-3-phosphocholine (Perkin-Elmer, Boston, MA) as previously described (30,37).

PtdCho-PLC activity was measured using the Amplex Red PtdCho-specific PLC assay kit (38) from Molecular Probes-Invitrogen (Eugene, OR). The principle of the assay is: PtdCho-PLC hydrolyzes PtdCho to DAG and phosphocholine. Phosphocholine is hydrolyzed to choline by alkaline-phosphatase. Choline is oxidized by choline oxidase to betaine and H₂O₂. Amplex red reagent is oxidized stoichiometrically by H₂O₂ in the presence of horseradish peroxidase to generate the fluorescent product resorufin. The reaction mixture contained 200 μM Amplex Red, 1 U of horseradish peroxidase/ml, 4 U alkaline phosphatase/ml, 0.1 U of choline oxidase/ml, 0.5 mM PtdCho in 50 mM Tris-HCl (pH 7.4)-140 mM NaCl-10 mM dimethylglutarate-2 mM CaCl₂, and an aliquot of tissue homogenate. Samples were incubated at 37 ° and fluorescence was measured (Ex 530 nm and Em 590 nm). Activity was calculated using purified bacterial PtdCho-PLC as a reference.

**CCT assay**

Brain tissue was homogenized in 50 mM Tris, pH 7.4, containing 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2 mM dithiothreitol, 0.025% sodium azide, and protease inhibitor cocktail (39). Homogenates were centrifuged at 400 x g for 10 minutes and the supernatant was centrifuged at 140,000 x g for 35 min. The resulting supernatant was taken as the cytosol. CCT activity was determined by measuring the formation of radioactive CDP-choline from [methyl-¹⁴C] phosphocholine (Amersham-GE, Piscataway, NJ) as previously described (31).

**Western blot analyses**

Brain tissue was processed as per the respective enzyme assays. 150 μg of protein was loaded onto polyacrylamide gels. SDS-PAGE was performed using the Criterion system (Bio-Rad) at a constant voltage of 200 V. Proteins were subsequently transferred to nitrocellulose at a constant voltage of 100 V for 1 h. Non-specific binding sites were blocked with 5% non-fat milk powder in 1x TBS with 0.05% Tween-20 (1x
TBST) at room temperature for 1 h. Blots were incubated with primary antibodies (diluted in 3% BSA in 1x TBST with 0.02% sodium azide) for overnight at 4 °C, washed with 1x TBST, then incubated with appropriate secondary antibodies for 1 h at room temperature. After washing, protein bands were visualized with SuperSignal for 5 min at room temperature and exposure to X-ray film. Relative changes in protein expression were estimated from the mean pixel density using Scion Image program (Scion Corporation, available as a free download at http://www.scioncorp.com), and normalized to ß-actin, and calculated as ipsilateral/contralateral ratios.

**Lipid analysis**

All solvents and extracts were purged with N2 during the extraction, TLC and methylation of lipids. Total lipids were extracted from brain tissue into CHCl3/MeOH (1:2 by volume) containing 0.01% BHT, separated by TLC, converted to methyl esters and quantitated using a Hewlett Packard 6890 gas chromatograph as described earlier (31,40).

**Ischemic injury volume**

Infarction volumes were measured using 2,3,5-triphenyltetrazolium chloride (TTC) staining as described previously (25,32). Brains were cut in 2 mm coronal slices, incubated with 2% TTC for 30 min at 37 °C, rinsed with saline and fixed in 4% paraformaldehyde. Stained sections were scanned and the ischemic injury volumes were computed by the numeric integration of data from individual slices using Scion Image program. To compensate for edema in the ischemic hemisphere, corrected infarction volumes were calculated as: corrected infarction volume = right hemisphere volume – (left hemisphere volume – measured infarction volume) (41).

**Statistical analyses**

Data were presented as mean ± SD (n=3-5 per group) and analyzed by ANOVA followed by Bonferroni’s multi-group comparisons post-test using Prism software (GraphPad, San Diego, CA). A value of p<0.05 was considered significant.

**RESULTS**

*CDP-choline attenuated the increase in sPLA2 IIA mRNA, protein expression, and PLA2 activity after tMCAO—Expression of sPLA2 IIA mRNA was measured by RT-PCR following 1 h MCAO and reperfusion for 1 d and 3 d using primers given in Experimental Procedures. sPLA2 IIA mRNA increased 4.7-fold and 6.2-fold in the ipsilateral (ischemic) cortex compared to contralateral (non-ischemic) cortex at 1 d and 3 d reperfusion (Fig. 1A), respectively. Treatment with CDP-choline (500 mg/kg i.p. at 0 and 3 h reperfusion, thereafter on days 1 and 2 for mRNA at 3 d) resulted in 39% and 37% reductions (to 2.87-fold and 3.9-fold increases compared to contralateral cortex) in sPLA2 IIA mRNA expression in the ipsilateral cortex at 1 d and 3 d reperfusion, respectively (p<0.05 compared to ipsilateral cortex, saline).

sPLA2 IIA protein expression following 1 h MCAO was determined by Western blotting using rabbit polyclonal anti-sPLA2 antibodies. An extract from rat platelets was used as an electrophoresis standard for sPLA2 IIA (42). sPLA2 IIA increased in the ipsilateral cortex beginning at 3 h reperfusion and remained elevated for up to 7 d (Fig. 1B). No significant changes in sPLA2 IIA protein levels were observed in the contralateral cortex over 7 d reperfusion. Treatment with CDP-choline attenuated sPLA2 IIA protein levels in the ipsilateral cortex nearly to contralateral cortex levels at 1 d and 3 d reperfusion (Fig. 1C). There was no statistically significant difference in sPLA2 expression in the contra cortex between saline and CDP-choline treatment. Western blots were also conducted using rabbit polyclonal anti-cPLA2 and anti-iPLA2 antibodies. No differences were observed in the cPLA2 or iPLA2 protein levels between ipsilateral and contralateral cortices over 7 d reperfusion after tMCAO (data not shown).

PLA2 activity was measured by the release of labeled arachidonic acid from 1-palmitoyl-2-[1-14C]-arachidonyl-sn-glycero-3-phosphocholine as described in Experimental Procedures (37). Most of the PLA2 activity required 5 mM Ca2+, characteristic of sPLA2 (data not shown). One hour MCAO resulted in 71%, 100%, and 130% increases in PLA2 activity in the ipsilateral cortex compared to contralateral cortex at 3 h, 6 h, and 1 d reperfusion, respectively (Fig. 1D). CDP-choline treatment attenuated PLA2 activities in the ipsilateral cortex by 31% (3 h), 29% (6 h) and 46% (1 d), compared to ipsilateral cortex/saline.
These differences in PLA₂ activity could not be attributed to dilution of the specific activity of the labeled PtdCho in the PLA₂ assay by PtdCho present in the tissue samples. PLA₂ activity (Fig 1D) was determined in the 18,000 x g supernatants of tissue homogenates, in which most of the PtdCho, which is localized to the membrane fraction, was removed. The PLA₂ assay contained 50 nmol PtdCho in 0.5 mL (37); the 18,000 x g supernatant (0.2 mg protein/assay) contributed approximately 3.7 nmol PtdCho, diluting the specific activity about 7%. While PtdCho levels in brain tissue declined following tMCAO (see later results section: “CDP-choline partially restored PtdCho levels after stroke”), these changes probably resulted in ≤1% variability in the PtdCho specific activity.

CDP-choline attenuated the loss of CCT protein and activity after tMCAO—Western blots for CCTα protein expression (goat polyclonal antibodies, Santa Cruz Biotech) showed that CCTα was decreased over 1 d to 7 d reperfusion in the ipsilateral cortex (Fig. 2A). There were no significant changes in CCTα levels in the contralateral cortex over 7 d reperfusion, and no significant differences between ipsilateral/contralateral ratios at 1 d, 3 d and 7 d (Fig. 2A). Treatment with CDP-choline significantly increased CCTα protein expression in the ipsilateral cortex at 1, 3, and 7 d reperfusion (Fig. 2B). The decrease in CCTα protein levels was reflected in a loss of CCT activity in the ipsilateral cortex. One hour MCAO and 1 d reperfusion resulted in 41% and 57% loss of CCT activity in the ipsilateral cortical total homogenate and cytosol, respectively, compared to contralateral cortex (Fig. 2C). CDP-choline treatment significantly increased CCT activity in the ipsilateral cortical total homogenate and cytosol (400 ± 40 mU/mg protein) at 1 d reperfusion following 1 h MCAO compared to contralateral cortex (100 ± 15 mU/mg protein, p < 0.01, n=4 per group). Activity in the assay was abolished by inclusion of the specific PtdCho-PLC inhibitor, D609 (44), thus confirming the activity as PtdCho-PLC. CDP-choline treatment significantly attenuated the PtdCho-PLC activity in the ipsilateral cortex to 150 ± 23, p < 0.05 compared to ipsilateral/saline (n=4 per group). Due to lack of commercially available antibodies for PtdCho-PLC, protein expression by Western blotting could not be pursued.

CDP-choline significantly restored PtdCho levels after stroke—Total lipids were extracted from cortical tissue using CHCl₃/MeOH 1:2 (by volume). PtdCho was separated by TLC, levels in normal rat brain are approximately 228 nmol/g tissue (43). Based on the amount of protein used in the CCT assay (60 µg/assay), the labeled phosphocholine in the assay (1.6 mM) is estimated to be at least 400-fold greater than the phosphocholine added by the aliquot of tissue homogenate.

PLD2 protein expression was unaffected by CDP-choline after stroke—Expression of PLD2 protein by Western blots (using rabbit polyclonal antibodies, Santa Cruz) demonstrated that PLD2 increased beginning at 3 h reperfusion and remained elevated up to 7 d (Fig. 3A). Caki-1 cell lysate (Santa Cruz) was used as an electrophoresis reference for PLD (designated as control). Treatment with CDP-choline did not alter PLD2 protein expression in either the contralateral cortex (Fig. 3B, lanes a, b) or the ipsilateral cortex (Fig. 3B, lanes c, d) at 1 d reperfusion compared to saline treatment. Expression of PLD1 (~120 kDa) was much lower than that of PLD2 and showed a slight increase at 1 d reperfusion, which was unaffected by CDP-choline treatment (data not shown).

CDP-choline significantly restored PtdCho-PLC activity after tMCAO—PtdCho-PLC activity was measured using the Amplex Red assay kit from Molecular Probes; activity in brain tissue homogenates was calculated using purified bacterial PtdCho-PLC as a reference (provided with the kit). PtdCho-PLC activity significantly increased in the 18,000 x g supernatant of ipsilateral cortex (400 ± 40 mU/mg protein) at 1 d reperfusion following 1 h MCAO compared to contralateral cortex (100 ± 15 mU/mg protein, p<0.01, n=4 per group). Activity in the assay was abolished by inclusion of the specific PtdCho-PLC inhibitor, D609 (44), thus confirming the activity as PtdCho-PLC. CDP-choline treatment significantly attenuated the PtdCho-PLC activity in the ipsilateral cortex to 150 ± 23, p < 0.05 compared to ipsilateral/saline (n=4 per group). Due to lack of commercially available antibodies for PtdCho-PLC, protein expression by Western blotting could not be pursued.

CDP-choline significantly restored PtdCho levels after stroke—Total lipids were extracted from cortical tissue using CHCl₃/MeOH 1:2 (by volume). PtdCho was separated by TLC,
converted to fatty acid methyl esters and quantitated by GC. PtdCho levels significantly decreased by 22% and 17% in the ipsilateral cortex at 1 d and 3 d reperfusion, respectively after 1 h MCAO (p<0.05 compared to contralateral cortex, Fig. 4). Treatment with CDP-choline resulted in a 17% and 13% increases in PtdCho levels in the ipsilateral cortex at 1 d and 3 d reperfusion, respectively (p<0.05 compared to ipsilateral/saline). PtdCho levels in ipsilateral cortices following CDP-choline treatment tended to be less than contralateral levels but these differences were not statistically significant. The effect of CDP-choline is not a time shift as CDP-choline treatment significantly attenuated the loss of PtdCho in the ipsilateral cortex compared to saline at 3 d reperfusion. There was no significant difference in PtdCho levels between ipsilateral cortex/saline treated at 1 d vs 3 d reperfusion. Similar losses were observed for total phospholipids (as the sum of PtdCho, phosphatidylethanolamine, phosphatidylerine, phosphatidylinositol, cardiolipin and sphingomyelin) at 1 d and 3 d reperfusion, which were partially restored by CDP-choline treatment (data not shown).

_CDP-choline decreased the infarction volume after stroke_—Cerebral infarction volumes were measured using TTC staining. Brain sections were scanned and the ischemic injury volumes were computed by the numeric integration of data from individual slices using the Scion Image program and corrected for edema. CDP-choline treatment significantly (p<0.01) reduced the infarction volume by 55% ± 5 compared to saline treated controls (Fig. 5).

**DISCUSSION**

In the present study, we report the findings that transient focal cerebral ischemia (a model closely parallels the clinical stroke condition) up-regulates sPLA2 IIA, PtdCho-PLC, and PLD2 and down-regulates CCT that collectively results in loss of PtdCho. CDP-choline, an intermediate in PtdCho synthesis, counteracts some of these changes and significantly preserves membrane PtdCho. To the best of our knowledge, this is the first report showing simultaneous up-regulation of sPLA2 IIA and down-regulation of CCT after tMCAO. This report also suggests part of the neuroprotective actions of CDP-choline in stroke may be due to preserving the membrane PtdCho by differentially affecting sPLA2 IIA, PtdCho-PLC and CCT protein expression. The effect of CDP-choline was determined over 1 to 7 d reperfusion since phospholipases (A2, PtdCho-PLC and D), CCT and PtdCho levels all showed significant changes in this period.

The release of free fatty acids as an indirect evidence for phospholipases activation has been shown in both global (45) and focal (46) cerebral ischemia (47) models. Most of the earlier studies on activation of PLA2 in cerebral ischemia focused on the cytosolic form (cPLA2) (33,48,49). Transgenic mice lacking cPLA2 were generated by targeted disruption of its gene. Following transient focal cerebral ischemia, cPLA2 deficient mice had smaller infarction volumes and fewer neurological deficits compared to wild type (48,50), demonstrating a role for cPLA2 in ischemic injury. It should be noted that mouse strains C57BL/6J and 129/SV used for transgenic studies have a naturally occurring mutation in the gene for sPLA2 IIA (51,52), and thus the cPLA2 knockout mice were deficient in both cPLA2 and sPLA2 IIA. Transgenic mice expressing the human sPLA2 IIA gene have been developed (53), but this mouse strain apparently has not yet been used in stroke research to assess the role of sPLA2 IIA.

Group IIA sPLA2, also known as inflammatory PLA2, is believed to play an important role in inflammation and injury (54,55) and its expression in rat brain has been shown both in global (56) and focal cerebral ischemia (54). Our studies showing increased sPLA2 IIA mRNA following MCAO and 1 and 3 d reperfusion (Fig. 1A) is consistent with a previous report demonstrating similar increases in male Long-Evans rats (54). In our studies, increased sPLA2 IIA protein expression by Western blots was observed as early as 3 h reperfusion, which persisted up to 7 d (Fig. 1B). The studies by Lin, et al. (54) also reported increased sPLA2 IIA expression by immunohistochemistry at 1 and 3 d, however immunoreactivity before 1 d following tMCAO could not be detected. The increase in sPLA2 IIA protein expression over 1 d reperfusion in our studies was reflected by increased PLA2 activity (Fig. 1D). Treatment with CDP-choline significantly decreased the sPLA2 IIA mRNA (Fig. 1A) and protein levels (Fig. 1C) and PLA2 enzyme
activity (Fig. 1D). We have previously demonstrated that CDP-choline attenuated the increase in PLA2 activity in transient forebrain (global) ischemia (30).

Tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) are up-regulated in the brain after cerebral ischemia (57-60). TNF-α and IL-1 are potent inducers of the transcription factor NF-κB and many of their effects are mediated through this transcription factor (61,62). Group II sPLA2 mRNA and protein expression are induced by TNF-α and IL-1, and inhibition of NF-κB activation suppresses this activation (63), indicating that NF-κB mediates cytokine-induced gene activation of sPLA2 IIA. NF-κB exists normally in an inactive form associated with inhibitory proteins IκB. Activation of NF-κB involves phosphorylation of IκB by IκB kinases (IKK) followed by ubiquitination and degradation to release active NF-κB (64). Several upstream kinases have been implicated in activation of IKK, including the mitogen-activated protein kinase (MAPK) family of enzymes. A recent study showed that CDP-choline attenuates MAPK signaling (65), suggesting that CDP-choline may decrease IKK phosphorylation, NF-κB activation, and subsequent transcriptional activation of sPLA2 IIA.

Exogenous CDP-choline is rapidly hydrolyzed to cytidine and choline (66), and it is these metabolites that reach the brain. Following a single bolus injection in rats, serum CDP-choline levels peaked at 1 min but declined to undetectable levels within 5 min (67). Plasma cytidine levels increased markedly at 1 min, then declined rapidly, but remained elevated for at least 1 h (67). Brain levels of choline showed a transient increase beginning 10 min after CDP-choline dosing which returned to near basal levels by 1 h (68). However, a significant increase in brain levels of CDP-choline could not be detected following CDP-choline treatment (69), even though increased incorporation of labeled fatty acids into brain lipids was demonstrated, indicating increased lipid biosynthesis after CDP-choline dosing (70). Since CDP-choline is the rate-limiting intermediate in the PtdCho synthesis, this is probably due to rapid utilization of CDP-choline, thus maintaining brain levels.

Due to rapid hydrolysis of exogenous CDP-choline, CDP-choline has to be re-synthesized in the brain from phosphocholine and cytidine triphosphate (CTP) by CCT (18,71). The status of CCT following cerebral ischemia would therefore be important with CDP-choline administration (25). CCTα protein expression (Fig. 2A) significantly decreased following tMCAO, which was reflected in loss of CCT activity (Fig. 2C). CCT activity decreased in the total cortical homogenate, indicating net loss of cellular CCT. CDP-choline treatment significantly attenuated loss of CCTα protein (Fig. 2B) and CCT activity (Fig. 2C). During apoptosis, CCTα has been shown to undergo caspase-mediated proteolytic cleavage that coincided with poly(ADP-ribose) polymerase (PARP) cleavage (72). It has been shown that CDP-choline reduced the expression of procaspases-1, -2, -3, -6, and -8 as well as expression of cleaved caspase-3 and caspase-cleaved products of PARP following MCAO (73). Thus, CDP-choline may have increased CCT in the ischemic cortex by attenuating caspase activation and proteolytic cleavage of CCT.

PLD catalyzes the hydrolysis of PtdCho to choline and phosphatidic acid. Two mammalian forms (74) have been identified, PLD1 (~120 kDa) and PLD2 (~100 kDa). Expression of PLD in forebrain ischemia (74) and other CNS injuries and disorders (75) has been demonstrated. PLD has been shown to be activated by TNF-α (76). CDP-choline increased PtdCho levels after tMCAO (Fig. 4), which may be attributable to increasing CCT activity (increased PtdCho synthesis) and attenuating PLA2 and PtdCho-PLC activation (decreased PtdCho hydrolysis). However, CDP-choline did not affect PLD2 expression (Fig. 3B) or PLD1 (data not shown).

CDP-choline significantly reduced the infarction volume following tMCAO (Fig. 5). It has been generally believed that CDP-choline, as a precursor for PtdCho, provides neuroprotection by increasing PtdCho synthesis and membrane integrity. The current study indicates that CDP-choline may affect PtdCho levels through additional mechanisms (2), particularly by attenuating PtdCho hydrolysis. In addition to preserving membrane PtdCho, attenuating PLA2 and PtdCho-PLC activation provides neuroprotection by reducing the release of free fatty acids (FFA) including arachidonic acid, and oxidative damage resulting from arachidonic acid.
metabolism (11,37). PtdCho-PLC releases phosphocholine and DAG; DAG subsequently can be hydrolyzed to FFA.

CDP-choline also provides choline for synthesis of the neurotransmitter acetylcholine (2). Cholinergic neurons are unique in the utilization of choline in two metabolic pathways: synthesis of PtdCho and acetylcholine (77,78). These two pathways compete for the available choline, with acetylation favored when neurons are physiologically active (79). If choline becomes depleted (for example by excessive neuronal stimulation due to release of excitatory amino acids (EAA) in cerebral ischemia), choline phospholipids, especially PtdCho, are hydrolyzed to provide a source of choline. This indicates that acetylcholine synthesis is favored when the available supply of choline is limited. Thus, neurotransmission is maintained, but at the expense of phospholipids, a process referred as “autocannibalism” that ultimately causes neuronal death (78,79). It has been shown in vitro that choline deficiency resulted in loss of membrane PtdCho and sphingomyelin, and induction of apoptosis (80). CDP-choline can thus prevent PtdCho hydrolysis and death in cholinergic neurons.

Activation of nicotinic acetylcholine receptors provided neuroprotection in focal cerebral ischemia (81), suggesting that neuroprotection by CDP-choline could involve stimulation of acetylcholine receptors. However brain levels of acetylcholine after transient cerebral ischemia were not affected by CDP-choline treatment (82), and thus it seems unlikely that this pathway is significant in CDP-choline neuroprotection.

Recent studies have shown that CDP-choline actions may extend beyond its effects on PtdCho levels. CDP-choline reduced the expression of pro-caspases and cleaved caspase-3 following MCAO (73), which may impact on PtdCho synthesis as discussed previously. As effectors of the apoptotic cascade, attenuation of caspase activation should also provide neuroprotection independent of effects on PtdCho synthesis. CDP-choline also reduced the phosphorylation of MAP-kinase family members ERK1/2 and MEK1/2, which may provide anti-inflammatory effects (65). To this extent, a hypothetical scheme (Scheme 1) is proposed based on the results from this study and integrating recent studies from the literature (65,73).

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FOOTNOTES

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1The abbreviations used are: CTP: cytidine triphosphate; CCT: CTP:phosphocholine cytidylyltransferase; CPT: CDP-choline:1,2-diacylglycerol choline phosphotransferase; D609: tricyclodecan-9-yl potassium xanthate; DAG; 1,2-diacylglycerol; FFA: free fatty acids; MAPK: mitogen-activated protein kinase; cPLA2: cytosolic phospholipase A2; iPLA2: calcium-independent phospholipase A2, sPLA2: secretory phospholipase A2; PARP: poly(ADP-ribose) polymerase; PEMT: phosphatidylethanolamine N-methyltransferase; PtdCho: phosphatidylcholine; PtdCho-PLC: phosphatidylcholine-phospholipase C; PLD: phospholipase D; SHR: spontaneously hypertensive rat; tMCAO: transient middle cerebral artery occlusion; TTC: 2,3,5 triphenyltetrazolium chloride.
FIGURE LEGENDS.

Fig. 1. Effect CDP-choline on sPLA₂ mRNA and protein expression, and PLA₂ activity.

A: Effect of CDP-choline treatment on sPLA₂ IIA mRNA expression determined by RT-PCR after 1 h MCAO and reperfusion for 1 day (1 d) and 3 days (3 d). mRNA levels in ipsilateral (ischemic) cortex were represented as fold increase compared to contralateral (non-ischemic) cortex levels, which were assigned a value of 1.0. Treatment with CDP-choline (500 mg/kg i.p. at 0 and 3 h reperfusion for 1 d mRNA, 0 h, 3 h, 1 d and 0 d for mRNA at 3 d) (23,25) resulted in significant reductions in sPLA₂ IIA mRNA expression in the ipsilateral cortex at 1 d and 3 d reperfusion (p<0.05 compared to ipsilateral cortex, saline, n=4 per group, ANOVA followed by Bonferroni’s multi-group comparisons post-test). There were no significant differences in sPLA₂ IIA mRNA expression in contralateral cortex compared to cortex of sham operated animals which underwent similar surgical procedures except the artery was not occluded. CC: contralateral cortex. IC: ipsilateral cortex. **p<0.01 compared to contralateral, #p<0.05 compared to respective IC, 1 d and 3 d saline treated groups (n=4 per group).

B: Western blot (using rabbit polyclonal anti-sPLA₂, Upstate) showing time course of sPLA₂ IIA protein expression in 18,000 x g supernatant of ipsilateral and contralateral cortical homogenates at various reperfusion times after 1 h MCAO. An extract of rat platelets was used as a reference source for sPLA₂ IIA (42). Bar graph: relative increases in sPLA₂ IIA protein expression were estimated from the mean pixel density (Scion Image program), normalized to β-actin, and calculated as ipsilateral/contralateral ratios. In sham animals, there was no difference between left and right cortex (ratio: 1). *p<0.05 and **p<0.01 compared to sham (n=5 per group).

C: Effect of CDP-choline (500 mg/kg i.p. at 0 and 3 h reperfusion for 1 d; 0 h, 3 h, 1 d and 2 d for 3 d reperfusion) on sPLA₂ IIA protein expression after 1 h tMCAO. A representative Western blot is shown for 1 d reperfusion. Bar graph: relative sPLA₂ IIA protein expression calculated as ipsilateral/contralateral ratios. CDP-choline significantly attenuated the sPLA₂ IIA expression in the ipsilateral cortex to contralateral levels at 1 and 3 d reperfusion. **p<0.01 compared to sham, ##p<0.01 compared to saline treated groups (n=4 per group). There was no statistically significant difference in sPLA₂ expression in the contra cortex between saline and CDP-choline treatment.

D: Effect of CDP-choline on PLA₂ activity in 18,000 x g supernatants of ipsilateral and contralateral cortical homogenates after 1 h MCAO and reperfusion for the indicated times. PLA₂ activity was determined by the release of labeled arachidonic acid from 1-palmitoyl-2[1-14C]-arachidonyl-sn-glycero-3-phosphocholine as described in Experimental Procedures (30,37). *p<0.05 and **p<0.01 compared to contralateral cortex, #p<0.05 and ##p<0.01 compared to ipsilateral cortex/saline (n=4 per group). CDP-choline attenuated sPLA₂ activity near to contralateral levels (no statistically significant differences between ipsilateral/CDP-choline group vs contralateral saline). CDP-choline did not affect PLA₂ activity in contralateral cortex. The PtdCho present in the tissue samples had minimal effect (≤1%) on the results of the PLA₂ assay, as elaborated in the results section.

Fig. 2. Effect CDP-choline on CCTα protein expression and CCT activity.

A: Western blot (goat polyclonal anti-CCTα, Santa Cruz) showing time course of CCTα protein expression in cytosol fractions (140,000 x g supernatant) of ipsilateral and contralateral cortex at various reperfusion times after 1 h MCAO. Bar graph: relative levels of CCTα protein expression [estimated from the mean pixel density (Scion Image program) and normalized to β-actin] are given as ipsilateral/contralateral ratios. There was significant loss of CCT protein in ipsilateral cortex over 1 d to 7 d reperfusion. *p<0.05 and **p<0.01 compared to contralateral cortex, #p<0.05 and ##p<0.01 compared to ipsilateral cortex/saline (n=4 per group). CDP-choline attenuated CCTα activity near to contralateral levels (no statistically significant differences between ipsilateral/CDP-choline group vs contralateral saline). CDP-choline did not affect PLA₂ activity in contralateral cortex. The PtdCho present in the tissue samples had minimal effect (≤1%) on the results of the PLA₂ assay, as elaborated in the results section.

B: Effect of CDP-choline on CCTα protein expression after 1 h tMCAO. A representative Western blot is shown for 1 d reperfusion. Bar graph: relative levels of CCTα protein expression are given as ipsilateral/contralateral ratios. CDP-choline (500 mg/kg i.p. at 0 and 3 h reperfusion, thereafter once daily until 1 d before euthanasia) significantly restored CCTα protein levels after 1 h MCAO and 1, 3, and 7 d reperfusion; *p<0.05 and **p<0.01 compared to sham, #p<0.05 compared to saline treatment (n=3-4 per group).
C: Effect of CDP-choline on CCT activity in total homogenates and cytosol fractions from ipsilateral and contralateral cortex after 1 h MCAO and 1 d reperfusion. CCT activity was determined by measuring the formation of radioactive CDP-choline from [methyl-14C] phosphocholine as described in Experimental Procedures (31). **p<0.01 compared to contralateral cortex; #p<0.05 compared to ipsilateral cortex, saline (n=4 per group). Phosphocholine present in the brain tissue homogenate did not significantly affect the results of the CCT enzyme assay as elaborated in the results section.

Fig. 3. CDP-choline did not alter PLD2 expression after stroke.

A: Western blot (rabbit polyclonal anti-PLD, Santa Cruz) showing time course of PLD protein expression in 18,000 x g supernatants from ipsilateral and contralateral cortical homogenates at various reperfusion times after 1 h MCAO. Caki-1 cell lysate (Santa Cruz) was used as a reference source for PLD (control). Bar graph: relative changes in PLD2 protein expression are given as ipsilateral/contralateral ratios. *p<0.05 compared to sham (n=4 per group).

B: Effect of CDP-choline (500 mg/kg i.p. at 0 and 3 h reperfusion) on PLD2 protein levels after 1 h MCAO and 1 d reperfusion (n=4 per group). a: contralateral cortex, saline; b: contralateral cortex, CDP-choline; c: ipsilateral cortex, saline; d: ipsilateral cortex, CDP-choline.

Fig. 4. CDP-choline significantly restored PtdCho levels after 1 h MCAO. Total lipids were extracted from cortices into CHCl3/MeOH, separated by thin-layer chromatography, converted to methyl esters and quantitated using a Hewlett Packard 6890 gas chromatograph as described earlier (31,40). PtdCho levels were calculated as one-half the sum of fatty acids derived from PtdCho since each PtdCho molecule contains two fatty acid residues. These values are therefore approximately one-half those reported by Narita et al. (46) since PtdCho levels in that report were given as the sum of fatty acids. PtdCho levels significantly decreased in ipsilateral cortex following 1 h MCAO and 1 d and 3 d reperfusion, *p<0.05 compared to contralateral (n=4 per group). CDP-choline (500 mg/kg i.p. at 0 h and 3 h for 1 d reperfusion, 0 h, 3 h, 1 d and 2 d for 3 d reperfusion) significantly restored PtdCho levels in the ipsilateral cortices, #p<0.05 compared to ipsilateral/saline (n=4 per group). PtdCho levels in ipsilateral cortices following CDP-choline treatment tended to be less than contralateral levels but these differences were not statistically significant. There was no significant difference between ipsilateral cortex/saline treated at 1 d vs 3 d reperfusion.

Fig. 5. CDP-choline treatment attenuated infarction volume after 1 h MCAO and 1 d reperfusion. Infarction volumes were measured using TTC staining (25,32). Brain sections were scanned and the ischemic injury volumes were computed by the numeric integration of data from individual slices using the Scion Image program with correction for edema as described in Experimental Procedures. A: saline, infarction volume 270 ± 38 mm³. B: CDP-choline treatment (500 mg/kg i.p. at 0 and 3 h reperfusion), infarction volume 121.5 ± 13.5 mm³, 55% ± 5 reduction, p<0.01 (unpaired t test, GraphPad Prism software) compared to saline (n=5 per group).

Scheme 1. Hypothetical actions of CDP-choline. CDP-choline may affect PLA2 (Fig. 1A, C, D) and CCT (Fig. 2B, C) by attenuating MAP kinases and caspase activation (65,73). The mechanism by which CDP-choline decreases PtdCho-PLC is yet to be elucidated. CDP-choline had no effect on PLD2 (Fig. 3B). As a result of these effects, CDP-choline partially restored PtdCho levels (Fig. 4) and attenuated cerebral infarction volume (Fig. 5) after stroke.
Figure 1.

**1A**

- sPLA$_2$ II A mRNA, fold increase
- CC, IC 1 d, IC 1 d CDP, IC 3 d CDP
- Graph showing mRNA expression levels over time.

**1B**

- Reperfusion time
- Ipsilateral cortex and control
- Image of Western blot with 14 kDa marker and β-actin.
- Bar graph showing sPLA$_2$ ipsi/contra ratio over time.
Figure 1 (continued).

**1C**

|        | Saline | CDP-choline |
|--------|--------|-------------|
| IC     | CC     | IC          | CC          |

14 kDa β-actin

**1D**

- **Contra./Saline**
- **Contra./CDP**
- **Ipsi./saline**
- **Ipsi./CDP**

| 3 h | 6 h | 1 d |
|-----|-----|-----|
| 30  | 60  | 100 |

**PLA2 activity, pmol/min/mg protein**

**IC**: IC choline; **CC**: CDP-choline.
Figure 2.

2A

Reperfusion time

| Ipsi-cortex | Contra- |
|-------------|---------|
| 0 | 1h | 3h | 6h | 1d | 3d | 7d |
| 0 | 1h | 3h | 6h | 1d | 3d | 7d |

42 kDa

β-actin

CCT, ipsi/contra ratio

| Sham | 0 h | 1 h | 3 h | 6 h | 1 d | 3 d | 7 d |
|------|-----|-----|-----|-----|-----|-----|-----|
|      |     |     |     |     |     |     |     |

* # # # #

2B

| Saline | CDP-choline |
|--------|-------------|
| IC | CC | IC | CC |
| 42 kDa | 42 kDa |
| β-actin | β-actin |

| Sham | 1 d CDP | 1 d CDP | 3 d CDP | 3 d CDP | 7 d CDP | 7 d CDP |
|------|---------|---------|---------|---------|---------|---------|
|      |         |         |         |         |         |         |

* # # #

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Figure 2 (continued).

![Bar chart showing CCT activity, pmol/min/mg protein](image)

- **Contra. Saline**
- **Contra. CDP**
- **Ipsi. Saline**
- **Ipsi. CDP**

CCT activity, pmol/min/mg protein

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Figure 3.

**3A**

| Reperfusion time | Ipsi-cortex | Contra |
|------------------|-------------|--------|
| 0h               | 1h          | 3h     | 6h    | 1d    | 3d | 7d | Control |
| 0.0              | 0.5         | 1.0    | 1.5   | 0.0   | 1.0| 7d |

100 kDa PLD2

β-actin

**3B**

| PLD2, ipsi/contra ratio |
|--------------------------|
| Sham | 1 d | 1 d/CDP |
| 0.0  | 1.5 | 1.7 |

PLD2, ipsi/contra ratio

β-actin

*"*
Figure 4.

Contra./saline  Contra. CDP  Ipsi. 1 d/saline  Ipsi. 1 d/CDP  Ipsi. 3 d/saline  Ipsi. 3 d/CDP

PtdCho, µmol/g tissue

*  #  *  #
Figure 5.
Scheme 1.

MAP kinases ↓ \[\text{CDP-choline} \rightarrow\] Activated caspases ↓

\[\downarrow\]

PtdCho-PLC ↓

\[\downarrow\]

PLA₂ ↓ \[\rightarrow\] PtdCho ↑ \[\leftarrow\] CCT ↑

\[\downarrow\]

Infarction ↓
CDP-choline significantly restores the phosphatidylcholine levels by differentially affecting phospholipase A2 and CTP-phosphocholine cytidylyltransferase after stroke
Rao Muralikrishna Adibhatla, James F. Hatcher, Eric C. Larsen, Xinzhi Chen, Dandan Sun and Francis H. Tsao

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