Effects of CDP-Choline on Neurologic Deficits and Cerebral Glucose Metabolism in a Rat Model of Cerebral Ischemia

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The effects of cytidine 5'-diphosphocholine (CDP-choline) on neurologic deficits and cerebral glucose metabolism were studied in a rat model of transient cerebral ischemia. Cerebral ischemia was induced by occluding both common carotid arteries for 20 or 30 minutes 24 hours after the vertebral arteries were permanently occluded by electrocautery. CDP-choline was administered intraperitoneally twice daily for 4 days after reestablishing carotid blood flow. CDP-choline at two dosages (50 and 250 mg/kg) shortened the time required for recovery of spontaneous motor activity in a dose-related manner; recovery time was measured early after reperfusion. Neurologic signs were observed for 10 days. High-dose CDP-choline improved neurologic signs in the rats within 20-30 minutes of ischemia. When cerebral glucose metabolism was assessed on Day 4, increases in the levels of glucose and pyruvate were accompanied by decreases in the synthesis of labeled acetylcholine from uniformly labeled [14C]glucose measured in the cerebral cortex of rats with 30 minutes of ischemia. High-dose CDP-choline also attenuated changes in these variables. CDP-[1,2-14C]choline injected intravenously 10 minutes after reperfusion was used for membrane lipid biosynthesis. These results indicate that CDP-choline has beneficial effects on brain dysfunction induced by cerebral ischemia, which may be due in part to the restorative effects of CDP-choline on disturbed cerebral glucose metabolism, probably by stimulating phospholipid biosynthesis. (Stroke 1988; 19:217-222)

Cytidine 5'-diphosphocholine (CDP-choline, Nicolin) is a key intermediary in the biosynthesis of phosphatidylcholine,1 an important phospholipid component of cell membranes. CDP-choline is widely employed in Japan and Europe to treat head injury and acute stroke. The therapeutic actions of CDP-choline are thought to be due to restorative effects on phospholipid synthesis in the damaged brain.2 Cerebral ischemia and hypoxia are known to affect membrane composition drastically by increasing free fatty acids (FFAs)3 and by decreasing phospholipid content.4 In addition, FFAs inhibit oxidative phosphorylation of mitochondrial preparations in vitro.4 Arachidonic acid, a major unsaturated FFA released during cerebral ischemia, induces brain edema in vitro5 and in vivo.6 Recent studies of animal models of cerebral ischemia7,8 reveal that CDP-choline attenuates these FFA increases and stimulates resynthesis of phospholipid. CDP-choline also restores the disruption of cerebral mitochondrial lipid metabolism induced by hypoxia.9 These findings strongly suggest the potential usefulness of CDP-choline as a therapeutic agent for treating brain damage induced by cerebral ischemia and hypoxia.

We investigated the effects of early CDP-choline treatment of cerebral ischemia on the recovery from neurologic deficits and on cerebral glucose metabolism in a rat model of temporary cerebral ischemia and reperfusion. Since acetylcholine (ACh) is synthesized by choline acetyltransferase from choline and acetyl coenzyme A via pyruvate from glucose, we also determined the synthesis of ACh as well as the levels of glucose metabolism. Likewise, we determined the use of labeled CDP-choline for lipid biosynthesis in the cerebral cortex of rats using CDP-[1,2-14C]choline ([14C]CDP-choline).

Materials and Methods

Preparation of Rats

Cerebral ischemia was induced in 8-9 week-old male Wistar rats by the method of Pulsinelli and Brierley.12 Under 35 mg/kg i.p. pentobarbital anesthesia, both vertebral arteries were occluded by cautery using a bipolar coagulator (Micro ID, Mizuhoika Kogyo, Tokyo, Japan). Silk sutures were placed around both common carotid arteries without interrupting the carotid blood flow. The next day, the rats were restrained and given local anesthesia; both carotid arteries were exposed by gently pulling the sutures, and the arteries were then occluded with clips. We used only those rats that lost the righting reflex immediately after bilateral carotid artery occlusion (BCAO) and never regained the reflex for the duration of BCAO. Reperfusion was established by removing both clips 20 or 30 minutes later.

Effects on Neurologic Deficits (Experiment 1)

Rats with 20 or 30 minutes of cerebral ischemia and reperfusion were used. Two dosages of CDP-choline...
(50 and 250 mg/kg i.p., low- and high-dose, respectively) dissolved in saline or saline was administered twice daily for 4 days. On Day 1, CDP-choline or saline (50 and 250 mg/kg i.p., low- and high-dose, respectively) dissolved in saline or saline was administered at 9:00 AM and 4:00 PM. We measured the time required for recovery of the righting reflex (RR time) and spontaneous motor activity (SM time) during the first 3 hours after reperfusion. We observed neurologic status for 10 days and scored it as: 0, normal; 1, decrease in spontaneous motor activity, piloerection, hyperirritability, and slight ataxia; 2, moderate to severe ataxia; 3, loss of righting reflex; 4, convulsion and coma; and 5, death.

Effects on Glucose Metabolism (Experiment 2)

We examined the effects of CDP-choline on cerebral glucose metabolism using three groups of rats: a control group of rats with bilateral occlusion of only the vertebral arteries and receiving saline, an ischemia-saline group of rats that received saline after 30 minutes of cerebral ischemia and reperfusion, and an ischemia—CDP-choline group of rats that received 250 mg/kg CDP-choline after 30 minutes of cerebral ischemia and reperfusion. CDP-choline was injected according to the schedule described above. On the morning of Day 4, the right femoral vein was cannulated under ether anesthesia. Uniformly labeled $[^{14}C]$glucose (52.6 μCi/40 μl/100 g, New England Nuclear, Boston, Massachusetts; 315 μCi/mmol) was injected intravenously 30 minutes after the final administration of CDP-choline; the rats were killed by microwave irradiation (5 kW, 1.5 seconds; model TMW 6402, Toshiba, Tokyo, Japan) 3 minutes later. Blood samples were collected from the abdominal vena cava by immediate laparotomy. After removing the brain, the effects of CDP-choline on glucose metabolism were evaluated in the cerebral cortex, which was homogenized in 3 ml of 0.3N perchloric acid (PCA) with 100 μl of 100 μM ethylhomocholine as an internal standard to determine the ACh and choline contents. After centrifuging at 40,000g for 30 minutes, the supernatant was used to assay the ACh and choline contents and the radioactivity of the labeled ACh. The remaining supernatant was stored at the adenosine triphosphate (ATP), lactate, pyruvate, and glucose contents and the radioactivities of the acid-soluble fraction of [14C]glucose.

The method for determining ACh and choline has been described. Briefly, 20 μl of 5 mM tetraethylammonium chloride as a coprecipitant and 200 μl of potassium periodide were added to 1 ml of the supernatant. After centrifuging at 10,000g for 5 minutes, the precipitate was dissolved in acetone-nitriile, and an anion exchange resin (AG1-X8 chloride form, Bio-Rad, Cambridge, Massachusetts; 100–200 mesh) was added to remove excess iodine. After centrifuging at 10,000g for 5 minutes, the supernatant was evaporated with a nitrogen stream. The residue was dissolved in 100 μl of 0.02 M citrate phosphate buffer (pH 3.5), and a 90-μl portion was analyzed by high-performance liquid chromatography equipped with an electrochemical detector (HPLC/ECD) (Ach analyzer, Bioanalytical Systems, Incorporated, West Lafayette, Indiana) as described by Potter et al with minor modification. After ACh and choline were determined, the eluates from HPLC/ECD were collected and the radioactivity of the ACh fraction was measured.

One milliliter of the remaining supernatant was neutralized with 100 μl of 2N KOH. ATP, lactate, pyruvate, and glucose were determined by the specific enzymatic fluorometric techniques of Lowry et al and Passonneau as reported by Folbergrová et al. The radioactivity of [14C]glucose was measured by the method of Gibson et al.

ACh synthesis was calculated by the method of Gibson and Duffy by using the equation:

$$ACh \text{ synthesis} = \frac{3 \times (dpm \text{ in ACh}) \times \text{nmol of glucose}}{g \text{ tissue} \times \text{dpm of glucose}}$$

where ACh synthesis is measured in nanomoles per gram per 3 minutes.

Incorporation of [14C]CDP-Choline Into Membrane Lipid (Experiment 3)

Rats with 30 minutes of cerebral ischemia and reperfusion were used. The right femoral vein was cannulated on the day before the experiment. [14C]CDP-choline (9.3 μCi/20 μl/100 g, Amersham, Arlington Heights, Illinois; 5.2 mCi/mmol) was dissolved in saline and administered intravenously 10 minutes after reperfusion. The rats were killed by microwave irradiation 3 or 60 minutes after the injection. Blood samples were collected, and the cerebral cortex was homogenized in 3 ml of 0.3N PCA. The homogenate was centrifuged at 40,000g for 30 minutes to separate the supernatant (acid-soluble fraction) and precipitate. The precipitate was rehomogenized in 5 ml chloroform-methanol mixture (CM, 1:1 vol:vol). The homogenate was centrifuged at 1,000g for 10 minutes, and the CM layer was transferred into a glass tube. After 1 ml of 0.5% NaCl solution was added, the mixture was centrifuged again at 1,000g for 10 minutes, and the upper layer was removed by aspiration. These procedures were repeated twice, and the CM layers were combined (lipid fraction).

Measurement of Radioactivity (Experiments 2 and 3)

Two hundred microliters distilled water and 50 μl of 1N NaOH were added to 50 μl blood, and the mixture was allowed to stand for 5 minutes. After 100 μl of 30% H2O2 was added, the mixture was allowed to stand for >30 minutes. The mixture was then neutralized by adding 10 μl acetic acid. After 12 ml scintillator (ACS-II, Amersham) was added, radioactivity was measured. The CM layer was evaporated to dryness with a nitrogen stream; 200 μl distilled water and 50 μl of 1N NaOH were added, and the mixture was treated as described above. Four milliliters scintillator...
was added to 100 μl of the acid-soluble, [14C]ACh, and [14C]glucose fractions.

Statistical Analysis
Rats with neurologic status scores >2 were analyzed for significant differences using the χ² test. The amounts of neurochemicals were compared using one-way analysis of variance with a posteriori comparisons using Tukey’s test.

Results
Effects on Neurologic Deficits (Experiment 1)
The effects of treatment with CDP-choline after ischemia on the RR and SM times are shown in Figure 1. In rats with 20 minutes of ischemia, CDP-choline shortened the SM time in a dose-related manner. The percent of rats with SM time of <40 minutes in the saline–treated group and the low- and high-dose CDP-choline–treated groups was 20, 40, and 70%, respectively. CDP-choline, however, did not shorten the RR time in rats with 20 or 30 minutes of ischemia.

The time course for development of neurologic deficits is shown in Figures 2 and 3. In rats with 20 minutes of ischemia, both dosages of CDP-choline attenuated the neurologic status score in a dose-dependent manner, and the effect of the high dose was significant at Days 7–10. The cumulative mortality rates for 10 days in the saline-treated and the low- and high-dose CDP-choline-treated groups were 40, 20, and 0%, respectively. In rats with 30 minutes of ischemia, high-dose CDP-choline attenuated the neurologic status score on Days 2, 4, and 8–10. The cumulative mortality rates in the saline– and high-dose CDP-choline–treated groups were 60 and 10%, respectively.

Effects on Glucose Metabolism (Experiment 2)
Radioactivity in the plasma 3 minutes after [14C]glucose administration was 3.78 ± 0.02, 3.75 ± 0.13, and 3.62 ± 0.07 dpm/μl in the control, ischemia–saline, and ischemia–CDP-choline groups, respectively. The corresponding radioactivity in the acid-soluble fraction was 1.46 ± 0.03, 1.65 ± 0.09, and 1.69 ± 0.11 dpm × 10⁴/g. No significant differences in these variables were observed among the three groups.

The effects of CDP-choline on the levels of glucose metabolites and the synthesis of ACh from glucose in the cerebral cortex are displayed in Table 1. Pyruvate and glucose levels were significantly increased in the ischemia–saline group compared with the control group. Treatment with CDP-choline tended to attenuate increases in pyruvate and glucose. The choline content in the ischemia–CDP-choline group was increased 2.3 times over that of the control group.

The radioactivity of [14C]glucose in the ischemia–saline group was increased 1.6 times over that of the control group, while the radioactivity of [14C]ACh synthesized from [14C]glucose was decreased to about 50% that of the control group. CDP-choline accelerated the utilization of [14C]glucose and restored the synthesis of [14C]ACh from [14C]glucose.

Incorporation of [14C]CDP-Choline (Experiment 3)
The incorporation of [14C]CDP-choline was studied in the cerebral cortex of rats with 30 minutes of ischemia (Figure 4). Three minutes after the injection, radioactivity in the blood and acid-soluble and lipid fractions was 248,000 dpm/ml and 93,000 and 172 dpm/g, respectively. At 60 minutes, radioactivity in the blood (41,000 dpm/ml) and acid-soluble fraction (34,000 dpm/g) was markedly decreased compared with those at 3 minutes. In contrast, radioactivity in the lipid fraction (6,800 dpm/g) was increased.

Discussion
We examined the effects of postischemic treatment with CDP-choline on neurologic deficits in cerebral ischemia–reperfused rats produced by the method of Pulsinelli and Brierley. In our study, blood gases, blood glucose, arterial blood pressure, and head temperature in the rats were not monitored. However,
the changes in physiologic, neurochemical, and behavioral variables in the acute phase of cerebral ischemia-reperfusion depend on the duration of ischemia, and the variation of values obtained within a group is small if rats that lose the righting reflex immediately after BCAO and never regain the reflex for the duration of BCAO are used. This model is useful for evaluating not only acute but also subacute and chronic effects of a drug on brain damage induced by cerebral ischemia and reperfusion because the duration of ischemia can be regulated precisely.

In ischemic rats, CDP-choline accelerated recovery from the acute ischemic state, as demonstrated by shortening of the SM time, and lessened the severity of neurologic deficits. The effects of preischemic or postischemic treatment with CDP-choline on neurologic deficits have also been studied in a different model using stroke-prone spontaneously hypertensive rats (SHRSP). BCAO causes severe brain damage in SHRSP but not in normotensive rats. CDP-choline administered before or after ischemia significantly prolonged the onset of unequivocal neurologic signs, "ischemic seizures," and survival times in SHRSP with BCAO. The results obtained in both models suggest that CDP-choline beneficially affects brain dysfunction induced by cerebral ischemia.

We investigated the effects of CDP-choline on glucose metabolism by determining the biosynthesis of labeled ACh from [14C]glucose as well as the levels of glucose metabolites because glucose is a donor of the acetyl carbons of ACh. Glucose and pyruvate contents were increased and the synthesis of [14C]ACh from [14C]glucose was decreased even on Day 4 in the cerebral cortex of rats with 30 minutes of ischemia and reperfusion. In support of these results, Pulsinelli and Duffy and Pulsinelli et al have found an increase in the glucose content and a decrease in glucose utilization measured by the 2-deoxy[14C]glucose method in the cerebral cortex, hippocampus, and striatum of rats after ischemia. Our study also showed that treatment with CDP-choline ameliorated the disruptions of cerebral glucose metabolism in the ischemic brain.

### Table 1. Effect of CDP-Choline on Glucose Metabolism in Cerebral Cortex of Rats After 30 Minutes of Cerebral Ischemia and Reperfusion

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Ischemia-saline</th>
<th>Ischemia-CDP-choline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine triphosphate (μmol/g)</td>
<td>1.19±0.14</td>
<td>1.03±0.02</td>
<td>1.04±0.05</td>
</tr>
<tr>
<td>Lactate (μmol/g)</td>
<td>1.96±0.14</td>
<td>2.30±0.22</td>
<td>1.94±0.09</td>
</tr>
<tr>
<td>Pyruvate (μmol/g)</td>
<td>0.06±0.004</td>
<td>0.09±0.01*</td>
<td>0.07±0.01†</td>
</tr>
<tr>
<td>Glucose (μmol/g)</td>
<td>1.56±0.05</td>
<td>1.97±0.12‡</td>
<td>1.84±0.04</td>
</tr>
<tr>
<td>Acetylcholine (nmol/g)</td>
<td>13.6±0.2</td>
<td>14.1±1.4</td>
<td>16.2±0.6</td>
</tr>
<tr>
<td>Choline (nmol/g)</td>
<td>45.4±2.4</td>
<td>53.1±3.1</td>
<td>104.2±5.3‡</td>
</tr>
<tr>
<td>[14C]glucose (dpm x 10^-1/g)</td>
<td>668±75</td>
<td>1084±119‡</td>
<td>998±78* (n = 4)</td>
</tr>
<tr>
<td>[14C]Acetylcholine (dpm x 10^-1/g)</td>
<td>1.77±0.10</td>
<td>1.19±0.07‡</td>
<td>1.82±0.14‡ (n = 4)</td>
</tr>
<tr>
<td>Acetylcholine synthesis (nmol/g/3 min)</td>
<td>12.9±1.1</td>
<td>6.7±0.6‡</td>
<td>9.8±0.1*§ (n = 4)</td>
</tr>
</tbody>
</table>

CDP-choline (cytidine 5'-diphosphocholine, 250 mg/kg i.p.) was administered twice a day for 4 days after reperfusion; on Day 4 52.6 μCi/40 μl/100 g [14C]glucose was injected i.v. 30 minutes after final dose of CDP-choline, and the rats were killed 3 minutes later. Values are mean ± SEM of 5 rats except where indicated.

*p<0.05, †p<0.01 vs. control; ‡p<0.05, §p<0.01 vs. ischemia-saline.
Several effects of CDP-choline on cerebral metabolism have been reported. Watanabe et al.27 found that CDP-choline injected into the perfused cat brain increased glucose incorporation into amino acids and decreased lactate production. Benzi et al.1 reported that intracarotid perfusion with CDP-choline increased the synaptosomal phosphorylation state in hypoxic dog brains.

Cerebral ischemia induces increased FFA contents,3 decreased phospholipid content,4,5 and uncoupling of mitochondrial oxidative phosphorylation.6,7 The effect of ischemia on mitochondrial function can be mimicked by exogenous FFAs.6 Furthermore, arachidonic acid reduces Na⁺,K⁺-ATPase activity8 and induces brain edema.9 In addition, recent evidence25 suggests that brain injury due to transient cerebral ischemia occurs, in part, during reperfusion. Free radical chain reaction leading to lipid peroxidation is one mechanism of ischemic brain damage, and accumulated polyunsaturated FFAs are converted to peroxide during reperfusion. These results indicate that inhibiting the release of FFAs and stimulating the removal of accumulated FFAs and resynthesis of phospholipid are important for ameliorating brain damage induced by ischemia.

The work of Trovarelli et al.9 and Dorman et al.10-32 on the effects of CDP-choline and CDP-ethanolamine in cerebral injuries suggests a role of direct precursors of essential membrane constituents in the restoration of damaged membranes. The source of FFAs during ischemia is not only phospholipid and diacylglycerol breakdown but also the reversal of the choline phosphotransferase reaction.33 The lack of high-energy compounds for phosphorylation of cytidine 5'-monophosphate may hinder phospholipid synthesis by reversal of phosphotransferase, with a subsequent production of diacylglycerols, which are degraded to FFAs and glycerol by diacylglycerol lipase. Pretreatment with CDP-choline and CDP-ethanolamine could direct the phosphotransferase reaction toward synthesis of phosphatidylcholine and phosphatidylethanolamine, preventing the release of FFAs and diacylglycerol associated with ischemia. Moreover, CDP-choline and CDP-ethanolamine stimulate the resynthesis of phospholipid and consequently remove the accumulated FFAs in the cerebral ischemia–reperfused brain. We show that [³⁵⁴C]CDP-choline, which was injected 10 minutes after reperfusion, is used for membrane lipid synthesis immediately after being injected. Alberghina et al.11 have also reported that CDP-choline injected 10 minutes before intraventricular administration of [¹⁴C]palmitate and [³H]glycerol has a stimulating effect particularly on the incorporation of both precursors into phospholipid in brain mitochondria, which were among the subcellular fractions affected most by hypoxic treatment. These results may explain the beneficial effects of CDP-
choline on mitochondrial dysfunction leading to disruption of glucose metabolism and indicate the role of CDP-choline in restoring the structural integrity of membrane impaired by cerebral ischemia.

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References

Key Words • cerebral ischemia • choline • metabolism, glucose • rats
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