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Phospholipase A_2, Hydroxyl Radicals, and Lipid Peroxidation in Transient Cerebral Ischemia

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ABSTRACT

Phospholipid degradation is an important promoter of neuronal death after transient cerebral ischemia. Phospholipid hydrolysis by phospholipase A_2 (PLA_2) after transient cerebral ischemia releases arachidonic acid. Arachidonic acid metabolism results in formation of reactive oxygen species, lipid peroxides, and toxic aldehydes (malondialdehyde, 4-hydroxynonenal, and acrolein). Citicoline (cytidine-5'-diphosphocholine), an intermediate in phosphatidylcholine synthesis, has undergone 13 phase III clinical trials for stroke, and is being evaluated for treatment of Alzheimer's and Parkinson's diseases. Here we examined the effect of citicoline on PLA_2 activity in relationship to attenuating hydroxyl radical (OH•) generation and lipid peroxidation after transient forebrain ischemia in gerbil. High Ca^{2+} dependency (millimolar range) of PLA_2 activity suggests that secretory PLA_2 is the predominant isoform in membrane and mitochondria. Citicoline attenuated the increase in PLA_2 activity in both membrane and mitochondrial fractions. *In vitro,* citicoline and its components choline and cytidine had no effect on the PLA_2 activity. Thus, citicoline is not a "direct PLA_2 inhibitor." Citicoline also significantly attenuated loss of cardiolipin and arachidonic acid release from phosphatidylcholine and phosphatidylethanolamine. Transient cerebral ischemia resulted in significant formation of OH• and malondialdehyde, and citicoline significantly attenuated their formation. These results suggest that citicoline provides neuroprotection by attenuating the stimulation of PLA_2. *Antioxid. Redox Signal.* 5, 647–654.

INTRODUCTION

FORMATION OF REACTIVE OXYGEN SPECIES (ROS) [superoxide radical ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH•)] and the ensuing oxidation of biological molecules have been proposed to be significant mechanisms of tissue damage in cerebral ischemia and reperfusion (6, 13, 30, 32). Phospholipase A_2 (PLA_2) activation following transient cerebral ischemia releases arachidonic acid from membrane phospholipids. Oxidative metabolism of arachidonic acid is considered to be a significant source of ROS (28, 45). Other important sources include metabolism of xanthine by xanthine oxidase, autooxidation of catecholamines, NADPH oxidation by neutrophils, and mitochondrial dysfunction (Scheme 1).

H_2O_2 formed by the dismutation of $O_2^{-\bullet}$ is in itself not very reactive, but in the presence of divalent metal ions, particularly Fe^{2+}, forms the highly reactive OH• via the Fenton reaction illustrated in Scheme 1 (6). Ferritin has a high affinity for Fe^{3+} at neutral pH, but readily releases iron at pH $\leq$ 6 (24), for example, by cellular acidosis resulting from excess lactate formation during cerebral ischemia (14, 24, 66). Released Fe^{3+} is reduced by $O_2^{-\bullet}$ to Fe^{2+}. During reperfusion, Fe^{3+} in ferritin can also be reduced by $O_2^{-\bullet}$ to Fe^{2+}, which is then released due to low affinity of ferritin for Fe^{2+}. The OH• in turn induces lipid peroxidation, resulting in formation of

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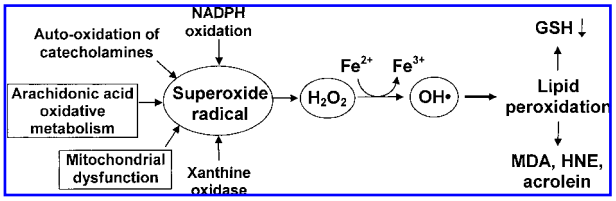


SCHEME 1. Sources of ROS, generation of OH•, lipid peroxidation, toxic aldehyde formation, and depletion of glutathione (GSH) (3) in ischemia and reperfusion.

toxic aldehydes, malondialdehyde (MDA), 4-hydroxynonenal (HNE), and acrolein (Scheme 1) (21, 61). These aldehydes, particularly HNE and acrolein, covalently bind preferentially to thiols and to a lesser extent to amine groups of proteins and alter their function (33), contributing to neuronal death (23, 52). We have previously shown localization of HNE in the CA_1 region of the hippocampus after transient cerebral ischemia in gerbil (45).

Citicoline (cytidine-5'-diphosphocholine) has provided beneficial effects in various CNS injuries and neurodegenerative diseases, including transient forebrain ischemia, and has undergone several phase III clinical trials (1, 2, 4, 5, 11, 18, 38, 43, 55, 59). However, its mode of action has not been clearly elucidated. It has been suggested that citicoline might affect the PLA_2 activation in cerebral injuries (9, 48), thus providing neuroprotection by attenuating the resultant oxidative damage. Here we examined the effect of citicoline on PLA_2 activity in relationship to attenuating the OH• generation and lipid peroxidation after transient forebrain ischemia of gerbil.

MATERIALS AND METHODS

Chemicals and reagents

1-Palmitoyl-2[1-^{14}C]-arachidonoyl-*sn*-glycero-3-phosphocholine was obtained from Perkin–Elmer Life Sciences (Boston, MA, U.S.A.). Nonradioactive 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine, 2,3-dihydroxybenzoic acid (2,3-DHBA), 2,5-DHBA, salicylate, protease inhibitor cocktail, malondialdehyde bis(dimethyl acetal), and common reagents were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). HPLC grade solvents and E. Merck silica gel 60 TLC plates were obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). Citicoline (CDP-choline) was from BioMol (Plymouth Meeting, PA, U.S.A.), and silica gel GHL TLC plates were from Analtech (Newark, DE, U.S.A.).

Induction of 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 by a neck incision, occluded for 10 min, and reperfused for up to 3 days (2, 6, 44, 46–48). Citicoline (500 mg/kg i.p., dissolved at 200 mg/ml in saline) was given to gerbils just after the end of ischemia, at 3 h of reperfusion, and daily thereafter until 24 h before euthanasia (3, 44). At the end of the reperfusion period, brains of anesthetized gerbils were frozen *in situ* (3); cortices and hippocampi were dissected at 0°C.

Mitochondria preparation (54)

Hippocampi or cortices were homogenized at 4°C in homogenization buffer (10 m*M* HEPES, pH 7.4, containing 0.15 *M* sucrose, 0.5 m*M* EDTA, 0.5 m*M* EGTA, and protease inhibitor cocktail). The homogenate was centrifuged at 2,000 *g* for 3 min and the pellet discarded. The supernatant was centrifuged (12,000 *g,* 8 min) to obtain the mitochondrial/synaptosomal pellet, which was resuspended in homogenization buffer and centrifuged again (12,000 *g,* 10 min). The supernatant was discarded, and the pellet was resuspended in 0.25 *M* sucrose and centrifuged (12,000 *g,* 10 min) to obtain the mitochondrial fraction. The mitochondrial pellet was resuspended in 0.25 *M* sucrose.

PLA_2 activity

Hippocampi were homogenized in 10 m*M* HEPES, pH 7.2, containing 0.5 m*M* EDTA, 0.5 m*M* EGTA, and protease inhibitor cocktail, and centrifuged (18,000 *g,* 10 min). The resulting pellet was resuspended in homogenization buffer and taken as the membrane fraction. PLA_2 activity was determined as the release of [1-^{14}C]arachidonic acid in a standard reaction mixture containing 100,000 dpm 1-palmitoyl-2[1-^{14}C]-arachidonoyl-*sn*-glycero-3-phosphocholine with some modifications (10). The labeled compound was diluted with the corresponding nonlabeled phosphatidylcholine (PtdCho) to give a final concentration of 100 μ*M* in 0.5 ml, prepared by sonication in 100 m*M* Tris-HCl, pH 8.5, containing 5 m*M* $CaCl_2$ and 50% glycerol. These conditions were determined to be optimum for the PLA_2 activity. The reaction was initiated by addition of 0.5 mg of protein (membrane or mitochondria), incubated at 37°C for 30 min, and then terminated by addition of 2 ml of 50 m*M* acetic acid. Samples were extracted twice with 1.5 ml of $CHCl_3$. The $CHCl_3$ layer containing the substrate and product was dried under nitrogen and redissolved in 50 μl of $CHCl_3$/methanol. The entire sample was applied to a silica gel TLC plate, which was developed in petroleum ether/diethyl ether/acetic acid (80:20:1, by volume) to separate [1-^{14}C]arachidonic acid from the starting material. The band corresponding to the arachidonic acid standard was scraped directly into a scintillation vial and counted. Negative controls included all reagents, with buffer replacing protein in the incubation mixture.

Lipid analysis

Brains of the anesthetized gerbils were frozen *in situ* and hippocampi were subjected to lipid analysis as described earlier (3). In brief, all solvents and extracts were purged with nitrogen during the extraction, TLC, and methylation of lipids. Total lipids were extracted into $CHCl_3$/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: (a) PtdCho and phosphatidylethanolamine (PtdEtn): Merck silica gel 60; $CHCl_3$/methanol/CH_3COOH/HCOOH/water (70:30:14:4:2); (b) Cardiolipin: silica gel GHL, $CHCl_3$/methanol/CH_3COCH_3/NH_4OH (60:28:20:2.5). The lipids were identified using authentic standards and converted to methyl esters by heating at 70°C for 30 min 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. The methyl esters were extracted into hexane and analyzed with a Hewlett Packard 6890 gas chromatograph.

In vivo *trapping of OH•*

Thirty minutes following injection of salicylate (300 mg/kg i.p.), anesthetized gerbils were decapitated; the hippocampi were rapidly dissected at 0°C and frozen in liquid nitrogen. Hippocampi were homogenized (purging with nitrogen during this process to avoid any OH• generation) in 70% ethanol containing 0.2 *M* $HClO_4$, 0.1 m*M* EDTA, 0.1 m*M* sodium metabisulfite, and then centrifuged at 18,000 *g*. This extracts >95% of salicylate and DHBAs from the tissue, and was found to be superior to previous extraction methods. We found that extraction with aqueous 0.2 *M* $HClO_4$ containing 0.1 m*M* EDTA, 0.1 m*M* sodium metabisulfite (37) did not quantitatively recover DHBAs and salicylate. Extraction with 100% ethanol (7) recovered DHBAs and salicylate, but also extracted components that eluted near the DHBA retention times in HPLC. The supernatant (300 µl) was mixed with an equal volume of 50 m*M* sodium acetate/50 m*M* citric acid, then filtered through a 0.2-µm cellulose acetate filter. The filtrate was injected into an Agilent (formerly Hewlett Packard) 1100 HPLC equipped with an ESA DHBA column and interfaced with an ESA Model 5200A Coulochem II electrochemical detector equipped with a Model 5010 dual-electrode analytical cell. Prior to the analyses, the HPLC was passivated with 6 *M* nitric acid according to Agilent's recommended procedure to remove trace metals. The electrode potentials were set to 300 mV (electrode 1, DHBAs) and 850 mV (electrode 2, salicylate). The HPLC was run isocratic at 0.5 ml/min with a premixed mobile phase on one channel of the LC pumping system consisting of 50 m*M* sodium acetate, 50 m*M* citric acid, 21% methanol, and 4% isopropanol; the pH was adjusted to 2.5 with phosphoric acid after the organic solvents were mixed with the aqueous buffer. Samples were quantitated against external standards of salicylate and 2,3-DHBA. The reaction of salicylate with OH• produces both 2,3-DHBA and 2,5-DHBA. As cytochrome P450 can hydroxylate salicylate to 2,5-DHBA, but not 2,3-DHBA, measurements were based on quantitation of 2,3-DHBA. Data were expressed as the ratio of 2,3-DHBA to salicylate to normalize DHBA levels to varying hippocampi levels of salicylate.

MDA measurements

Lipid peroxidation was determined as 2-thiobarbituric acid (TBA) reactive species (TBARS) (26). MDA forms a chromogenic adduct (2 molecules of TBA + 1 molecule of MDA) with a molar extinction coefficient 153,000 at 535 nm (20). Hippocampi were homogenized at 4°C in 15% trichloroacetic acid, then 0.75% TBA in 0.5% sodium acetate was added, and the sample was incubated at 70°C for 45 min. After cooling to room temperature, TBARS were extracted into *n*-butanol, and the absorbance was read at 535 nm. The MDA concentration was calculated against a standard curve using malondialdehyde bis(dimethyl acetal).

RESULTS

PLA_2 activity was measured over the range of 0–25 m*M* Ca^{2+} in the assay buffer to determine the calcium requirement for PLA_2 in gerbil brain. To obtain sufficient mitochondria, pooled cortices and pooled hippocampi from ischemic gerbils were used. Figure 1 shows the Ca^{2+} dependency of PLA_2 activity in membrane and mitochondrial fractions. The Ca^{2+} response was similar between mitochondria from cortex or hippocampus. Membrane and mitochondria also showed similar Ca^{2+} response toward the PLA_2 activity. Very little PLA_2 activity was detected in the absence of Ca^{2+}. Maximum activity was observed at 5 m*M* Ca^{2+}, and subsequent experiments used this concentration for the rest of the PLA_2 studies.

Following 10-min ischemia and over 24-h reperfusion period, hippocampal PLA_2 activity significantly increased in both membrane ($p < 0.05$) and mitochondrial ($p < 0.01$) fractions (Fig. 2). The increase in the mitochondria was greater than in the membrane fraction. Citicoline treatment resulted in significant decreases ($p < 0.05$ and $p < 0.01$) in PLA_2 activity in membrane and mitochondria. PLA_2 activity in membrane and mitochondrial fractions after 10-min ischemia with no reperfusion (10-min permanent ischemia) were 47.8 ± 6.1 and 73.4 ± 1.6 pmol/min/mg of protein, respectively.

The increase in PLA_2 activity in membrane and mitochondria was reflected in a decrease in the arachidonic acid content (expressed as percentage of total fatty acids) of PtdCho and PtdEtn, and a decrease in the cardiolipin levels at 24-h reperfusion following 10-min ischemia (Table 1). Citicoline (500 mg/kg at 0- and 3-h reperfusion) significantly restored

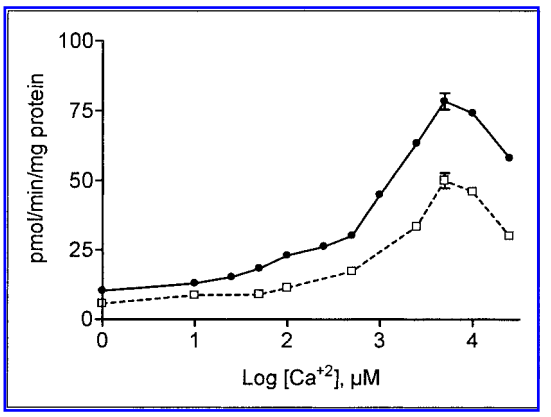


FIG. 1. Ca^{2+} dependency of PLA_2 activity in membrane (dashed line) and mitochondria (solid line) from ischemic gerbil hippocampus. Average of $n = 3$ experiments and typical standard deviation are shown.

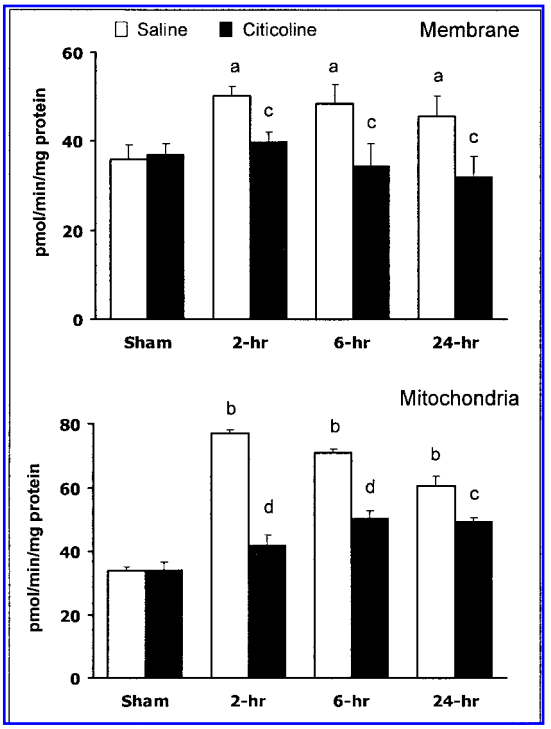


FIG. 2. Effect of citicoline on PLA_2 activity in gerbil hippocampal membrane and mitochondrial fractions after transient forebrain ischemia (n = 5 animals per group). $^{a}p < 0.05$ and $^{b}p < 0.01$, compared with sham; $^{c}p < 0.05$ and $^{d}p < 0.01$, compared with saline-treated ischemic group.

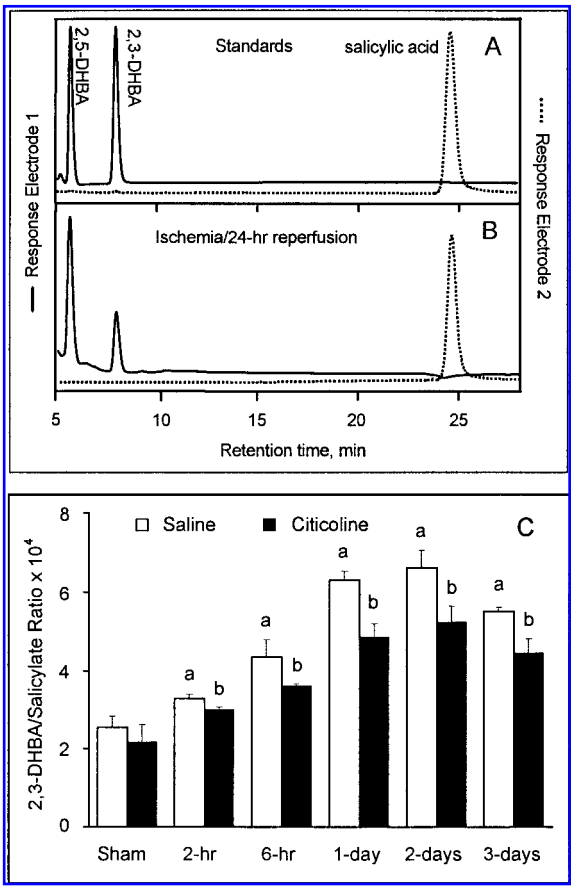


FIG. 3. HPLC profile of (A) 2,5-DHBA and 2,3-DHBA and salicylic acid standards and (B) 10-min ischemia and 24-h reperfusion in gerbil. **(C)** OH• formation in gerbil hippocampus over 3 days of reperfusion after 10-min forebrain ischemia measured by salicylate trapping and effect of citicoline (n = 5 per group). $^{a}p < 0.01$, compared with sham; $^{b}p < 0.05$, compared with saline-treated ischemic group.

cardiolipin levels and the arachidonic acid content in PtdCho and PtdEtn.

To determine OH• radical generation following transient cerebral ischemia and effect of citicoline, 2-hydroxybenzoic acid (salicylic acid) trapping was used and the formation of 2,3-DHBA was quantitated by HPLC with electrochemical detection. Figure 3A shows the HPLC profiles of 2,5-DHBA, 2,3-DHBA, and salicylate after 10-min ischemia and 24-h reperfusion. The authenticity of the peaks was confirmed in comparison with the standards (Fig. 3B). No peaks corresponding to DHBAs or salicylate were detected in hippocampal extracts of gerbils that were not treated with salicylate (data not shown). Based on this method, OH• formation was significantly increased in gerbil hippocampus following 10 min of ischemia and over 3 days of reperfusion (Fig. 3C). OH• generation was significantly attenuated by citicoline, but did not return to sham levels (Fig. 3C). MDA levels (an indicator for lipid peroxidation) were significantly elevated over 3 days of

TABLE 1. EFFECT OF CITICOLINE ON ARACHIDONIC ACID (20:4) COMPOSITION OF PTDCHO AND PTDETN AND ON CARDIOLIPIN FOLLOWING FOREBRAIN ISCHEMIA AND 24-H REPERFUSION IN GERBIL HIPPOCAMPUS

	Arachidonic (20:4) (% of total fatty acids)		*Cardiolipin (μmol of fatty acids/g of tissue)*	
	PtdCho	*PtdEtn*	*Arachidonic acid*	*Total*
Sham (n = 12 animals)	6.1 ± 0.45	15.8 ± 0.97	0.22 ± 0.07	1.60 ± 0.29
Sham + citicoline (n = 8)	6.3 ± 0.51	15.9 ± 0.83	0.21 ± 0.07	1.54 ± 0.26
I/24-h R (n = 8)	5.0 ± 0.39*	12.9 ± 0.49*	0.16 ± 0.05	1.25 ± 0.17*
I/24-h R + citicoline (n = 8)	6.0 ± 0.37‡	15.0 ± 0.46‡	0.21 ± 0.05	1.63 ± 0.20‡

*$p < 0.01$, compared with sham; ‡$p < 0.01$, compared with saline-treated ischemia (I) and 24-h reperfusion (R).

reperfusion after transient ischemia, and citicoline significantly attenuated these ($p < 0.05$ compared with saline), but did not return MDA to sham levels (Fig. 4).

DISCUSSION

Phospholipid degradation due to the activation of phospholipases is an important promoter of neuronal death after cerebral ischemia/reperfusion. Expression of PLA_2 mRNA has been demonstrated in both neurons and glia (31). Increased PLA_2 immunoreactivity demonstrated a precise overlap with neuropathological changes in several types of CNS injury, including focal and global cerebral ischemia (16, 60). PLA_2 immunoreactivity was increased in the hippocampal CA_1 region undergoing neuronal death (16, 60, 62). PLA_2 isozymes occur in multiple forms in the mammalian cell and are classified as calcium independent, cytosolic ($cPLA_2$), and secretory ($sPLA_2$) (39, 58, 65). Transgenic 129/Sv mice deficient in $cPLA_2$ developed smaller infarcts after transient ischemia (12). Interestingly, this mouse strain is spontaneously deficient in type II $sPLA_2$ due to a point mutation in exon 3 of the gene encoding $sPLA_2$ protein (29, 35). Hence, the 129/Sv $cPLA_2$ transgenic mice (12) were deficient in both $cPLA_2$ and $sPLA_2$, but still developed infarcts, consistent with ischemic injury being multidimensional, and PLA_2 contributing to neuronal damage after ischemia and reperfusion.

We selected 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine as the substrate for the PLA_2 assay because PtdCho containing arachidonic acid in the *sn*-2 position is the preferred substrate for $cPLA_2$, and with the expectation that there would be significant changes in $cPLA_2$ activity in the hippocampus following transient forebrain ischemia. Although the preferred substrate for $sPLA_2$ is PtdEtn, $sPLA_2$ has no preference for the fatty acid in the *sn*-2 position and also acts on PtdCho (39). Thus, activities of both forms of PLA_2 ($cPLA_2$ and $sPLA_2$) could be determined using this substrate.

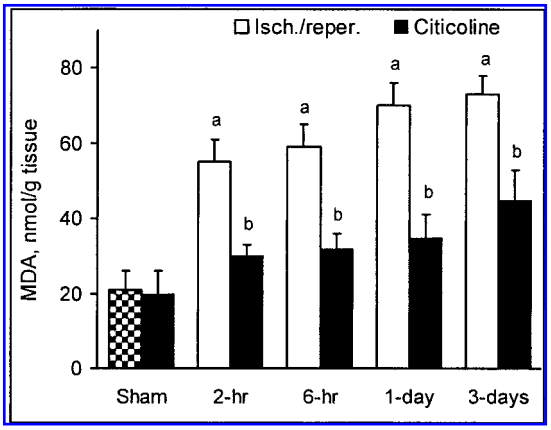


FIG. 4. MDA levels, an indicator of lipid peroxidation, in the hippocampus over 3 days of reperfusion and effect of citicoline following 10-min forebrain ischemia ($n = 5$ per group). [a]$p < 0.01$, compared with sham; [b]$p < 0.05$, compared with saline-treated ischemic group.

$cPLA_2$ is stimulated by micromolar Ca^{2+} concentrations, whereas $sPLA_2$ requires millimolar Ca^{2+} for optimal activity. Based on the Ca^{2+} dependency of PLA_2 activity (Fig. 1), the predominant isoform present in gerbil hippocampus is $sPLA_2$. In the absence of Ca^{2+}, there was very little PLA_2 activity, indicating that there is no significant amount of the Ca^{2+}-independent form. There was very little increase in PLA_2 activity at 10–100 μM Ca^{2+}, the range where $cPLA_2$ is activated, suggesting that this isoform is also present in gerbil hippocampus at very low concentrations. In this regard, it has been reported that the level of $cPLA_2$ in rat brain is very low (65). Our results are consistent with those of a previous report (50) demonstrating that the major PLA_2 in mitochondria of gerbil brain has a molecular mass of 14 kDa, characteristic of $sPLA_2$. Previous studies indicate that the mitochondrial PLA_2 is a group IIA isoform that acts on PtdCho, PtdEtn, and cardiolipin (40, 67). $sPLA_2$ activity increased in the membrane and mitochondrial fractions of the hippocampus over 24 h of reperfusion (Fig. 2).

Citicoline treatment *in vivo* resulted in a decrease in PLA_2 activity in both membrane and mitochondrial fractions (Fig. 2). It is unlikely that citicoline attenuated PLA_2 activity by directly inhibiting the enzyme, because *in vitro,* citicoline and its components choline and cytidine at 0.5 mM in the PLA_2 assay had no effect on the enzyme activity and, as such, citicoline is not a PLA_2 inhibitor (1). It has been suggested that citicoline prevented the activation of mitochondrial PLA_2 (9, 48). However, "preventing the activation" has sometimes been misinterpreted as "PLA_2 inhibition." As citicoline does not directly inhibit PLA_2, our data suggest that citicoline may act on upstream events that regulate $sPLA_2$. It is not known if the effect of citicoline on $sPLA_2$ involves phosphorylation. Although $cPLA_2$ is activated by phosphorylation, it has been generally believed that $sPLA_2$ isoforms in general lack phosphorylation sites (19). However, a recent study reported significant stimulation of PLA_2 activity by phosphorylation *in vitro* of a purified type IIA $sPLA_2$ by casein kinase (57). It has been shown that proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) are induced in gerbil hippocampus at 1–6 h and 24 h following transient ischemia (51). TNF-α has been shown to regulate expression of $sPLA_2$ (8, 56) and caspase activation (41). The stimulation of PLA_2 by TNF-α can be mediated through PLA_2 activating protein (15, 49).

Inhibition of PLA_2 attenuated TNF-α cytotoxicity (25, 64), indicating that PLA_2 mediates TNF-α cytotoxic signaling. TNF-α has also been shown to inhibit cytidine triphosphate phosphocholine cytidylyltransferase (PCCT) (36), the rate-limiting enzyme in PtdCho synthesis. Thus, increases in TNF-α may simultaneously stimulate PtdCho hydrolysis and inhibit its synthesis. Inhibition of PtdCho synthesis is sufficient in itself to cause cell death (17). However, the role of TNF-α in PLA_2 induction and lipid metabolism in cerebral ischemia has not been demonstrated. It was recently shown that citicoline decreased activation of caspases and poly(ADP-ribose) polymerase-cleaved products of caspase activation after focal cerebral ischemia (34). It is possible that citicoline prevented stimulation of $sPLA_2$ and caspase activation by attenuating induction of TNF-α, although the precise entry point of citicoline in the sequence of events following cerebral ischemia is not yet clear. We hypothesize that TNF-α is responsible for

the loss of PtdCho through modulation of PLA_2 (8), PtdCho-specific phospholipase C (53), and PCCT (36) following transient cerebral ischemia (Scheme 2).

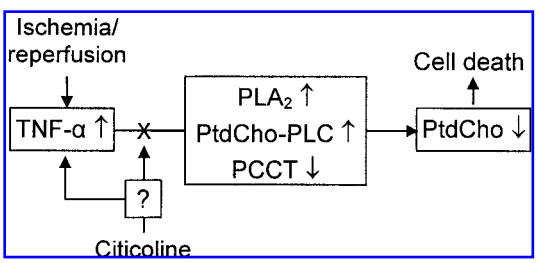
Our studies demonstrated significant decrease in the arachidonic acid composition of PtdCho and PtdEtn and loss of cardiolipin in the gerbil hippocampus following ischemia and 24 h of reperfusion, which were prevented by citicoline treatment (Table 1). Cardiolipin is an exclusive inner mitochondrial phospholipid enriched with unsaturated fatty acids and is essential for mitochondrial electron transport (30); its loss could disrupt the mitochondrial respiratory chain, resulting in increased ROS generation. Citicoline may have prevented hydrolysis of PtdCho, PtdEtn, and cardiolipin by attenuating stimulation of $sPLA_2$.

Our studies also demonstrated an increase in OH• generation at 24 h of reperfusion after forebrain ischemia (Fig. 3C). Citicoline treatment decreased formation of OH•. Citicoline has been described as an antioxidant or free-radical scavenger (27), but this apparently was based on the ability of citicoline to attenuate arachidonic acid release. There is no evidence that citicoline directly interacts with or detoxifies oxygen radicals. TNF-α increases generation of ROS, which is mediated by stimulation of PLA_2 (63). The decrease in OH• may reflect the effects of citicoline on TNF-α and/or $sPLA_2$, thus decreasing mitochondrial dysfunction and limiting the amount of arachidonic acid available for oxidative metabolism by cyclooxygenases/lipoxygenases, pathways that generate ROS (Scheme 1). Citicoline did not bring the OH• generation to sham levels, indicating that other pathways also contribute to ROS generation.

Citicoline also attenuated the increased levels of MDA (Fig. 4) and conjugated dienes (22), indicators of lipid peroxidation, following transient cerebral ischemia. The effect of citicoline on MDA was greater than its effect on OH• (Fig. 3C). This may be due to the fact that MDA levels reflect accumulation of the aldehyde over time and/or detoxification by glutathione *S*-transferase, whereas the 2,3-DHBA/salicylate ratio is a measure at a specific time point of OH• levels, which cannot accumulate due to its extreme reactivity. We have also shown in the past that citicoline treatment increased the total glutathione and glutathione reductase activity and decreased the glutathione oxidation ratio, an indicator of glutathione redox status (3).

A transgenic derivative of the mouse strain C57BL/6 has been developed that expresses type II $sPLA_2$ to compensate for the defective gene in the "normal" C57BL/6 (42), which might provide an interesting model to assess the role of $sPLA_2$ in ischemic brain injury.

In conclusion, citicoline neuroprotection in cerebral ischemia (1, 44) may be partly due to limiting the stimulation of PLA_2, resulting in a decrease in hydrolysis of phospholipids such as PtdCho, PtdEtn, and cardiolipin, thereby limiting the ROS generation and lipid peroxidation.



SCHEME 2. Proposed action of citicoline on TNF-α-mediated events after transient cerebral ischemia (8, 36, 52). PCCT (36) is the rate-limiting enzyme of PtdCho synthesis. ↑ = increase, ↓ = decrease. PtdCho-PLC, PtdCho-specific phospholipase C.

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ABBREVIATIONS

citicoline, cytidine-5'-diphosphocholine; $cPLA_2$, cytosolic phospholipase A_2; DHBA, dihydroxybenzoic acid; HNE, 4-hydroxynonenal; H_2O_2, hydrogen peroxide; MDA, malondialdehyde; $O_2^{-\bullet}$, superoxide anion radical; OH• hydroxyl radical; PCCT, cytidine triphosphate phosphocholine cytidylyltransferase; PLA_2, phospholipase A_2; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; ROS, reactive oxygen species; $sPLA_2$, secretory phospholipase A_2; TBA, 2-thiobarbituric acid; TBARS, 2-thiobarbituric acid reactive species; TNF-α, tumor necrosis factor-α.

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