Using a rat model of stroke, we examined the effects of focal cerebral ischemia on the metabolism of polyphosphoinositides by injecting $^{32}$Pi into both the left and right cortices. After equilibration of the label for 2–3 hours, ischemia induced a significant decrease ($p<0.001$) in the concentrations of labeled phosphatidyl 4,5-bisphosphates (66–78%) and phosphatidylinositol 4-phosphate (64–67%) in the right middle cerebral artery cortex of four rats. The phospholipid labeling pattern in the left middle cerebral artery cortex, which sustained only mild ischemia and no permanent tissue damage, was not different from that of two sham-operated controls. However, when $^{32}$Pi was injected 1 hour after the ischemic insult, there was a significant decrease ($p<0.01$) in the incorporation of label into the phospholipids in both cortices of four ischemic rats compared with four sham-operated controls. Furthermore, differences in the phospholipid labeling pattern were observed in the left cortex compared with the sham-operated controls. The change in labeling pattern was attributed to the partial reduction in blood flow following ligation of the common carotid arteries. We provide a sensitive procedure for probing the effects of focal cerebral ischemia on the polyphosphoinositide signaling pathway in the brain, which may play an important role in the pathogenesis of tissue injury. (Stroke 1991;22:495-498)

A

Although stimulation of neuronal activity, including the process of neurotransmitter release, is one of the early events of cerebral ischemia, the mechanism leading to irreversible tissue damage is not well understood. A number of neurotransmitters are known to transduce their signals through stimulated hydrolysis of phosphatidylinositol 4,5-bisphosphates (PIP$_2$). In turn, inositol trisphosphates can serve as second messengers for the mobilization of intracellular calcium stores. Therefore, stimulation of this cell signaling process may have important consequences toward explaining the alteration of calcium homeostasis underlying ischemic tissue damage.

Previous studies by Ikeda et al$^3$ as well as those from our own laboratory$^4$ have indicated a rapid decrease in the levels of polyphosphoinositides (poly-PI) in rat brain after global cerebral ischemia induced by decapitation. Poly-PI were also degraded following global cerebral ischemia induced by ligation of the common carotid arteries (CCAs) in gerbils.$^5$ Since these compounds are present in trace quantities in the brain, a more sensitive procedure to study the ischemia-induced breakdown of poly-PI can be achieved by prelabeling the brain with $^{32}$Pi.$^6-7$ or $[^3H]$inositol.$^8$ Although the tracer technique has been used to examine poly-PI metabolism in a model of global ischemia, the response in brain tissue after a focal ischemic insult has not been examined. We used a rat model of focal cerebral ischemia to examine poly-PI metabolism after ligation of the middle cerebral artery (MCA). This model closely resembles human ischemic stroke in that a well-defined infarct is consistently produced in the cerebral cortex irrigated by the right MCA.$^9,10$ With this model, it is also possible to examine and compare poly-PI metabolism in the right MCA cortex with that in the left MCA cortex, which sustains only a mild ischemic insult without indication of permanent tissue injury.$^9$

**Materials and Methods**

Adult male Long-Evans hooded rats (Charles River Laboratories, Inc., Wilmington, Mass.) weighing 250–300 g were given lab chow and water ad libitum until surgery. Under anesthesia with 100 mg/kg i.p. ketamine and 6 mg/kg i.m. xylazine, a sagittal incision was made to expose the skull. Two burr holes were drilled stereotactically 5.5 mm lateral
to the sagittal suture and 1.5 mm dorsal to the coronal suture for $^{32}$Pi injections into the right and left MCA cortices. The $^{32}$Pi (20 μCi in the form of $^{32}$PiPO$_4$, carrier free, 285 Ci/mg; New England Nuclear, Boston, Mass.) was delivered with a 10-μl microsyringe at a depth of 3 mm beneath the calvaria. The $^{32}$Pi injections were made at precise time intervals either before or after the ischemic insult.

The procedure to induce severe focal cerebral ischemia leading to a consistently large infarct in the right MCA cortex has been described in detail. Ligation of the right MCA and temporary occlusion of both CCAs for 30 minutes or longer consistently resulted in coagulation necrosis of the cortex in the right MCA territory but sparing of the subcortical structures and the left hemisphere. Cerebral blood flow was less than 20% of control in the ischemic right MCA cortex and approximately 60–80% of control in the left MCA cortex. At precise time intervals after the ischemic insult, the rats were decapitated and the heads were dropped into liquid nitrogen for rapid freezing of the brain tissue. The left and right MCA cortices were identified and punched out using a cylindrical 1 cm in diameter, and subcortical tissue was dissected out with a scalpel.

Brain tissues were homogenized in a buffer containing 0.32 M sucrose with 1 mM ethylenediaminetetraacetic acid and 50 mM Tris-HCl (pH 7.4). Aliquots of the tissue homogenate were taken for counting of radioactivity. Lipids from the tissue homogenate were extracted by a stepwise procedure with neutral and acidified chloroform:methanol (2:1, vol:vol) as described previously. Lipids were separated by the two-dimensional high-performance thin-layer chromatography procedure as described by Sun and Lin. After solvent development, lipid spots on the plate were visualized by exposure to iodine vapors and then scraped into scintillation vials for counting of radioactivity. Lipids from the tissue homogenate were taken for counting of lipid radioactivity. There were no differences in the labeling pattern. Nevertheless, 3 minutes of global cerebral ischemia elicited significant decreases compared with control in the labeling of PIP$_2$ (64%), phosphatidylinositol 4-phosphate (PIP) (58%), and phosphatidic acids (60%) in both the left and right MCA cortices (Figure 1). In addition, some decreases in the labeling of phosphatidylcholines (12%) and ethanolamine plasmalogens (31%) were also observed in the ischemic brain.

The phospholipid labeling patterns obtained from the left and right MCA cortices of the sham-operated controls and rats that sustained focal cerebral ischemia by ligation of the right MCA and both CCAs for 30 minutes are shown in Figure 2, top. Two hours after the injection of $^{32}$Pi into the brain, labeled PE comprised approximately 7–8% of the total phospholipid radioactivity. There were no differences in the phospholipid labeling pattern between the left and right MCA cortices of the sham-operated controls and the left MCA cortex of the ischemic rats. However, 30 minutes of ischemia resulted in a significant decrease ($p<0.001$) in the labeling of PIP$_2$ (66%) and PIP (64%) in the ischemic right MCA cortex, which sustained severe injury. The labeling of other phospholipids in the ischemic right MCA cortex was not appreciably altered. When the duration of the ischemia was extended to 2 hours, there was a similar decrease in the labeling of PIP$_2$ and PIP (Figure 2, bottom) and a small but significant increase (143%).

Results

When the left and right cortices were prelabeled with $^{32}$Pi for 2.7 hours and the brain tissue was frozen immediately after decapitation, we observed no obvious differences in the phospholipid labeling pattern between the two MCA cortices (data not shown). There were some variations (approximately 35%) in the amount of label delivered into the brain tissue, but this variance did not alter the phospholipid labeling pattern. Nevertheless, 3 minutes of global cerebral ischemia elicited significant decreases compared with control in the labeling of PIP$_2$ (64%), phosphatidylinositol 4-phosphate (PIP) (58%), and phosphatidic acids (60%) in both the left and right MCA cortices (Figure 1). In addition, some decreases in the labeling of phosphatidylcholines (12%) and ethanolamine plasmalogens (31%) were also observed in the ischemic brain.

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in the labeling of phosphatidylinositols compared with that in the left MCA cortex and that in the sham-operated controls.

When the rats were subjected to focal cerebral ischemia for 1 hour prior to the injection of $^{32}$Pi, measurement of the radioactivity in brain homogenates from the MCA cortices of sham-operated controls and the left and right MCA cortices of ischemic rats gave values of $1.0\pm0.33\times10^5$ (n=4), $1.8\pm0.46\times10^5$ (n=6), and $7.3\pm4.1\times10^4$ (n=5) cpm, respectively. Thus, although the left MCA cortex of the ischemic rats sustained only mild ischemia with no obvious pathological consequence, there was a significant decrease ($p<0.01$) in the uptake of labeled precursor during ischemia. When phospholipids in the left MCA cortex of the ischemic rats were analyzed, we were surprised to find twofold increases in the labeling of PIP and PIP$_2$ compared with the sham-operated controls (Figure 3). On the other hand, the labeling of poly-PI in the ischemic right MCA cortex showed large variances and was significantly less than that in the left MCA cortex.

**Discussion**

Previous studies from our laboratory as well as from others have demonstrated a rapid decrease in the level of poly-PI in brain during the early phase of global cerebral ischemia. In most instances, poly-PI degradation is marked by a concomitant increase in the concentrations of diacylglycerols and inositol phosphates, suggesting that poly-PI is hydrolyzed through the receptor-mediated phospholipase C pathway.

To facilitate transport of the labeled precursor to the MCA cortex, $^{32}$Pi was injected into both the left and right cortices. As indicated by the results of the first...
not as dramatic as the right MCA cortex; the left MCA cortex also showed a decrease in the uptake of label, probably due to the partial reduction of blood flow. These results suggest an apparent relation between cerebral blood flow and the transport of metabolites to various brain regions. Under this condition of partial reduction of blood flow, the left MCA cortex also showed differences in the labeling of poly-PI compared with the sham-operated controls. After ligation of the right MCA, the ability of the ischemic brain region to incorporate the label into phospholipids was severely reduced. Furthermore, there was unusually large variance in the labeling of poly-PI in this brain region, although the labeling was also greatly reduced compared with that in the left MCA cortex.

In summary, the results of these experiments demonstrate the feasibility of the radiotracer technique to examine poly-PI metabolism in a specific brain region (MCA cortex) and to assess changes caused by focal ischemic insults. Stimulation of the signal transduction mechanism coupled to poly-PI breakdown was observed in the right MCA cortex, which sustained severe ischemic damage. Stimulation of this signaling process is known to result in an increase in the cytoplasmic free calcium, which in turn may contribute to the underlying mechanism of tissue injury.

References


KEY WORDS • animal models • phospholipids • rats

FIGURE 3. Bar graph of phospholipid labeling patterns in rat middle cerebral artery (MCA) cortices after 1 hour of focal cerebral ischemia induced 1 hour prior to injection of 32P. Procedure for phospholipid analysis is as described in Figure 1. Radioactivity recovered in brain region was 1.9±0.33×10^4 cpm (n=4) for both left and right MCA cortices of sham-operated controls and 1.7±0.46×10^4 (n=6) and 7.3±4.1×10^4 (n=5) for left and right MCA cortex of ischemic rats, respectively, a, p<0.01 different from left MCA cortex by analysis of variance. b, p<0.01 different from sham-operated controls by analysis of variance. PE, phosphatidylethanolamine; PIP2, phosphatidylinositol 4,5-bisphosphates; PIP, phosphatidylinositol 4-monophosphate; PA, phosphatidic acids; PI, phosphoinositides; PC, phosphotidyl cholines; PE, ethanolamine plasmalogens.
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T N Lin, T H Liu, J Xu, C Y Hsu and G Y Sun

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