A Flavonoid Inhibitor of 5-Lipoxygenase Inhibits Leukotriene Production Following Ischemia in Gerbil Brain

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Leukotrienes C4 and D4 are arachidonic acid metabolites that constrict blood vessels and enhance vascular permeability; their biosynthesis is initiated by the reaction of arachidonic acid with 5-lipoxygenase enzyme. After bilateral carotid artery occlusion for 15 minutes and reperfusion of the gerbil brain for 15 minutes, we determined the brain tissue concentrations of leukotrienes C4 and D4 by radioimmunoassay; they had increased from a baseline concentration of <1 to a mean±SEM concentration of 12.8±3.9 pmol/g brain. We also studied the effect of a flavonoid 5-lipoxygenase inhibitor on leukotriene production in the reperfused gerbil brain. A water-soluble flavonoid (5-hexyloxy-3',4'-dihydroxy-6,7-dimethoxyflavone 4'-disodium phosphate) was administered intravenously at a dose of 200 mg/kg body wt; 15 minutes later, both carotid arteries were clamped. The enhanced production of leukotrienes C4 and D4 in the reperfused brain was reduced by approximately 80% (from a mean±SEM of 12.8±3.9 to 2.2±1.3 pmol/g brain) in the presence of the 5-lipoxygenase inhibitor. The flavonoid did not affect the production of prostaglandin D2, the concentration of which also increased in the reperfused ischemic brain. (Stroke 1989;20:248-252)

Leukotrienes (LTs) are known to be chemical mediators of anaphylaxis and inflammation.1 Among the various LTs, LTC4 and LTD4 are potent vasoconstrictors of human cerebral arteries in vitro and increase vascular permeability.3,4 Moskowitz et al demonstrated enhanced synthesis of LTC4 and LTD4 in gerbil brain after ischemia and reperfusion; therefore, locally generated LTC4 and LTD4 may be involved in the pathologic change in blood flow and the pathogenesis of edema.

In view of the proposed pathophysiologic roles of LTs, a number of compounds have been studied and developed as selective inhibitors of 5-lipoxygenase, the enzyme initiating LT biosynthesis from arachidonic acid. In previous studies of various flavonoids, we found that cirsiliol (3',4',5-trihydroxy-6,7-dimethoxyflavone) potently inhibited 5-lipoxygenase; furthermore, we studied its structure-activity relation6,7 and screened more potent derivatives.8 A major disadvantage of cirsiliol and other flavonoids for in vivo studies is their hydrophobicity. Based on earlier work showing the advantages of a synthetic phosphate ester of baicalin for in vivo studies,9 we synthesized a watersoluble derivative of cirsiliol (5-hexyloxy-3',4'-dihydroxy-6,7-dimethoxyflavone 4'-disodium phosphate) and examined the effects of this inhibitor in a model of brain ischemia.

Materials and Methods

Mongolian gerbils (Meriones unguiculatus) were obtained from Inoue Experimental Animals Center (Kumamoto, Japan). Prostaglandins (PGs) B2 and D2, LTC4, and LTD4 were provided by Ono Research.
To obtain its monophosphate, 500 mg of 5-hexyloxy-3',4'-dihydroxy-6,7-dimethoxyflavone was condensed with 19 g of polyphosphoric acid; the monophosphate was then neutralized with sodium hydroxide to give the 4'-disodium phosphate (360 mg, 60% yield). Identification of the compound was confirmed by ultraviolet absorption spectroscopy, elemental analysis, and nuclear magnetic resonance spectroscopy.

Arachidonate 5-lipoxygenase was purified from porcine leukocytes by immunofinity chromatography, and enzyme activity was assayed with carbon-14-labeled arachidonic acid as the substrate in a standard reaction mixture fortified with calcium ions and ATP as described previously.

Frozen gerbil forebrain was homogenized in four volumes of cold 80% ethanol with a Polytron Model CH-6010 homogenizer (Kinematica, Luzern, Switzerland). After centrifugation at 9,500g for 20 minutes at 20°C, 2 ml of the supernatant was evaporated in vacuo. The dried residue was resuspended in 1 ml of a buffer (10 mM sodium phosphate buffer, pH 7.3, containing 0.1 M NaCl, 1 mM MgCl₂, and 0.1% NaN₃) and then centrifuged at 15,000g for 10 minutes. Recoveries of PGD₂ and LTC⁴ in the extraction procedure were approximately 70% and 40%, respectively, using tritium-labeled compounds as internal standards. The 80% ethanol extract was further purified by the use of a Sep-Pak C₁₈ column (0.8x 10 cm) was obtained from Waters Chromatography Division (Milford, Massachusetts), bovine γ-globulin from Nakarai (Kyoto, Japan), polyethylene glycol-6000 from Wako (Osaka, Japan), and gelatin from Sigma Chemical Co. (St. Louis, Missouri). All other chemicals were of reagent grade.

For PGD₂ radioimmunoassay, anti-PGD₂, [5,6,8,9,12,14,15-³H(N)]PGD₂ (160 Ci/mmol), [1-¹⁴C]-arachidonic acid (59.6 mCi/mmol), and [14,15-³H(N)]LTC₄ (25 Ci/mmol) from Amersham International (Amersham, United Kingdom). Anti-PGD₂ antiserum was a gift from Dr. Y. Watanabe of Osaka Medical College (Osaka, Japan), and anti-LTC₄ antiserum was a gift from Dr. J. Rokach of Merck Frosst Canada (Pointe-Claire/Dorval, Canada). Sep-Pak C₁₈ cartridges and Nova-Pak C₁₈ Radial Pak cartridges were obtained from Waters Chromatography Division (Milford, Massachusetts), bovine γ-globulin from Nakarai (Kyoto, Japan), polyethylene glycol-6000 from Wako (Osaka, Japan), and gelatin from Sigma Chemical Co. (St. Louis, Missouri). All other chemicals were of reagent grade.

Our previous study of the structure–activity relations of flavonoids as 5-lipoxygenase inhibitors indicated an important role of the catechol structure in the B ring of flavone. Since one of the catecholic hydroxy groups was esterified with a phosphate in the water-soluble flavonoid used in this work (5-hexyloxy-3',4'-dihydroxy-6,7-dimethoxyflavone 4'-disodium phosphate), its inhibitory effect on 5-lipoxygenase purified from porcine leukocytes was tested, using the unesterified form as a control. The esterified form was less active than the free form by one order of magnitude (Figure 1). In view of the reported effect of baicalin 6-phosphate in experi-
Arachidonate 5-lipoxygenase reaction inhibited by 5-hexyloxy-3',4'-dihydroxy-6,7-dimethoxyflavone (□) and its 4'-disodium phosphate (●). Purified enzyme (3.3 μg protein) was allowed to react with carbon-14-labeled arachidonic acid as described in “Materials and Methods.” Methanol solution of 4 μl of both types of inhibitor was added to 200 μl of assay mixture and preincubated with enzyme for 3 minutes before start of reaction by addition of arachidonic acid. Data are mean ± standard error of the mean (n=3).

Mental asthma, we applied the water-soluble flavonoid to an in vivo study using Mongolian gerbils as a model of brain ischemia. Production of PGD₂ and LTC₄ in ischemic gerbil brain was assessed by radioimmunoassay, the validity of which was examined in two types of experiments. There was a linear relation between the volume of brain extract and the amount of PG or LT measured by radioimmunoassay. On the other hand, when a given amount of brain extract was mixed with various amounts of PGD₂ or LTC₄ and the mixtures were subjected to radioimmunoassay, a nearly linear relation was observed between the amount of PG or LT added and the result of the radioimmunoassay.

When ethanol extract from the brain of a gerbil subjected to ischemia for 15 minutes and reperfusion for 15 minutes was purified using Sep-Pak C₁₈ cartridges and analyzed by HPLC, increased production of PGD₂ (Figure 2C) and LTC₄ and LTD₄ (Figure 2G) was observed; such increases were not observed in sham-occluded gerbils (Figure 2B for PGD₂, Figure 2F for LTC₄ and LTD₄). Production of LTC₄ and LTD₄ was markedly reduced when the gerbils received the flavonoid 5-lipoxygenase inhibitor before carotid occlusion and reperfusion (Figure 2H); administration of the flavonoid did not affect PGD₂ concentration (Figure 2D).

Reperfusion increased the production of LTC₄ and LTD₄ from a baseline concentration of <1 to a mean±SEM concentration of 12.8±3.9 pmol/g brain (n=6) and the PGD₂ concentration from <3 to a mean±SEM concentration of 174.7±20.1 pmol/g brain (n=6). Inhibition of enhanced LT synthesis depended on the dose of flavonoid, and maximum inhibition was observed at a flavonoid dose of 200 mg/kg body wt (Figure 3). PGD₂ synthesis was almost unaffected by increasing doses of flavonoid. LT synthesis but not PGD₂ synthesis was inhibited by administration of the flavonoid 5–30 minutes before bilateral carotid artery occlusion (Figure 4).

The metabolic fate of the flavonoid 4'-phosphate (of >99% purity as the esterified form) was also studied, expecting removal of its phosphate in vivo after its intravenous administration to gerbils. Only approximately 7% of the flavonoid appearing in the blood stream after 15 minutes was in an unesterified form, which was more potent in inhibiting 5-lipoxygenase (Table 1).

Discussion

Mongolian gerbils have been used by many investigators as an animal model for brain ischemia. A number of articles have been published reporting
increased production of various PGs (PGE₂, PGF₂α, PGD₂, PGI₁, and their metabolites) and thromboxane in the gerbil brain after transient ischemia followed by reperfusion. More recently an increase of the LT concentration was demonstrated in the ischemic gerbil brain after reperfusion and in subarachnoid hemorrhage and concussive brain injury. The pathologic roles of these PGs and LTs have been discussed; special attempts were made to correlate the development of brain edema with increased synthesis of PGs and LTs. Since the development of brain edema was not prevented by treatment of the gerbils with indomethacin, which inhibited cyclooxygenase and reduced PG production, a possible role of LTs with a biologic activity to enhance vascular permeability was sought in the pathogenesis of brain edema. Indeed, an article reported that direct application of LTs to brain parenchyma enhanced Evans blue extravasation in rats. Thus, it was desirable to develop a drug to block the biosynthesis of LTs and their receptor interaction.

We have been developing various flavonoids as selective inhibitors of arachidonate 5-lipoxigenase. While chemically modifying these flavonoids, we developed a water-soluble flavonoid (5-hexyloxy-3',4'-dihydroxy-6,7-dimethoxyflavone 4'-disodium phosphate) as a 5-lipoxigenase inhibitor. Intravenous injection of the water-soluble flavonoid before bilateral carotid artery occlusion markedly reduced LT production, which was enhanced after reperfusion of the ischemic brain. Maximum inhibition required a dose as high as 200 mg/kg body wt. The need for such a high dose may be attributed to slow deesterification of the less potent phosphate ester form to a more active free form. Blood analysis demonstrated that a small portion of the injected prodrug was actually converted to an active unesterified form in the circulating blood at the end of ischemia and reperfusion (Table 1). To circumvent some of these problems, we are now developing a more potent 5-lipoxigenase inhibitor. Alternative methods of drug administration must also be investigated.

The 5-lipoxigenase inhibitor thus developed must be applied to study of the pathogenesis of brain edema following ischemia and reperfusion with special reference to a possible role of LTs. A previous report suggested the roles of two types of PGs in the two phases of pathogenesis of brain edema: PGE₂ in cytotoxic edema examined by specific gravity measurement and PGI₁ in vasogenic edema followed by Evans blue staining. Therefore, a precise time relation must be investigated between LT biosynthesis and edema formation, and the flavonoid 5-lipoxigenase inhibitor must be used in this line of study.

Several more problems remain to be investigated further. We still do not know whether LTs are produced by neuronal cells, glial cells, or infiltrating leukocytes. A precise knowledge of the regional

### Table 1. In Vivo Metabolism of Flavonoid 5-Lipoxygenase Inhibitors in Gerbils

<table>
<thead>
<tr>
<th>Flavonoid</th>
<th>Time after administration</th>
<th>Time after administration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15 min (n=3)</td>
<td>30 min (n=3)</td>
</tr>
<tr>
<td>Esterified flavone</td>
<td>860.3±73.1</td>
<td>491.7±50.9</td>
</tr>
<tr>
<td>Unesterified flavone</td>
<td>60.4±8.5</td>
<td>23.3±3.7</td>
</tr>
</tbody>
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Data are mean±SEM μg/ml in plasma after injection of 200 mg/kg body wt 5-lipoxigenase inhibitor; plasma was analyzed by reverse-phase high-performance liquid chromatography as described in "Materials and Methods."
distribution of 5-lipoxygenase in brain is also required for understanding how the drug functions as an inhibitor of the in vivo synthesis of LTs in brain tissue.

References

Key Words: cerebral ischemia • prostaglandins • leukotrienes • gerbils
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