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 occluded. 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.

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 vitro² and increase vascular permeability.³⁴ 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 relation⁶⁷ 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 baikalein 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

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Institute (Osaka, Japan). We purchased [5,6,8,9,12, 14,15-\textsuperscript{3}H(N)]PGD\textsubscript{2} (160 Ci/mmol), [1-\textsuperscript{14}C]-arachidonic acid (59.6 mCi/mmol), and [14,15-\textsuperscript{3}H(N)]LTC\textsubscript{4} (25 Ci/mmol) from Amersham International (Amersham, United Kingdom). Anti-PGD\textsubscript{2} antiserum was a gift from Dr. Y. Watanabe of Osaka Medical College (Osaka, Japan), and anti-LTC\textsubscript{4} antiserum was a gift from Dr. J. Rokach of Merck Frosst Canada (Pointe-Clair/Dorval, Canada). Sep-Pak C\textsubscript{18} cartridges and Nova-Pak C\textsubscript{18} Radial Pak cartridges were obtained from Waters Chromatography Division (Milford, Massachusetts), bovine \gamma-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.

To obtain its monophosphate, 500 mg of 5-hexyloxy-3',4'-dihydroxy-6,7-dimethoxyflavone\textsuperscript{4'} 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 immunoaffinity chromatography,\textsuperscript{10} 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.\textsuperscript{10}

Frozen gerbil forebrain was homogenized in four volumes of cold 80% ethanolic 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 reconstituted in 1 ml of a buffer (10 mM sodium phosphate buffer, pH 7.3, containing 0.1 M NaCl, 1 mM MgCl\textsubscript{2}, and 0.1% NaN\textsubscript{3}) and then centrifuged at 15,000g for 10 minutes. Recoveries of PGD\textsubscript{2} and LTC\textsubscript{4} 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 C\textsubscript{18} column essentially by the method of Powell.\textsuperscript{11} High-performance liquid chromatography (HPLC) using a Nova-Pak C\textsubscript{18} Radial Pak column (0.8×10 cm) was performed to identify LTC\textsubscript{4} and LTD\textsubscript{4} (at 280 nm) and PGD\textsubscript{2} (at 290 nm) and PGD\textsubscript{2} (at 192 nm). PGD\textsubscript{2} was eluted with acetonitrile:water:acetic acid 300:100:400:0.8 by vol, adjusted to pH 5.7 with ammonium hydroxide, at a flow rate of 1.0 ml/min.

For PGD\textsubscript{2} radioimmunoassay, anti-PGD\textsubscript{2}, [5,6,8,9,12,14,15-\textsuperscript{3}H(N)]PGD\textsubscript{2}, and the sample to be tested were mixed and incubated. Radioactivity in the immunoprecipitate collected by the addition of polyethylene glycol-6000 was counted in a liquid scintillation counter. For radioimmunoassay of LTC\textsubscript{4} and LTD\textsubscript{4}, anti-LTC\textsubscript{4} and [14,15-\textsuperscript{3}H(N)]LTC\textsubscript{4} were used. The anti-LTC\textsubscript{4} antibody cross-reacted with LTD\textsubscript{4} by 55.5%.

Adult Mongolian gerbils (60–80 g) were maintained in accordance with the guidelines of the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Pub. No. NIH78-23, revised 1978). Mongolian gerbils lack the anastomosis between the carotid and vertebral arteries, and temporary occlusion of both carotid arteries produces forebrain ischemia.\textsuperscript{12}

Both common carotid arteries were exposed under an operative microscope while the gerbils were briefly anesthetized with diethyl ether; tracheotomy was done to prevent respiratory difficulties. An aneurysm clip was applied to each common carotid artery simultaneously, maintained in place for 15 minutes, and then removed to allow reperfusion. After 15 minutes of reperfusion, the whole gerbil was frozen in liquid nitrogen and decapitated. Sham-occluded gerbils had their common carotid arteries exposed under ether anesthesia but not occluded. A polyethylene catheter was inserted into the left femoral vein for sampling of blood and intravenous injection of 200 μl of the flavonoid 5-lipoxygenase inhibitor or its vehicle (physiological saline). The flavonoid was dissolved in vehicle, and the pH was adjusted to 7.4 with 1N sodium hydroxide.

Plasma from the blood sample was mixed with PGB\textsubscript{2} as an internal standard, and then the mixture was extracted with ethyl acetate:methanol 2:1 by vol. After centrifugation, the supernatant was evaporated to dryness in vacuo. The extract was analyzed by reverse-phase HPLC using acetonitrile:methanol:water 300:100:250 by vol, containing 111 mM phosphoric acid, at a flow rate of 1.0 ml/min. The average retention time was 9.0 minutes for PGB\textsubscript{2}, 5.8 minutes for the ester form, and 17.4 minutes for the free form of the flavonoid. For quantification of the flavonoid, its peak area (325 nm) and that of PGB\textsubscript{2} (278 nm) were calculated, and the flavonoid:PGB\textsubscript{2} ratio was plotted against varying amounts of flavonoid to obtain a standard curve.

Results

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.\textsuperscript{7} 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 baicalein 6-phosphate in expen-
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 (PGE_2, PGF_2α, PGD_2, PGI_2, 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-lipoxygenase. 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-lipoxygenase 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-lipoxygenase inhibitor. Alternative methods of drug administration must also be investigated.

The 5-lipoxygenase 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: PGE_2 in cytotoxic edema examined by specific gravity measurement and PGE_2 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-lipoxygenase 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

**Figure 3.** Synthesis of prostaglandin D_2 (PGD_2) and leukotrienes C_4 and D_4 (LTC_4/D_4) by ischemic gerbil brain after 15-minute pretreatment with flavonoid 5-lipoxygenase inhibitor at various doses before 15-minute bilateral carotid artery occlusion followed by 15-minute reperfusion. The 80% ethanol extract was analyzed for PGD_2 and LTC_4 by radioimmunoassay as described in "Materials and Methods." Data are mean + or − standard error of the mean. Number of gerbils is indicated in parentheses. LTC_4/D_4 values at 5, 15, and 30 minutes were significantly different (p<0.01) from control value at 0 minute.

**Figure 4.** Synthesis of prostaglandin D_2 (PGD_2) and leukotrienes C_4 and D_4 (LTC_4/D_4) by ischemic gerbil brain after pretreatment with 200 mg/kg body wt i.v. flavonoid 5-lipoxygenase inhibitor for various time intervals before 15-minute bilateral carotid artery occlusion followed by 15-minute reperfusion. The 80% ethanol extract was analyzed for PGD_2 and LTC_4 by radioimmunoassay as described in "Materials and Methods." Data are mean + or − standard error of the mean. Number of gerbils is indicated in parentheses. LTC_4/D_4 values at 5, 15, and 30 minutes were significantly different (p<0.01) from control value at 0 minute.

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

<table>
<thead>
<tr>
<th>Flavonoid</th>
<th>Time after administration</th>
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<tr>
<td></td>
<td>15 min (n=3)</td>
</tr>
<tr>
<td>Esterified flavone</td>
<td>860.3±73.1</td>
</tr>
<tr>
<td>Unesterified flavone</td>
<td>60.4±8.5</td>
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</tbody>
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Data are mean±SEM μg/ml in plasma after injection of 200 mg/kg body wt 5-lipoxygenase 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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