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REVIEW

Citicoline: neuroprotective mechanisms in cerebral ischemia

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Abstract

Cytidine-5′-diphosphocholine (citicoline or CDP-choline), an intermediate in the biosynthesis of phosphatidylcholine (PtdCho), has shown beneficial effects in a number of CNS injury models and pathological conditions of the brain. Citicoline improved the outcome in several phase-III clinical trials of stroke, but provided inconclusive results in recent clinical trials. The therapeutic action of citicoline is thought to be caused by stimulation of PtdCho synthesis in the injured brain, although the experimental evidence for this is limited. This review attempts to shed some light on the properties of citicoline that are responsible for its effectiveness. Our studies in transient cerebral ischemia suggest that citicoline might enhance reconstruction (synthesis) of PtdCho and sphingomyelin, but could act by inhibiting the destructive processes (activation of phospholipases). Citicoline neuroprotection may include: (i) preserving cardiolipin (an exclusive inner mitochondrial membrane component) and sphingomyelin; (ii) preserving the arachidonic acid content of PtdCho and phosphatidylethanolamine; (iii) partially restoring PtdCho levels; (iv) stimulating glutathione synthesis and glutathione reductase activity; (v) attenuating lipid peroxidation; and (vi) restoring Na^+/K^+-ATPase activity. These observed effects of citicoline could be explained by the attenuation of phospholipase A_2 activation. Based on these findings, a singular unifying mechanism has been hypothesized. Citicoline also provides choline for synthesis of neurotransmitter acetylcholine, stimulation of tyrosine hydroxylase activity and dopamine release.

Keywords: *S*-adenosyl-L-methionine, lipid peroxidation, mitochondria, phospholipases, phospholipids, stroke.

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Cytidine-5′-diphosphocholine (citicoline or CDP-choline) was originally identified as the intermediate in phosphatidylcholine (PtdCho) synthesis by Eugene Kennedy in 1956 (Kennedy and Weiss 1956). In 1983 22 articles were published that described the physico-chemical properties, pharmacokinetics, toxicity and bioavailability of this agent (Anonymous 1983). In 1995 there were two review articles that discussed the beneficial effects of this drug in CNS injury (Secades and Frontera 1995; Weiss 1995). Although much work has been carried out investigating citicoline absorption and metabolism, its mechanism of neuroprotection has not been experimentally delineated in CNS injury models including cerebral ischemia. Citicoline has shown beneficial effects in a variety of CNS injury models and neurodegenerative diseases, suggesting a common underlying mechanism associated with the loss of membrane integrity (Table 1). Citicoline neuroprotection is thought to be a result of increased PtdCho synthesis in the injured brain, but the experimental evidence is limited. The present review takes an overview of citicoline neuroprotective actions based on our recent findings.

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Abbreviations used: AdoMet, *S*-adenosyl-L-methionine; ArAc, arachidonic acid; citicoline, cytidine-5′-diphosphocholine; CMP, cytidine 5′-monophosphate; DAG, 1,2-diacylglycerol; GSH, glutathione (reduced); GSSG, glutathione (oxidized); nSMase, neutral sphingomyelinase; PCCT, cytidine triphosphate-phosphocholine cytidylyltransferase; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine: PLA_2, phospholipase A_2; PLC, phospholipase C; PLD, phospholipase D; ROS, reactive oxygen species; TBI, traumatic brain injury.

Table 1 Recent studies (since 1995) investigating the action of citicoline in neuropathological conditions

Study	Effect/Outcome	Reference
Transient forebrain ischemia/gerbil	Effects on lipids, phospholipases, glutathione, blood–brain barrier dysfunction, edema and neuronal death (details in Table 2)	(Adibhatla *et al*. 2001; Rao *et al*. 1999a,b, 2000a, 2001)
Transient middle cerebral artery occlusion/rat	Decreased edema and infarct volume	(Schabitz *et al*. 1996)
	Citicoline + basic fibroblast growth factor reduced infarct volume	(Schabitz *et al*. 1999)
	Citicoline + MK-801 reduced the infarct volume	(Onal *et al*. 1997)
Embolic focal cerebral ischemia/rat	Citicoline + *rt*PA; promoted functional recovery and reduced infarction	(Andersen *et al*. 1999)
	Citicoline and (citicoline + urokinase): improved neurobehavioral score and reduced infarct volume	(Shuaib *et al*. 2000)
Traumatic brain injury/rat	Improved cognitive deficits, increased acetylcholine levels	(Dixon *et al*. 1997)
	Attenuated blood–brain barrier dysfunction and edema	(Baskaya *et al*. 2000)
Aged rats	Increased PCCT activity	(Gimenez *et al*. 1999)
Intracerebral hemorrhage/mice	Improved functional outcome, reduced ischemic injury volume and no effect on hematoma volume	(Clark *et al*. 1998)
β-amyloid deposit + hypoperfusion/rat	Attenuated hippocampal neuronal apoptosis and degeneration	(Alvarez *et al*. 1999)
Dog	Increased memory and learning	(Bruhwyler *et al*. 1998)
Clinical studies	Improved functional outcome and reduced neurological deficit in stroke patients	(Clark *et al*. 1997)
	Improved functional outcome in moderate-to-severe stroke patients	(Clark *et al*. 1999)
	Improved the neurological score	(Bruhwyler *et al*. 1997)
	No significant difference in lesion volume change with citicoline, determined by diffusion-weighted magnetic resonance imaging	(Warach *et al*. 2000)
	Improved memory performance in elderly subjects	(Alvarez *et al*. 1997)
	Improved mental performance in Alzheimer's disease	(Cacabelos *et al*. 1996)

Citicoline metabolism

Citicoline is composed of two essential moieties, cytidine and choline, linked by a diphosphate bridge (Fig. 1), and serves as the phosphocholine donor to 1,2-diacylglycerol (DAG) to form PtdCho. Exogenous citicoline is hydrolyzed and absorbed as cytidine and choline (Secades and Frontera 1995; Weiss 1995). Following absorption, choline and cytidine are re-phosphorylated and citicoline is synthesized from cytidine triphosphate and choline monophosphate by cytidine triphosphate-phosphocholine cytidylyl transferase (PCCT) (Fig. 2a) (Kent and Carman 1999). As the rate-limiting intermediate in PtdCho biosynthesis, it was believed that citicoline administration would provide benefit in pathological conditions such as CNS injury where membrane damage contributes to neuronal death.

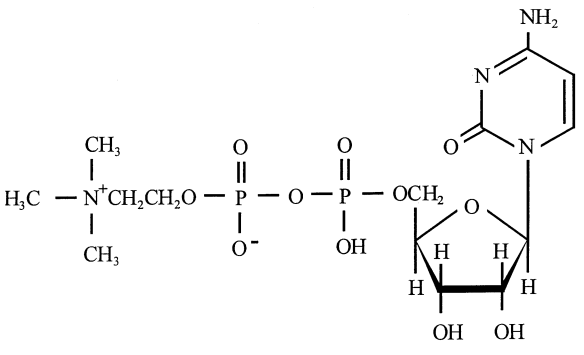

Fig. 1 Citicoline structure.

During PtdCho synthesis, choline monophosphate is incorporated into PtdCho and cytidine 5′-monophosphate (CMP) is released. CMP can be utilized for synthesis of RNA, or of DNA as the deoxyribonucleotide. The choline moiety from citicoline can also be acetylated to the neurotransmitter acetylcholine, or metabolized to betaine, which serves as a source of methyl groups in the synthesis of methionine and *S*-adenosyl-L-methionine (AdoMet) (Fig. 2a). AdoMet is the methyl donor in the methylation of proteins and nucleotides, and the conversion of phosphatidyl-ethanolamine (PtdEtn) to PtdCho (Fig. 2a). The product *S*-adenosyl-L-homocysteine can be metabolized further to glutathione (GSH) (Adibhatla *et al*. 2001).

Pharmacokinetic studies have shown that orally administered citicoline is nearly completely absorbed with very little of the dose excreted (Agut *et al*. 1983). Brain uptake of

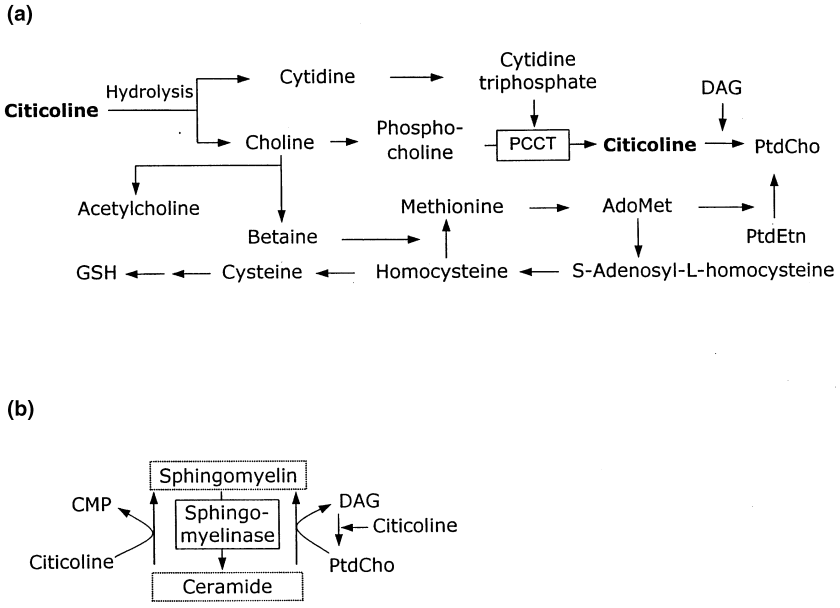

Fig. 2 Citicoline: (a) acetylcholine, PtdCho, AdoMet and GSH biosynthesis and (b) sphingomyelin synthesis.

citicoline metabolites was demonstrated as early as 30 min after administration (Galletti *et al.* 1991). Blood levels of citicoline metabolites slowly increased and peaked at 6 h after oral administration (Agut *et al.* 1983). Previous studies (Anonymous 1983) have reported the uptake and clearance of radioactivity after the administration of labeled citicoline. In those studies, only total radioactivity was measured, and thus it is not known which metabolites were present. Clearance of the radiolabel may represent slow turnover after the incorporation of choline, cytidine or their metabolites into proteins, phospholipids and nucleic acids. To the best of our knowledge, no study has measured cerebral levels of citicoline itself after its administration, so it is not known to what extent brain tissue levels are altered at any given dose.

When labeled citicoline is administered orally, only ~0.5% of the total radioactivity is incorporated into the brain (Agut *et al.* 1983). Brain uptake increased to ~2% of the total radioactivity when citicoline was administered i.v. (Fresta *et al.* 1995). Cerebral levels of citicoline or its metabolites could be greatly enhanced in ischemic rats (~23% of total dose) by the incorporation of citicoline into liposomes (Fresta *et al.* 1995). Transport of the citicoline-loaded liposomes into the brain depends on disruption of the blood–brain barrier, which occurs following cerebral ischemia (Fresta *et al.* 1995). Liposome encapsulation suggests a possible strategy to increase the citicoline levels in the CNS and enhance its clinical effectiveness (Fresta *et al.* 1994, 1995; Fresta and Puglisi 1996, 1997, 1999).

Recent studies (since 1995) examining the effects of citicoline in experimental models of CNS injury and neurodegenerative disorders are summarized in Table 1. It is worthwhile understanding the action(s) of citicoline as very few agents have such versatile beneficial effects with virtually no observed side-effects.

Acetylcholine and dopamine

The central cholinergic system plays a crucial role in learning and memory, and interacts with other neurotransmitter systems (Blusztajn and Wurtman 1983). Cholinergic neurons are unique in the utilization of choline in two metabolic pathways: synthesis of PtdCho and the neurotransmitter acetylcholine (Blusztajn and Wurtman 1983; Klein 2000). These two pathways compete for the available choline, with acetylation being favored when neurons are physiologically active (Wurtman 1992). If choline becomes depleted (for example by excessive neuronal stimulation caused by the release of excitatory amino acids in cerebral ischemia), choline phospholipids, especially PtdCho, are hydrolyzed to provide a source of choline. Stimulation of acetylcholine release in rat striatal slices caused a decrease in membrane phospholipids including PtdCho, which was prevented by the addition of choline to the incubation medium (Ulus *et al.* 1989). 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 to as 'autocannibalism' that ultimately causes neuronal death (Wurtman 1992; Klein 2000). It has been shown *in vitro* that choline deficiency resulted in the loss of membrane PtdCho and sphingomyelin, and the induction of apoptosis (Yen *et al.* 1999). Inhibition of PtdCho synthesis is sufficient in itself to cause cell death (Cui *et al.* 1996). Citicoline as a source of supplemental choline can prevent PtdCho hydrolysis and death in cholinergic neurons.

Loss of PtdCho and other membrane phospholipids caused by the stimulation of acetylcholine release implies that phospholipases are activated to release choline from PtdCho (and sphingomyelin). The specific phospholipases that are activated under these conditions have not been delineated (see the section entitled Phospholipases).

Citicoline was shown to stimulate tyrosine hydroxylase activity and dopamine release (Secades and Frontera 1995), which may be a result of increases in brain acetylcholine because choline administration also produced the same effects (Blusztajn and Wurtman 1983). Transient cerebral ischemia results in decreases in glucose utilization, acetylcholine synthesis (Kakihana *et al*. 1988) and choline acetyltransferase immunoreactivity (Ishimaru *et al*. 1994, 1995). Citicoline ameliorated the disruption of glucose metabolism, increased brain choline levels and stimulated acetylcholine synthesis (Kakihana *et al*. 1988).

Cerebral ischemia

Cerebral ischemia is caused by reduced blood supply to the brain and can be focal (regional) or global (forebrain). Energy failure, ATP loss, glutamate release and stimulation of glutamate receptors results in phospholipases activation (Siesjo 1992; Siesjo *et al*. 1995; Lipton 1999; Rao *et al*. 1999b), phospholipid hydrolysis and arachidonic acid (ArAc, 20 : 4) release (Rao *et al*. 1999c).

Interestingly, even though citicoline has undergone 12 clinical trials for the treatment of stroke, there have been no studies examining phospholipid changes including PtdCho or the effect of citicoline on lipid alterations in either permanent or transient *focal* ischemia models. There have been a number of studies conducted with citicoline in stroke models, showing a decrease in infarction volume or improvement in behavioral parameters (D'Orlando and Sandage 1995; Aronowski *et al*. 1996; Schabitz *et al*. 1996, 1999; Onal *et al*. 1997; Clark *et al*. 1998; Andersen *et al*. 1999; Shuaib *et al*. 2000) (Table 1), but no mechanistic data has been presented.

Phospholipids

Non-pathological conditions

Normal rats treated with citicoline (500 mg/kg per day) showed no significant increase in cortical PtdCho levels after 21 days of administration. PtdCho levels were significantly elevated (~22%) only after 42 days of treatment (Lopez-Coviella *et al*. 1995). This suggests that under non-pathological conditions, the synthesis of PtdCho is regulated to maintain normal brain levels.

Permanent global ischemia

Until recently only one paper had been published that examined the effects of citicoline on phospholipid changes in cerebral ischemia (Trovarelli *et al*. 1981). This study was conducted in permanent ischemia (10-min global ischemia in gerbil with no reperfusion). Of the phospholipids examined, only PtdCho showed a significant decrease. Loss of ATP during ischemia results in the accumulation of CMP, which is normally phosphorylated to the triphosphate in the presence of ATP. It is generally believed that the accumulation of CMP contributes to PtdCho loss through reversal of the PtdCho synthesis pathway in the reaction (Dorman *et al*. 1983; Murphy and Horrocks 1993): CMP + PtdCho $\rightarrow$ DAG + citicoline.

Intracerebroventricular injection of citicoline 5 min prior to ischemia partially but significantly restored PtdCho levels and decreased the release of free fatty acids. This effect was attributed to the stimulation of the choline phosphotransferase reaction to increase the incorporation of DAG into PtdCho. In these studies, citicoline was injected directly into the brain and thus cerebral levels of the drug were likely to be much higher than when the drug was administered systemically. Systemic pretreatment with citicoline (i.p.) did not significantly alter free fatty acid levels after 10 min of permanent ischemia (no reperfusion) in gerbil (Rao *et al*. 1999a).

Transient global ischemia

In 10-min transient forebrain ischemia in gerbil, hippocampal CA_1 neurons undergo delayed death beginning on day 3 and culminating on day 6 (Kirino and Sano 1984; Rao *et al*. 2000b). In our studies, one dose of citicoline administered at the onset of reperfusion did not show neuroprotection. Two doses at 0 and 3 h provided significant but incomplete neuroprotection (Rao *et al*. 1999b, 2001). Maximum neuroprotection was obtained when the treatments were continued over days 1–5 and neuronal survival was assessed on day 6 (Hatcher *et al*. 1999; Rao *et al*. 1999a).

Citicoline treatment attenuated ArAc release in hippocampus after 10-min forebrain ischemia/1-day reperfusion in gerbil (Rao *et al*. 1999a). Citicoline can decrease ArAc levels by either increasing the synthesis of PtdCho (Cui *et al*. 1996) or preventing the activation of phospholipase A_2 (PLA_2) (Arrigoni *et al*. 1987; Rao *et al*. 2001). Citicoline therefore may affect the levels of many lipids following ischemia and reperfusion. Whether citicoline directly inhibits PLA_2 or prevents its activation requires further investigation.

The 10-min ischemia with 0- (permanent ischemia) or 1-day reperfusion (transient ischemia) resulted in significant decreases in levels of PtdCho, PtdIns, PtdSer and sphingomyelin, but not PtdEtn (Rao *et al*. 2000a). ArAc levels also significantly decreased in PtdCho, PtdIns and PtdSer after ischemia/0-day reperfusion (permanent global ischemia), but not in PtdEtn.

In addition to theses changes, there were significant decreases in cardiolipin and ArAc levels in PtdEtn following ischemia/1-day reperfusion (transient ischemia) (Rao *et al*.

2000a; Adibhatla *et al*. 2001). Citicoline significantly restored cardiolipin, sphingomyelin and both the ArAc levels and total fatty acids of PtdCho in 1-day reperfusion, but had no significant effect on the levels of PtdEtn, PtdIns or PtdSer. There were significant alterations in the composition of PtdCho and PtdEtn. Thus, although the total levels of PtdEtn fatty acids were unchanged following ischemia/1-day reperfusion, the ArAc content as a percentage of the total fatty acids showed a significant decline. This change in composition was observed also in PtdCho, in addition to the decrease in the level of total fatty acids. Treatment with citicoline significantly restored the ratio of ArAc to total fatty acids in both PtdCho and PtdEtn (Rao *et al*. 2000a).

The significant decrease in the levels of ArAc and the proportion of ArAc in total fatty acids in PtdEtn and PtdCho may be caused by the activation of PLA_2, which selectively hydrolyzes ArAc at the *sn*-2 position of PtdCho and PtdEtn (Rao *et al*. 2000a, 2001). The loss of PtdIns suggests the activation of a PtdIns-specific phospholipase C (PLC) (Rhee and Bae 1997). Both the total level of PtdCho and the ArAc content of PtdCho and PtdEtn, but not of PtdIns, were restored after ischemia/1-day reperfusion by the administration of citicoline. Citicoline could also have restored PtdCho levels by preventing the activation of PLA_2, which may account for the effect of citicoline on restoring the ArAc content of PtdEtn. The observation that citicoline did not restore PtdIns suggests it had no effect on PtdIns-PLC.

Citicoline may increase the level of PtdCho via two pathways: (Rao *et al*. 1999a) (i) transfer of phosphocholine to DAG in order to form PtdCho and (ii) choline liberated from citicoline can be utilized in biosynthesis of methionine and AdoMet (Fig. 2a). AdoMet serves as a methyl donor in the conversion of PtdEtn to PtdCho. Because PtdEtn contains a much higher proportion of docosahexaenoic acid (22 : 6, ~37% of total fatty acids) compared with PtdCho (4%), conversion of PtdEtn to PtdCho following citicoline treatment might be reflected in an increase in the 22 : 6 content of PtdCho. Citicoline did not alter the proportion of 22 : 6 in PtdCho, indicating that it did not significantly increase PtdEtn conversion to PtdCho (Rao *et al*. 2000a). These data are consistent with observations that PtdEtn-*N*-methyltransferase activity is high in liver, but is generally very low in other tissues (Walkey *et al*. 1998).

Cardiolipin

Cardiolipin is an exclusive inner mitochondrial phospholipid enriched with unsaturated fatty acids and is essential for mitochondrial electron transport (Hoch 1992). Citicoline 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*. 1991, 1992) (see the section entitled Phospholipases). It is conceivable that citicoline stimulated cardiolipin synthesis by increasing cytidine diphosphodiacylglycerol, a precursor in the biosynthesis of both PtdIns and cardiolipin (Vance 1998). However, as citicoline treatment had no effect on PtdIns, it appears unlikely that citicoline increased biosynthesis of cytidine diphosphodiacylglycerol, and may therefore have prevented cardiolipin hydrolysis (Rao *et al*. 2001).

Sphingomyelin and ceramide

Citicoline completely restored sphingomyelin levels after ischemia/1-day reperfusion. Sphingomyelin can be synthesized using either PtdCho or citicoline as the phosphocholine donor to ceramide (Fig. 2b) (Stoffel and Melzner 1980; Vos *et al*. 1997; Goswami and Dawson 2000). Alternatively, sphingomyelinase is stimulated by tumor necrosis factor-α (Levade and Jaffrezou 1999; Liu *et al*. 1999), which is induced over a period of 1–6 h following transient forebrain ischemia (Saito *et al*. 1996). Activation of neutral sphingomyelinase may be mediated through PLA_2 and the release of ArAc (Jayadev *et al*. 1994). If citicoline modulates PLA_2 activity, activation of sphingomyelinase could in turn be affected.

Even though sphingomyelin levels declined following ischemia with no or 1-day reperfusion, the sphingomyelinase product ceramide did not show corresponding accumulation (Rao *et al*. 2000a). Ceramide levels may be highly regulated (Kolesnick and Fuks 1995), and further metabolism may preclude its accumulation. Alternatively, sphingomyelin might be hydrolyzed by a phospholipase that cleaves the fatty acid residue to form sphingosylphosphorylcholine (lysosphingomyelin) (Zeisel 1993). In contrast, ceramide levels were significantly elevated after 3 and 6 days of reperfusion, without a significant decrease in sphingomyelin, but it should be noted that the sphingomyelin pool is much larger than the levels of ceramide (Adibhatla *et al*. 2001). Although ceramide has been implicated in the induction of apoptosis (Green and Reed 1998; Goswami and Dawson 2000), its role in neuronal death remains debatable (Hofmann and Dixit 1998; Kolesnick and Hannun 1999). The increase in ceramide could be a signal of impending neuronal death (which begins after 3 days; Rao *et al*. 2000b) as apoptosis is usually accompanied by a late phase of ceramide production (Tepper *et al*. 2000). Ceramide levels further increased after 6 days and could be the result of neuronal death, which is nearly complete at this time. However, citicoline treatment did not alter ceramide levels on day 3 or day 6, even though it provided neuroprotection (Rao *et al*. 1999a), and thus ceramide levels did not correlate with neuronal death.

Under normal conditions plasma membrane phospholipids have an asymmetrical distribution: neutral phospholipids such as PtdCho and sphingomyelin are located in the exofacial membrane, whereas anionic phospholipids (PtdEtn, PtdIns and PtdSer) are localized in the cytofacial leaflet

(Devaux and Zachowski 1994; Wattiaux-De Coninck and Wattiaux 1994; Wood *et al*. 1996). Alterations of the plasma membrane structure caused by translocation of phospholipids between the exo- and cytofacial leaflets, a process known as phospholipid scrambling, induces apoptosis (Martin *et al*. 1995; Rimon *et al*. 1997; Suzuki *et al*. 1999; Kagan *et al*. 2000). Loss of sphingomyelin *per se* may contribute to membrane damage because it has a high affinity for cholesterol, and these lipids are major determinants of the membrane integrity. Sphingomyelin hydrolysis following phospholipid scrambling results in redistribution of cholesterol to intracellular compartments, causing major changes in membrane structure and fluidity (Tepper *et al*. 2000).

Phospholipases

PtdCho can be hydrolyzed (Exton 1994; Tronchere *et al*. 1994) by PtdCho-PLC (Li *et al*. 1998), PtdCho-phospholipase D (PLD) (Thompson *et al*. 1991, 1993; Klein *et al*. 1995), or PLA_2 (Farooqui *et al*. 1997a,b; Six and Dennis 2000). There is substantial evidence that PLA_2 is activated in ischemia/reperfusion and contributes to neuronal damage (Bonventre *et al*. 1997; Farooqui *et al*. 1999, 2000a,b,c; Rao *et al*. 1999c). PtdCho is hydrolyzed to provide choline to maintain neurotransmission when acetylcholine release is stimulated, but there is currently no clear evidence that PLA_2 is activated in response to the requirement for acetylcholine synthesis (see the Acetylcholine and Dopamine sections).

Our data on the effects of citicoline on phospholipids following transient ischemia are consistent with an effect on PLA_2 activation. However, there has been only *one* study (Arrigoni *et al*. 1987) directly examining the effect of citicoline on PLA_2, which demonstrated that citicoline prevented the increase in mitochondrial PLA_2 activity following cryogenic brain injury in rabbit, in which energy failure does not occur as it does in ischemia. It was concluded that citicoline prevented the activation of PLA_2 instead of directly inhibiting the enzyme as citicoline had no effect on PLA_2 activity in non-injured controls and restored PLA_2 activity to control levels in the injured group.

Previous studies have indicated that the mitochondrial PLA_2 is a Ca^{2+}-dependent 14-kDa group IIA secretory PLA_2 ($sPLA_2$) isoform that acts on PtdCho, PtdEtn and cardiolipin (Nakahara *et al*. 1991, 1992; Rordorf *et al*. 1991; Zhang *et al*. 1999). It is possible that citicoline prevented the cardiolipin hydrolysis by inhibiting the activation of this isoform. Post-ischemic activation of a mitochondrial 14-kDa PLA_2 was demonstrated in transient forebrain ischemia in gerbil (Rordorf *et al*. 1991), but the effect of citicoline has not been determined. Our data also suggests that citicoline did not affect activities of phospholipase C or D because citicoline had no effect on PtdIns levels, and PtdCho levels were only partially restored (Rao *et al*. 2001).

Sustained activation of phospholipases over 7 days has been reported earlier in gerbil 5-min transient ischemia (Abe *et al*. 1989). However, our recent studies showed that all major phospholipids returned to sham levels after a 2–6-day reperfusion following 10-min ischemia (Adibhatla *et al*. 2001), suggesting that phospholipases are not significantly activated over this time. This is in agreement with previous studies which showed that phospholipases are down-regulated during this period (Lauritzen *et al*. 1994).

Lipid peroxidation and glutathione

Formation of reactive oxygen species (ROS) (including superoxide radical, hydrogen peroxide and hydroxyl radical) and the ensuing oxidation of biological molecules is a well-recognized mechanism of tissue damage in ischemia/reperfusion (Werns and Lucchesi 1990; Coyle and Puttfarcken 1993; Globus *et al*. 1995; Yamaguchi *et al*. 1998; Chan 2001). ROS induce lipid peroxidation, resulting in the formation of malondialdehyde and 4-hydroxynonenal (Esterbauer *et al*. 1991). 4-Hydroxynonenal induces neuronal apoptosis by covalently cross-linking with proteins (Uchida and Stadtman 1992; Kruman *et al*. 1997). One previous study showed that citicoline decreased lipid peroxidation following transient cerebral ischemia (Fresta *et al*. 1994), suggesting that citicoline neuroprotection may include the attenuation of oxygen radical formation.

Glutathione

Glutathione (GSH, reduced) is one of the primary endogenous antioxidant defense systems in the brain that removes hydrogen peroxide and lipid peroxides (Coyle and Puttfarcken 1993). Increased GSH may contribute to neuroprotection by attenuating lipid peroxidation (Rao *et al*. 2000a; Adibhatla *et al*. 2001). Choline liberated from citicoline can be metabolized to GSH through the AdoMet pathway (Fig. 2a). Exogenous AdoMet provided significant neuroprotection (Rao *et al*. 1997) and increased GSH levels (De la Cruz *et al*. 2000).

Total glutathione levels [GSH + glutathione (oxidized)] remained unaltered during 6-h reperfusion after ischemia, but decreased between 1 and 3 days. Several factors could contribute to this decrease, such as the cleavage of GSH to cysteine (Slivka and Cohen 1993), the decreased synthesis of GSH or the formation of mixed disulfides with GSSG (Shivakumar *et al*. 1995). Citicoline administration resulted in transient increases in total glutathione over 1-day reperfusion. Two considerations suggest these increases represent an increase in GSH synthesis (Lu 1999): GSSG levels were not altered during this time and the levels in citicoline treated groups exceeded sham levels. This suggests that citicoline did not simply prevent oxidation of GSH. Citicoline treatment beyond 1 day did not

significantly alter total glutathione levels (Adibhatla *et al*. 2001).

Changes in total glutathione represent alterations in GSH levels as GSSG levels accounted for only 2–4% of the total and showed very small changes (Adibhatla *et al*. 2001). Low levels of GSSG have been shown in other ischemia models, which were not altered during reperfusion (Cooper *et al*. 1980; Rehncrona *et al*. 1980). Citicoline treatment decreased the GSSG levels as well as the glutathione oxidation ratio ($2 \times$ GSSG/total glutathione, an indicator of the redox status of glutathione), suggesting that citicoline attenuated the oxidative stress (Adibhatla *et al*. 2001).

Glutathione reductase

The activity of GSSG reductase decreases after transient ischemia (Shivakumar *et al*. 1995; Adibhatla *et al*. 2001). Loss of GSSG reductase activity may be a result of the inactivation of the enzyme by oxygen radicals generated during reperfusion (Chan *et al*. 1998). Changes in GSSG reductase activity were not accompanied by changes in GSSG levels, suggesting that either the reductase activity did not become limiting, or that excess GSSG was excreted or reacted with protein thiols to form mixed disulfides (Lu 1999). Administration of citicoline resulted in significant increases in reductase activity after transient ischemia. If the GSSG reductase is inactivated by ROS following ischemia, it is conceivable that citicoline prevented this inactivation by attenuating ROS formation.

Blood–brain barrier dysfunction and edema

Citicoline attenuated cerebral edema in transient focal (Schabitz *et al*. 1996) and global (Rao *et al*. 1999a,b) ischemia and traumatic brain injury (TBI) (Baskaya *et al*. 2000). Citicoline restored Na^+/K^+-ATPase activity (following cold injury in rabbits) (Rigoulet *et al*. 1979) and thus may have attenuated cytotoxic edema. Because ArAc inhibits Na^+/K^+-ATPase (Chan and Fishman 1978), restoration of this activity could be mediated through the prevention of PLA_2 activation and the subsequent decrease in ArAc release. Alternatively, citicoline had a direct stimulatory effect on Na^+/K^+-ATPase activity *in vitro* (Plataras *et al*. 2000). Citicoline also attenuated blood–brain barrier dysfunction following TBI (Baskaya *et al*. 2000) or transient forebrain ischemia (Rao *et al*. 1999a) thus attenuating vasogenic edema.

The known effects of citicoline, to the best of our knowledge are summarized in Table 2. Many of the effects of citicoline could be explained by the attenuation of PLA_2 activation (Fig. 3). If citicoline primarily acts by preventing PLA_2 activation, the effects of citicoline may be limited to those cell types wherein PLA_2 is activated. *In situ* hybridization studies indicated that the expression of cytosolic PLA_2 (Kishimoto *et al*. 1999) and type-II *s*PLA_2 (Lauritzen *et al*. 1994) in the hippocampus was primarily neuronal. To the best of our knowledge virtually no literature exists on lipid alterations or the biochemical actions/mechanisms of citicoline in focal cerebral injury. One of the common

Table 2 Citicoline mechanisms of action

Function/System	Action	Reference
Lipids		
Affected	Restored cardiolipin and sphingomyelin levels	(Rao *et al*. 2000a)
	Partially restored PtdCho	(Rao *et al*. 2000a)
	ArAc composition of PtdCho and PtdEtn	(Rao *et al*. 2000a)
	ArAc release	(Rao *et al*. 1999a; Trovarelli *et al*. 1981)
	Leukotriene C_4 formation	(Rao *et al*. 1999a)
Unaffected	PtdSer, PtdIns and ceramide	(Rao *et al*. 2000a)
	Methylation of PtdEtn to PtdCho	(Rao *et al*. 2000a)
Phospholipases:	Affecting the activation of PLA_2	(Arrigoni *et al*. 1987; Rao *et al*. 2001)
	May not affect PLC and PLD	(Rao *et al*. 2001)
Antioxidant systems:	Increase GSH levels and GSSG reductase activity	(Adibhatla *et al*. 2001)
	Attenuated glutathione oxidation ratio	(Adibhatla *et al*. 2001)
	Attenuated lipid peroxidation	(Fresta and Puglisi 1996; Fresta *et al*. 1994)
Neurotransmitter systems:	Increase in acetylcholine synthesis	(Kakihana *et al*. 1988; Dixon *et al*. 1997)
	Increase in tyrosine hydroxylase activity and dopamine levels	(Secades and Frontera 1995)
Ion transport:	Restored Na^+/K^+-ATPase activity	(Rigoulet *et al*. 1979)
Physiological:	Decreased edema	(Baskaya *et al*. 2000; Rao *et al*. 1999a; Schabitz *et al*. 1996)
	Attenuated blood–brain barrier dysfunction	(Baskaya *et al*. 2000; Rao *et al*. 1999a)

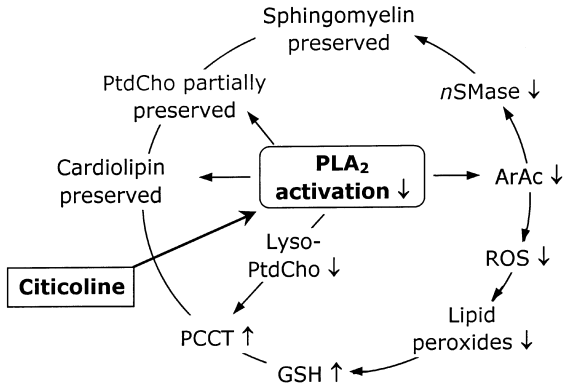

Fig. 3 Proposed major pathway of citicoline neuroprotection. ArAc, arachidonic acid; GSH, glutathione; *n*SMase, neutral sphingomyelinase; PLA_2 phospholipase A_2; PtdCho, phosphatidylcholine; ROS, reactive oxygen species. ↑ indicates increase; ↓ indicates decrease.

features of cerebral ischemia, whether global or focal, is energy failure and activation of phospholipases (Siesjo 1992; Siesjo *et al*. 1995). Thus, the effects of citicoline on PLA_2 activation may be applicable to focal ischemia models also. This hypothesized singular unifying mechanism (Fig. 3) needs to be investigated further.

Current status in clinical trials

Of the neuroprotective agents undergoing phase-III clinical trials in acute stroke (De Keyser *et al*. 1999; Fisher and Schaebitz 2000), citicoline has shown beneficial effects with virtually no side-effects, whereas all the other treatments have given negative results with regard to the primary outcome (functional and/or cognitive) measure (Clark *et al*. 1999; De Keyser *et al*. 1999; STAIR-II 2001). However, the most recent clinical trials of citicoline did not provide conclusive results.

There have been 12 clinical trials of citicoline since 1980 (nine in Europe and Japan and three in the USA) (Boudouresques and Michel 1980; Goyas *et al*. 1980; Hazama *et al*. 1980; Corso *et al*. 1982; Franceschi *et al*. 1982; Tazaki *et al*. 1988; Bruhwyler *et al*. 1997; Clark *et al*. 1997, 1999; Warach *et al*. 2000). The European clinical trials showed that citicoline improved global and neurological function and promoted earlier motor and cognitive recovery. A large multicenter study in Japan found that citicoline showed improvement in the global outcome rating scale (Tazaki *et al*. 1988). Three major clinical trials in the USA were conducted in 1997 (Clark *et al*. 1997), 1999 (Clark *et al*. 1999) and 2000 (Warach *et al*. 2000). In the first study, 259 patients were enrolled with citicoline treatment initiated within 24 h of stroke onset (mean time to treatment was 14.5 h). Citicoline improved the functional outcome and reduced the neurologic deficit with 500 mg appearing to be the optimal dose. However, the second study involving 394 patients (Clark *et al*. 1999) failed to demonstrate improvement in the outcome. In *post-hoc* analysis, citicoline was shown to provide beneficial effects in a subgroup of moderate–severe stroke cases (Clark *et al*. 1999). In the third study (Warach *et al*. 2000), although a large difference in the percentage change in lesion volume was observed in favor of citicoline (34% for citicoline vs. 180% for placebo group), the large variance in the placebo group precluded statistical significance. Owing to the inconclusive results of some of the clinical trials, further studies are necessary to obtain clear results on the efficacy of citicoline for stroke therapy.

Although citicoline clinical trials were initiated based on the positive outcome in animal models, a recent study showed that citicoline metabolism in humans (Wurtman *et al*. 2000) differs from that in rodents (Lopez-Coviella *et al*. 1995). In rodents, blood plasma levels of cytidine and choline are increased after oral citicoline (Lopez-Coviella *et al*. 1995). However, in humans blood plasma levels of uridine, but not cytidine, are increased after oral citicoline as a result of cytidine deaminase in the gastrointestinal tract and liver (Wurtman *et al*. 2000). Uridine must then enter the brain, become phosphorylated to uridine triphosphate, which in turn is converted to cytidine triphosphate.

As a result of the multiple pathways involved in ischemic injury, no single agent is likely to provide complete neuroprotection following transient ischemia (White *et al*. 2000). Citicoline restored cardiolipin and sphingomyelin, partially restored PtdCho, and completely restored the ArAc composition of PtdCho and PtdEtn, which would help stabilize the cellular membrane and restore mitochondrial function. Citicoline also decreased lipid peroxidation and increased GSH. It is likely that all of these effects contribute to citicoline neuroprotection as there is substantial evidence that loss of phospholipids (Siesjo and Katsura 1992; Siesjo *et al*. 1995; Farooqui *et al*. 1997a,b; Rao *et al*. 1999b) and generation of ROS (Siesjo *et al*. 1989; Chan 2001) contribute to ischemic injury. Citicoline had no effect on cytofacial phospholipids (PtdIns or PtdSer), and did not fully prevent the loss of PtdCho. Thus, its ability to completely restore membrane integrity may be limited. Most of the observed biochemical consequences could be attributable to the inhibition of PLA_2 activation (Fig. 3).

Combining agents with different mechanisms of action will probably be necessary for full recovery (De Keyser *et al*. 1999). Citicoline in combination with NMDA receptor antagonist MK801 (Onal *et al*. 1997), thrombolytic agents (recombinant tissue plasminogen activator) (Andersen *et al*. 1999) or urokinase (Shuaib *et al*. 2000), or basic fibroblast growth factor (Schabitz *et al*. 1999) showed synergistic benefit in experimental ischemia models. Identifying the mechanism(s) by which citicoline provides neuroprotection is crucial to develop more efficient treatment strategies for stroke.

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Note added in proof

Recently it has been shown that the other hydrolysis product of PtdCho by PLA_2, lyso-PtdCho could inhibit the PCCT activity (Boggs *et al.* 1995; Awasati *et al.* 2001) resulting in impairment of PtdCho synthesis. Citicoline may increase PCCT activity (Gimenez *et al*. 1999) by inhibiting PLA2 activation and limiting the formation of lyso-PtdCho.

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