A Phospholipase C Inhibitor Ameliorates Postischemic Neuronal Damage in Rats
Atsushi Umemura, MD; Hideo Mabe, MD; and Hajime Nagai, MD

Background and Purpose: The hypothesis of calcium-induced neuronal damage has been proposed regarding brain ischemia. Phospholipase C is an enzyme that catalyzes the phosphodiesteratic cleavage of phosphatidylinositol. The cleavage of phosphatidylinositol 4,5-bisphosphate by phospholipase C yields 1,4,5-inositol triphosphate, which mediates intracellular release of calcium, and 1,2-diacylglycerol, which is an activator of protein kinase C. We examined the effect of phenylmethylsulfonyl fluoride, a phospholipase C inhibitor, on delayed neuronal damage after transient forebrain ischemia in the hippocampal CA1 subfield in rats to assess the role of phospholipase C in postischemic neuronal damage.

Methods: Twenty-minute forebrain ischemia was induced using the method of Pulsinelli and Brierley. We measured the neuronal density of the hippocampal CA1 subfield 7 days after reperfusion. The effect of phenylmethylsulfonyl fluoride was tested in both pretreatment and posttreatment groups.

Results: In the vehicle treatment group (n=13), neuronal density was 51±42/mm (mean±SD). The neuronal densities in the 50-mg/kg (n=12) and 100-mg/kg (n=14) phenylmethylsulfonyl fluoride pretreatment groups and the 100-mg/kg (n=10) phenylmethylsulfonyl fluoride posttreatment group were 99±50, 150±55, and 143±63/mm, respectively. These values were significantly higher than that of the vehicle treatment group (p<0.05, p<0.01, and p<0.01, respectively).

Conclusions: It is suggested that the activation of phospholipase C has an important role in postischemic delayed neuronal damage. (Stroke 1992;23:1163-1166)

KEY WORDS • cerebral ischemia • neuronal damage • rats

It is well known that neurons in the CA1 subfield of the hippocampus are selectively vulnerable to ischemic insult. Slowly progressing neuronal death occurs in the CA1 subfield after transient forebrain ischemia (delayed neuronal death). The hypothesis of calcium-induced neuronal damage has been proposed regarding brain ischemia. Some specific events, including activation of intracellular lipases and proteases, generation of free radicals, depletion of energy reserves by activation of Ca2+-adenosine triphosphatase, and impairment of mitochondrial oxidative phosphorylation, are triggered by excess intracellular Ca2+. Some studies have proposed that excessive release of glutamate may play a role in delayed neuronal death. Glutamate can lead to a high Ca2+ influx directly through chemically gated channels linked to N-methyl-D-aspartate (NMDA) receptors.

Phospholipase C is an enzyme that catalyzes the phosphodiesteratic cleavage of phosphatidylinositol (PI). Phospholipase C is activated by a receptor-mediated mechanism coupled to a guanosine triphosphate (G protein). The cleavage of phosphatidylinositol 4,5-bisphosphate (PIP2) by phospholipase C yields two second messengers, 1,4,5-inositol triphosphate (IP3) and 1,2-diacylglycerol (DG). IP3 interacts with the endoplasmic reticulum membrane to release Ca2+, while DG is an activator of protein kinase C (PI turnover).

During brain ischemia, many kinds of neurotransmitters are released from synaptosomes in large amounts by membrane depolarization. Some neurotransmitter receptors including muscarinic cholinergic, adrenergic, histaminergic, and serotonergic receptors activate phospholipase C by a receptor-mediated mechanism coupled to a G protein and linked to PI turnover. Recently it has become evident that glutamate, which may play an important role in neurotoxicity in ischemia, has such a receptor activating PI turnover. The activation of phospholipase C may lead, through IP3-mediated Ca2+ mobilization from intracellular stores, to increased cytosolic calcium concentration. It therefore seems possible that the activation of phospholipase C by the excessive release of neurotransmitters in ischemia is related to neuronal damage.

We previously reported the effect of phenylmethylsulfonyl fluoride (PMSF), a phospholipase C inhibitor, on the release of free fatty acids in brain ischemia. The results showed that the release of free fatty acids in the early period of ischemia can be attributed mostly to the action of phospholipase C, and that the activation of phospholipase C further influences the release of fatty acids by phospholipase A2 in the subsequent course. This effect seemed to be related to IP3-mediated Ca2+ release. The activation of phospholipase C seems to trigger some specific events, such as the activation of phospholipase A2 in ischemia.
TABLE 1. Neuronal Density of Hippocampal CAl Subfield After 20 Minutes of Forebrain Ischemia

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Neuronal density (mm)</th>
<th>Rectal temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham control</td>
<td>9</td>
<td>194±7</td>
<td>37.5±0.2</td>
</tr>
<tr>
<td>Vehicle treatment</td>
<td>13</td>
<td>51±42</td>
<td>37.2±0.4</td>
</tr>
<tr>
<td>PMSF pretreatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 mg/kg</td>
<td>12</td>
<td>99±50*</td>
<td>37.2±0.4</td>
</tr>
<tr>
<td>100 mg/kg</td>
<td>14</td>
<td>150±55†</td>
<td>36.9±0.6</td>
</tr>
<tr>
<td>PMSF posttreatment</td>
<td>10</td>
<td>143±63†</td>
<td>37.1±0.6</td>
</tr>
</tbody>
</table>

Values are mean±SD. PMSF, phenylmethylsulfonyl fluoride. *p<0.05, †p<0.01 compared with vehicle.

The present study examined the effect of PMSF, a phospholipase C inhibitor, on neuronal damage after transient forebrain ischemia in the hippocampal CAl subfield in rats. The role of phospholipase C in postischemic neuronal damage is then discussed.

Materials and Methods
Phenylmethylsulfonyl fluoride was obtained from Sigma Chemical Co., St. Louis, Mo. It was mixed with 1 ml of 4% gum acacia suspension before use.

Fifty-eight male Wistar rats weighing 250–300 g were used in this study. Transient forebrain ischemia was induced using the method described by Pulsinelli and Brierley. Under 2% halothane anesthesia, both vertebral arteries of each rat were electrocoagulated. The following day both common carotid arteries were gently exposed under 2% halothane anesthesia. Three minutes after the halothane was discontinued both common carotid arteries were clamped with aneurysm clips for 20 minutes. Signs such as unresponsiveness and cataleptic postures were thought to be indicative of forebrain ischemia.

Five experimental groups were formed. In the pretreatment group, rats were treated with 50 mg/kg (n=12) or 100 mg/kg (n=14) PMSF intraperitoneally 60 minutes before ischemia. In the posttreatment group, rats were treated with 100 mg/kg PMSF intraperitoneally immediately after recirculation (n=10). In the vehicle treatment group, rats were treated with the vehicle (1 ml of 4% gum acacia suspension) 60 minutes before ischemia (n=13). Sham-operated control rats (n=9) were prepared and handled similarly, except that their common carotid arteries were not occluded. The rectal temperature of the rats was maintained at 36°C using a heating pad and a heating lamp during forebrain ischemia. The rats were killed 7 days after recirculation, and the brains were removed quickly, fixed by 10% formalin for 4 days, and embedded in paraffin. Coronal sections (2 μm thick) containing dorsal hippocampus were stained with hematoxylin and eosin, and the number of neurons in the linear length (1 mm) of the hippocampal CAl subfield was counted in each specimen.

Statistical analysis of differences in the neuronal density between the vehicle and PMSF treatment groups was made with the two-tailed t test. Values are given as mean±SD.

Results
The neuronal densities of the CAl subfield are shown in Table 1 and Figure 1. In the sham-operated control group, the neuronal density of the CAl subfield was 194±7/mm. In the vehicle treatment group, extensive neuronal damage was observed; neuronal density of the CAl subfield was 51±42/mm. In preliminary experiments, seven rats with vehicle treatment were killed 2 days after recirculation. Neuronal damage 2 days after recirculation was minimal; neuronal density was 152±57/mm. Thus, the neuronal damage that occurred in this forebrain ischemia seemed to be slowly progressing neuronal damage (delayed neuronal death).

The neurons in the CAl subfield were well preserved by both pretreatment and posttreatment with PMSF. The neuronal densities in the 50- and 100-mg/kg PMSF pretreatment groups and the 100-mg/kg PMSF posttreatment group were 99±50, 150±55, and 143±63/mm, respectively. These neuronal densities were significantly lower than those in the vehicle treatment group.
higher than that of the vehicle treatment group ($p<0.05$, $p<0.01$, and $p<0.01$, respectively).

The rectal temperature during forebrain ischemia in each group is shown in Table 1. Phenylmethylsulfonyl fluoride did not decrease the rectal temperature significantly.

**Discussion**

Activation of phospholipase C yields two intracellular second messengers, IP$_3$ and DG, that control a variety of cellular functions by the ability of the former to mobilize Ca$^{2+}$ from intracellular stores and by the ability of the latter to activate protein kinase C. Many kinds of neurotransmitters activate phospholipase C by a receptor-mediated mechanism coupled to G protein. Phenylmethylsulfonyl fluoride is a serine esterase inhibitor. Walenga et al$^{10}$ reported that PMSF blocked the stimulus-induced mobilization of arachidonic acid in platelets by inhibiting PI-specific phospholipase C. It is known that PMSF can pass the blood–brain barrier freely.$^{11}$ We also reported that PMSF inhibited the increase of arachidonic and stearic acids that seemed to be released from PI during the early period of ischemia in rat neocortex.$^{8}$ From the result, it is certain that PMSF inhibits phospholipase C in rat brain. However, PMSF is not a specific inhibitor for phospholipase C. Phenylmethylsulfonyl fluoride also inhibits serine proteases. It seems likely that some proteases are activated in ischemia. Proteolysis by calcium-activated neutral protease has been proposed in connection with ischemia,$^{12}$ but PMSF does not inhibit calcium-activated neutral protease, which is a cysteine protease.

Recently an elevated intracellular Ca$^{2+}$ level has been considered to be attributable to ischemic delayed neuronal damage. There are several mechanisms of intracellular Ca$^{2+}$ influx during ischemia: through voltage-sensitive and agonist-operated (NMDA receptor-gated) calcium channels, IP$_3$-mediated intracellular release of Ca$^{2+}$ from the endoplasmic reticulum, reversed 3Na$^+$/Ca$^{2+}$ antiporter, and a nonspecific Ca$^{2+}$ channel.$^3$ Among these mechanisms, the NMDA subclass of glutamate receptor may be the predominant route of ischemia-induced lethal Ca$^{2+}$ entry.$^4$ In the present study, PMSF pretreatment at any dose significantly ameliorated ischemic delayed neuronal damage in the rat hippocampal CA1 subfield. Inhibition of phospholipase C by PMSF seems to suppress IP$_3$ production from PIP$_2$. In addition, it seems that the neuroprotective effect of PMSF in the present study is due to inhibition of IP$_3$-mediated Ca$^{2+}$ release. Our results suggest that IP$_3$-mediated Ca$^{2+}$ release from endoplasmic reticulum also plays a predominant role in lethal intracellular Ca$^{2+}$ increase during ischemia. The cleavage of PIP$_2$ by phospholipase C also yields DG, which is an activator of protein kinase C. Hara et al$^{13}$ reported that staurosporine, a protein kinase C inhibitor, prevents postischemic neuronal damage in rat hippocampus. Therefore the effect of PMSF may be partially attributed to the inhibition of DG production. Phospholipase C is activated by a receptor-mediated mechanism coupled to a G protein. Several neurotransmitters, including glutamate released from synaptosomes by ischemia, activate phospholipase C.$^6$ This suggests that the neurotoxicity of glutamate may be due not only to an NMDA receptor-mediated mechanism but also to an IP$_3$-mediated mechanism.

According to previous studies, arachidonic acid increase during the early stage of ischemia is attributed to the breakdown of PI by phospholipase C and the diacylglycerol lipase system.$^{14,15}$ This indicates that phospholipase C is activated during the early stage of ischemia. It is known that delayed neuronal death in the hippocampal CA1 subfield occurs after even a brief period of forebrain ischemia. So it is suggested that phospholipase C, which is activated during the early period of ischemia, plays an important role in delayed neuronal death.

In the present study, PMSF posttreatment also ameliorated delayed neuronal damage in the hippocampal CA1 subfield. In the pretreatment series, inhibition of phospholipase C during the early period of ischemia is considered to be attributable to the neuroprotective effect of PMSF. But in the posttreatment series, this neuroprotective effect cannot be explained by the same mechanism. Abe et al$^{16}$ investigated regional arachidonic acid and PI metabolism during transient ischemia and long after reperfusion in the parietal cortex and the hippocampal CA1 subfield using a forebrain ischemia model of Mongolian gerbils. According to their results, the levels of polyphosphoinositides in the cortex (an area resistant to delayed neuronal damage) rapidly recovered during reperfusion periods, but those in the CA1 subfield (a vulnerable area) did not recover. The amount of arachidonic acid in the CA1 subfield continued to increase up to 1 day of reperfusion. These results suggest that a breakdown of polyphosphoinositides by phospholipase C continues even after the reperfusion period in the CA1 subfield. Phenylmethylsulfonyl fluoride posttreatment in the present study seems to influence this postischemic activation of phospholipase C. The levels of neurotransmitters that activate phospholipase C decrease after reperfusion. The mechanism of this continuous breakdown of polyphosphoinositides is not well understood. It is well known that there is a continuous firing of CA1 cells after reperfusion.$^{17}$ There may be some relation between these results.

We conclude that the activation of phospholipase C during or after ischemia seems to have an important role in postischemic delayed neuronal damage. In addition, not only glutamate but also some other neurotransmitters that activate phospholipase C may cause neuronal damage.

**References**

1. Kirino T: Delayed neuronal death in the gerbil hippocampus following ischemia. *Brain Res* 1982;239:57–69
The article by Umemura et al presents interesting data showing that phenylmethylsulfonyl fluoride (PMSF) given before or immediately after transient ischemia in rats prevents neuronal cell death in the CA1 subfield of the hippocampus. The authors review evidence that PMSF inhibits phospholipase C. Phospholipase C cleaves phosphatidylinositol 4,5-bisphosphate to yield 1,4,5-inositol trisphosphate (IP₃) and 1,2-diacylglycerol (DG). IP₃ is known to mediate intracellular release of Ca²⁺, while DG activates protein kinase C. The authors suggest that after ischemia, phospholipase C-induced release of IP₃ raises intracellular Ca²⁺, long believed to contribute to cell death. The initiating mechanism by which phospholipase C is activated is unclear but may include neurotransmitters such as glutamate.

The data presented in this article are very remarkable and the discussion enlightening. Some caution might be advised, however, because phospholipase C activity was not directly measured and PMSF may have other effects in addition to inhibition of phospholipase C. These studies support the utility of continuing studies of PMSF and phospholipase C activity. Future studies will no doubt consider the effect of PMSF on phospholipase C and correlation of the degree of phospholipase inhibition with degree of cell preservation.

Earl F. Ellis, PhD, Guest Editor
Department of Pharmacology and Toxicology
The Medical College of Virginia
Richmond, Va.
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A Umemura, H Mabe and H Nagai

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