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

Akira Nishida, MD, PhD; Katsuya Emoto, MD; Masami Shimizu, PhD; Touru Uozumi, MD, PhD; Shigeto Yamawaki, MD, PhD

**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 \(^3\)H-labeled exogenous substrate.

**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 \(V_{\text{max}}\) value of PLD in ischemia was one half of that in the control without changes in \(K_m\) value.

In recent years, phosphatidylcholine (PC)–phospholipase D (PLD) has been demonstrated to couple with receptors in many types of mammalian cells and constitutes a novel pathway for signals across the plasma membrane. PLD hydrolyzes PC to produce phosphatidic acid and choline. There are several reports implicating phosphatidic acid in cellular processes such as Ca\(^{2+}\) entry into cells or activation of phospholipase C (PLC). In addition to the physiological role of PLD in providing an intracellular signal, PLD may have an important role in the source of choline for acetylcholine formation in brain tissue. Another intracellular signal pathway, phosphatidylinositol-PLC, also has been found to generate two intracellular second messengers, inositol triphosphate (IP\(_3\)) and diacylglycerol, from hydrolysis of phosphatidylinositol-4,5-bisphosphate. While IP\(_3\) causes the release of Ca\(^{2+}\) from intracellular stores, diacylglycerol stimulates protein kinase C.

The biological mechanisms leading to neuronal injury and subsequent cell death after global cerebral ischemia remain controversial. Among many hypotheses proposed to explain the ischemic damage, one of the best supported hypotheses is that disruption of neuronal Ca\(^{2+}\) homeostasis leads to sustained increases in intracellular Ca\(^{2+}\) concentration. Because both PLC and PLD are important for maintenance of the intracellular Ca\(^{2+}\) concentration, modifications of these enzymes could be an underlying mechanism for the disordering of neuronal Ca\(^{2+}\) homeostasis. Indeed, several studies have indicated a decrease in the levels of polyphosphoinositides and an increase in the concentrations of diacylglycerols and inositol phosphates in rat brains after global ischemia by decapitation, suggesting that polyphosphoinositide is hydrolyzed through PLC after ischemia. However, little is known about the effects of ischemia on the PC-PLD system. In this study, we have examined the effects of global ischemia on PLD in the membrane fraction of rat brains after decapitation or four-vessel occlusion compared with the effect of ischemia on phosphatidylinositol-PLC. Our results demonstrate that basal PLD activity was decreased in a time-dependent manner (5 to 30 minutes) in the hippocampus and that the apparent \(V_{\text{max}}\) value of PLD in ischemia was lower than that in the control without changes in \(K_m\) value. The decrease in PLD activity after ischemia was also shown in another ischemic condition in a four-vessel occlusion study. In contrast to PLD, global ischemia by decapitation had no effect on basal activity of PLC.
PLC activity or the amounts of PLCβ2, 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[7H]-choline (dipalmitoyl-1H[4]PC, 73 Ci/mmol) and [inositol-2-3H(N)]-phosphatidylinositol-4,5-biphosphate (‘‘H]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 [1H]choline generated from [1H]PC. [1H]Choline-labeled PC can be obtained only as dipalmitoyl PC in the ready-made isotopes. Phosphatidylinositol-4,5-biphosphate was obtained from Sigma Chemical Co. Antibodies to PLCβ2 were from Upstate Biotechnology. Chemicals for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting 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 diethiothreitol, 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 100 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, atraumatic aneurysm clips were placed across both carotid arteries. After access and occlusion. Twenty-four hours later, atraumatic aneurysm clips were placed across both carotid arteries. After after decapitation. We thus examined the effect of ischemic condition (incubation in the Krebs-HEPES buffer) and electrophoretically transferred onto nitrocellulose. 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 PLCβ2 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 43% 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 apparent V_{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_{max} value and lack of change in K_{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). 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 hypothermic protection against neuronal damage caused by cerebral ischemia. 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^{2+}-dependent protease by elevation of intracellular calcium levels, because the calcium elevation was blocked by hypothermic treatment in the hippocampus after ischemia.

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 pathologic significance of PLD in the human brain, as shown in a study of PLD activity decrease in Alzheimer's-diseased brain. Our findings also suggest the possibility that the PLD activity may serve as a good biochemical marker for assessing ischemic injury because of the simple procedure for assaying PLD activity.

Although there is growing evidence of marked perturbation in inositol lipid metabolism due to cerebral ischemia, 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, 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 PLCβ_{2} 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^{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^{2+} signal transduction 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. 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. Interestingly, this distribution pattern resembles that of choline acetyltransferase rather than that of PLC, 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
5. No reference found.
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.

Robert G. Lamb, PhD, Guest Editor
Department of Pharmacology and Toxicology
Medical College of Virginia
Richmond, Va
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A Nishida, K Emoto, M Shimizu, T Uozumi and S Yamawaki

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