Brain Ischemia Decreases Phosphatidylcholine–Phospholipase D but Not Phosphatidylinositol–Phospholipase C in Rats

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Background and Purpose
Phosphatidylcholine (PC)-phospholipase D (PLD) is an important intracellular signaling pathway in response to a variety of agonists, but little is known about the effects of brain ischemia on the PC-PLD system. We thus have examined the effects of global cerebral ischemia on PLD in rats.

Methods
We have examined the effects of global ischemia (decapitation or four-vessel occlusion) on PLD and PLC activity in the membrane fraction of rat brains. We measured the PLD and PLC activity in detergent-mixed micelle assay systems using 

Results
The results demonstrate that basal PLD activity showed a gradual decrease with increased duration (5 to 30 minutes) of ischemia by decapitation in the hippocampus; after 30 minutes of ischemia, PLD activity was significantly decreased compared with the control. Lineweaver-Burk plots showed that the apparent V_max value of PLD in ischemia was one half of that in the control without changes in K_m value.

Ischemia by decapitation significantly decreased PLD activity in the brain stem as well as the hippocampus, whereas in four-vessel occlusion study, ischemia significantly decreased PLD activity in the hippocampus but not in the brain stem. Lowered temperature (30°C and 22°C) during ischemic incubation did not reverse the ischemia-induced PLD activity decrease. In contrast to PLD, ischemia by decapitation had no effect on basal phosphatidylinositol-phospholipase C activity or the amount of phospholipase C4 in the membrane fractions from 30-minute ischemic hippocampus by immunoblots probed with the antibody.

Conclusions
These results suggest that PC-PLD is one of the target enzymes of ischemia; its decrease may cause a perturbation of PC hydrolysis and/or disorders of intracellular transduction of signals for choline metabolism for acetylcholine formation in brain. (Stroke. 1994;25:1247-1251.)

Key Words • cerebral ischemia • choline • rats • phospholipase
PLC activity or the amounts of PLCβ, in the membrane fractions from ischemic hippocampi. The ischemia-induced alteration of PLD activity is likely to result in a perturbation of PC hydrolysis and disorder of intracellular transduction of signals or choline metabolism for acetylcholine formation in the brain.

Materials and Methods

1,2-Dipalmitoyl-sn-glycero-3-phospho[methy-3H]choline (dipalmitoyl [3H]PC, 73 Ci/mmol) and [inositol-2-3H(N)]-phosphatidylinositol-4,5-bisphosphate ([3H]PIP2, 8.8 Ci/mmol) were obtained from NEN. 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (dipalmitoyl PC) was obtained from Avanti Polar Lipids Inc. Although dipalmitoyl PC is not common in brain, we assayed PLD activity by measuring [3H]choline generated from [3H]PC. [3H]choline-labeled PC can be obtained only as dipalmitoyl PC in the ready-made isopotes. Phosphatidylinositol-4,5-bisphosphate was obtained from Sigma Chemical Co. Antibodies to PLCβ were from Upstate Biotechnology. Chemicals for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunobots were obtained from Bio-Rad. Other materials and chemicals were obtained from Sigma or Wako Pure Chemicals.

Male Wistar rats were killed by decapitation, and the brains were removed. In control brains, the dissected brain areas (hippocampus and brain stem) were immediately homogenized at 4°C in a buffer consisting of 40 mmol/L Na-4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, pH 7.2), 0.5 mmol/L ethylenediamine tetraacetate (EDTA), 1 mmol/L disodium EDTA, 0.1 mmol/L phenylmethylsulfonyl fluoride, and 0.32 mol/L sucrose with a Potter-Elvehjem homogenizer. The homogenate was centrifuged initially at 400g for 5 minutes, and the resultant postnuclear supernatant was centrifuged at 10 000g for 60 minutes to obtain membrane fraction. In ischemic brains by decapitation, the dissected brain areas were incubated in glucose-free Krebs-HEPES buffer (127 mmol/L NaCl, 1.6 mmol/L KCl, 1.24 mmol/L KH2PO4, 1.3 mmol/L MgSO4, 26 mmol/L NaHCO3, 2.4 mmol/L CaCl2, 10 mmol/L HEPES-NaOH, pH 7.2, equilibrated by bubbling 95% nitrogen/5% CO2 for 30 minutes) for the indicated period. After incubation at the indicated temperatures, the dissected brain areas were immediately homogenized and prepared for membrane fraction by the same method for the control group. A four-vessel occlusion study was done as previously described.12 With rats under pentobarbital anesthesia, the vertebral arteries were coagulated, and the carotid arteries were loosely tagged for subsequent rapid access and occlusion. Twenty-four hours later, atrumatic aneurysm clips were placed across both carotid arteries. After 30 minutes of ischemia, the rats were killed by decapitation, and the brains were removed; membrane fractions were prepared in the same manner as in the decapitation experiment. These membrane fractions were stored at −80°C until assay.

PLD activity was assayed using dipalmitoyl [3H]PC as a substrate for PLD.14 For the standard assay, the reaction mixture contained 40 mmol/L Na-HEPES (pH 7.2), 100 mmol/L dipalmitoyl [3H]PC (0.1 μCi), 0.1% (vol/vol) Triton X-100, 5 mmol/L p-nitrophenylphosphate, and 20 μg of protein in a total volume of 100 μL. Incubations were carried out at 37°C for 15 minutes. The reaction was stopped by adding 500 μL of chloroform and methanol (7:3, by volume) and 100 μL of 5 mmol/L ethylene glycol bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetate (EGTA) in 0.2 mol/L KCl. The aqueous phase was analyzed as described previously.14 A phosphatase inhibitor, p-nitrophenylphosphate, was included to prevent the conversion of phospho-[3H]choline formed by the PC-specific PLC to [3H]choline by phosphatidylinositol phosphatase.15

PLC activity was assayed using [3H]PIP2 as a substrate for PLC as described previously with modification.16 The reaction mixture contained 20 mmol/L 2-amino-2-hydroxyethylpropane-1,3-diol (Tris)–maleate (pH 6.5), 0.2 mmol/L [3H]PIP2 (0.1 μCi), 0.1% (by volume) sodium deoxycholate, 80 mmol/L KCl, 1.8 mmol/L CaCl2, 2 mmol/L EGTA, and 0.5 μg of protein in a total volume of 50 μL. Incubations were carried out at 37°C for 5 minutes. The reaction was stopped by adding 250 μL of chloroform-methanol-concentrated HCl (100:100:0.6, by volume) and 10 μL of 0.005 mol/L EGTA in the HCl. The water-soluble radioactivity released into the upper phase was quantitated by scintillation counting.

For immunoblot analysis, 25 μg of each of the membrane fractions was separated by SDS-PAGE on 8% acrylamide gels and electrophoretically transferred onto nitrocellulose. Incubation with monoclonal antibodies specific to PLCβ was carried out at room temperature for 1 hour. Immunostaining was developed using the DAKO LSAB kit.

The statistical evaluations of all experiments were carried out by the Kruskal-Wallis test or by the Mann-Whitney U test. The results were expressed as mean±SD. Significance was accepted at P<.01.

Results

PLD activity showed a gradual decrease with increased duration (5 to 30 minutes) of ischemia and was significantly (P<0.01) decreased compared with the control after 30 minutes of ischemia (control, 10.58±2.42 nmol/h per milligram protein; 30-minute ischemia, 5.28±1.94 nmol/h per milligram protein; n=4, mean±SD) (Fig 1). To test the possibility that the decrease in PLD activity was due to the decrease in the amount of PLD, we examined the concentration dependency of PLD activities on PC concentration. The PLD activities were increased with the increase of PC concentration. Lineweaver-Burk plots showed that the apparent Vmax value of PLD in ischemia was approximately half of that in the control without changes in Km value (Fig 2).

After 30 minutes of ischemia, PLD activity was significantly (P<0.01) decreased by 41% compared with the control in the hippocampus (control, 8.60±0.42 nmol/h per milligram protein; ischemia, 5.11±0.41 nmol/h per milligram protein; n=4) and by 20% in the brain stem (control, 8.29±0.43 nmol/h per milligram protein; ischemia, 6.66±0.43 nmol/h per milligram protein; n=8) (Fig 3A). It was necessary to exclude the possibility that the decrease in PLD activity in the decapitation experiment arose artificially from the ischemic condition (incubation in the Krebs-HEPES buffer) after decapitation. We thus examined the effect of four-vessel occlusion on PLD activity to confirm the
decrease in PLD activity as found in the experiment of ischemia by decapitation. After 30 minutes of ischemia by four-vessel occlusion, PLD activity was significantly (P<.01) decreased by 23% in the hippocampus (control, 8.79±1.61 nmol/h per milligram protein; ischemia, 6.74±1.77 nmol/h per milligram protein; n=8), although PLD activity was unchanged in the brain stem (control, 6.58±1.09 nmol/h per milligram protein; ischemia, 6.33±1.58 nmol/h per milligram protein; n=8) (Fig 3B).

Hypothermia has been shown to ameliorate neuronal damage caused by cerebral ischemia. We thus examined the influence of temperature on ischemia-induced PLD activity decrease. Two lowered-temperature (of Krebs-HEPES buffer) groups were studied: 30°C and 22°C. However, these lowered temperatures did not reverse the ischemia-induced PLD activity decrease (Fig 4).

After 30 minutes of ischemia by decapitation, PLC activity was unchanged in the hippocampus (control, 203.9±17.8 nmol/min per milligram protein; ischemia, 205.9±13.9 nmol/min per milligram protein; n=8) in contrast to the significant decrease in PLD activity in the hippocampus (control, 8.87±0.97 nmol/h per milligram protein; ischemia, 4.10±1.07 nmol/h per milligram protein; n=8). This result suggests that the decrease in PLD activity is a specific phenomenon in ischemic injury but not overall proteolytic incidents.

Discussion

Results from this study reveal susceptibility of PLD but not PLC to cerebral ischemic insult. The decrease in PLD activity is dependent on the duration of ischemia, indicating that this event is secondary to those occurring during the initial phase of the insult. The mechanism of decrease in PLD activity by global ischemia is still unclear. The decrease in apparent V_max value (Fig 2) suggests at least two possible explanations for the observed reduction in PLD activity. One possibility is that PLD was destroyed. In this case, the decrease of

The membrane-bound activity of PLC is mostly derived from PLCβ isoforms rather than other isoforms such as PLCγ or PLCδ. We thus analyzed the amounts of PLCβ in the membrane fractions from controls and ischemia subjects by immunoblotting probed with antibody. The reactivity was not significantly different between control and ischemia samples (Fig 5), in agreement with the result that ischemic injury did not cause any changes in PLC activity.

Figure 2. Graph showing Lineweaver-Burk plots for phosphatidylcholine (PC) hydrolysis by phospholipase D. Assay condition is described in "Materials and Methods." The results represent the mean of duplicate determinations. Similar results were obtained in three other experiments.

Figure 3. Bar graphs showing effect of 30-minute ischemia by decapitation (A) or by four-vessel occlusion (B) on phospholipase D (PLD) activity in hippocampus and brain stem. Procedures and assay condition of PLD are described in "Materials and Methods." The results represent the mean±SD (bars) values from eight independent experiments of duplicate determinations. *P<.01 different from control.

Figure 5. Immunoblots showing effects of 30-minute ischemia by decapitation on immunoreactivity of phospholipase Cβ (PLCβ) in hippocampus. Lanes 1 and 2 represent the control samples, and lanes 3 and 4 represent the ischemic samples. Lane 5 shows that the immunoblot was performed without first antibody. Immunostaining was developed using the DAKO LSAB kit. This figure shows the representative results. Similar results were obtained in three other experiments.
the apparent V\textsubscript{max} value of PLD in ischemia indicates that the decrease in PLD activity was due to the decrease of the amount of PLD. Another possibility is that interferences with PLD were generated after ischemia, and in this case, the decrease of the V\textsubscript{max} value and lack of change in K\textsubscript{m} value suggest that the interferences inhibit PLD in a noncompetitive manner.

There are two different results between the decapitation experiment and the four-vessel occlusion study. First, the degree of the decrease in PLD activity in the four-vessel occlusion study is relatively small compared with that of the decapitation experiment. Second, in the brain stem, PLD activity was decreased in the decapitation ischemia but not in the four-vessel occlusion. These different results probably arise from the fact that blood flow after four-vessel occlusion was, in part, only just enough for attenuating the decrease in PLD activity, rather than from the difference of incubation conditions, because a radiotracer study during four-vessel occlusion showed local cerebral blood flow to be markedly reduced in the hippocampus (3% to 7% of control) but not in the brain stem (25% to 30% of control).\textsuperscript{17} The contrastive result in the brain stem between the decapitation and the four-vessel occlusion experiments suggests the importance of blood flow in the decrease in PLD activity.

A number of reports have shown the presence of hypothemic protection against neuronal damage caused by cerebral ischemia.\textsuperscript{18,19} However, lowered temperature, as examined, lacked the effect on the PLD activity decrease (Fig 4). One possible explanation is that PLD plays little role in neuronal injury in ischemia; however, the lack of hypothermic effect is explainable on the assumption that hypothermic condition may not completely protect against damage to the brain during ischemia and that the decrease in PLD activity may be caused by only slight ischemic damage in the hypothermic condition because of susceptibility of PLD to ischemia. Another possible explanation for the lack of hypothermic effect is that the decrease in PLD activity may have a protective reaction rather than a deteriorating role in brain ischemia. The lack of hypothermic effect suggests that ischemia-induced PLD activity decrease may not be due to the activation of the Ca\textsuperscript{2+}-dependent protease by elevation of intracellular calcium levels, because the calcium elevation was blocked by hypothermic treatment in the hippocampus after ischemia.\textsuperscript{20}

Our finding that the PLD activity in ischemia remained decreased even at 22°C (room temperature) suggests the need for prudent investigation of PLD activity in the human brain after death. However, careful research on PLD may lead to a new pathological condition because of susceptibility of PLD to ischemia. Another possible explanation for the lack of hypothermic effect is that the decrease in PLD activity may have a protective reaction rather than a deteriorating role in brain ischemia. The lack of hypothermic effect suggests that ischemia-induced PLD activity decrease may not be due to the activation of the Ca\textsuperscript{2+}-dependent protease by elevation of intracellular calcium levels, because the calcium elevation was blocked by hypothermic treatment in the hippocampus after ischemia.\textsuperscript{20}

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Although there is growing evidence of marked perturbation in inositol lipid metabolism due to cerebral ischemia,\textsuperscript{11,12,22} an effect of ischemia on PLC was not seen in our experiment. This divergence may be due to the difference of the time course after ischemia. The perturbation in inositol lipid metabolism occurs at the initial phase (probably seconds) of ischemic insult,\textsuperscript{11,12,22} whereas no effect of PLC by ischemia in this experiment was seen after 30 minutes of ischemia. It is more probable that this divergence may be due to the differences in experimental methods between other investigations and ours. We measured basal PLC activity and PLC\textsubscript{B} content in this experiment, while other investigations indicated PLC activation by calculating the levels of polyphosphoinositides, diacylglycerols, or inositol phosphates after ischemia. These divergences of time courses and methods of measuring PLC activity between other investigations and ours probably account for the different results of the effect of ischemia on PLC.

The significance of decrease in PLD activity caused by global ischemia is unclear. The ischemia-induced alteration of PLD activity is likely to result in a perturbation of PC hydrolysis as well as disordered intracellular signal transduction and regulation of intracellular Ca\textsuperscript{2+} concentration. In turn, this decrease may contribute to the pathogenesis of neuronal cell death. In contrast to this pathogenetic hypothesis, the decrease in PLD activity by ischemia may have a homeostatic role in ischemic insult by reducing the overflow of Ca\textsuperscript{2+} signal transduction\textsuperscript{10} and stabilizing the cell function after global ischemia.

In addition to the physiological role of PLD related to providing an intracellular signal, PLD has an important role in the source of choline for acetylcholine formation in brain tissue.\textsuperscript{5} The rank order of PLD activity in rat brain was hippocampus>brain stem>cortex>cerebellum (data not shown), and this result is compatible with the previous report.\textsuperscript{23} Interestingly, this distribution pattern resembles that of choline acetyltransferase\textsuperscript{24} rather than that of PLC,\textsuperscript{10} suggesting the significance of PLD in acetylcholine synthesis in brain. The decrease in PLD activity after brain ischemia also might cause an imbalance of acetylcholine turnover.

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References


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Editorial Comment

There is considerable interest in understanding the mechanisms by which ischemia produces cell injury and death. One potential mechanism is that of ischemia disrupting normal cellular functions, such as calcium homeostasis, by altering the phospholipase (A, C, and D)-mediated hydrolysis of membrane phospholipids. In their article, Nishida and colleagues demonstrate that two models (decapitation and four-vessel occlusion) of global cerebral ischemia produce a time-dependent decrease in phospholipase D (PLD) activity. Ischemia reduced the amount of active PLD, since the V_max of PLD was lowered without altering the K_m. Therefore, the authors conclude that PLD activity is rapidly reduced by ischemia and that changes in PLD activity may be a good measure of ischemic injury. However, conditions such as hypothermia that reduce ischemic injury did not alter the ischemia-dependent reduction in PLD activity.

It is not known whether ischemia-induced alterations in cellular phospholipase activity increase cell death, decrease cell death, or do both. However, membrane phospholipids and their hydrolysis products (arachidonate, diacylglycerol, inositol phosphates, phosphatidate, etc) regulate important cellular functions, and significant changes in their normal cellular levels could result in cell injury and death. The ischemia-dependent alterations in phospholipase activity are more likely a cause rather than a result of cell injury because they are rapidly and significantly altered. Nevertheless, additional studies are required to determine the role of phospholipases in ischemia-mediated cell injury and death.

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