Sodium 5-(3'-pyridinylmethyl)benzofuran-2-carboxylate (U-63557A) potentiates protective effect of intravenous eicosapentaenoic acid on impaired CBF in ischemic gerbils

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Eicosapentaenoic acid (EPA) has been reported to improve postischemic cerebral blood flow (CBF). The present study was designed to determine whether sodium 5-(3'-pyridinylmethyl)benzofuran-2-carboxylate (U-63557A), a selective thromboxane synthetase inhibitor, could potentiate the effects of EPA on CBF in ischemic gerbils. Ischemia was produced by bilateral carotid artery occlusion for 15 minutes followed by reperfusion for 2 hours. Immediately after ischemia, gerbils were given either an intravenous bolus of 0.167 mg of EPA followed by a continuous infusion of EPA at 1 mg/hr, or U-63557A (10 mg/kg intraperitoneally), or U-63557A and EPA, or a saline infusion. Regional CBF was measured by the hydrogen clearance method, and brain water by the specific gravity technique. Brain prostaglandins were measured by radioimmunoassay. Preischemic CBF's ranged from 27.4 to 29.5 ml/100 gm/min for the four animal groups. After ischemia and 2 hours of reperfusion, CBF in the saline-infused gerbils was significantly decreased to 19.2 ml/100 gm/min. Gerbils treated with either EPA or U-63557A alone had a CBF of 23.7 and 21.6 ml/100 gm/min, respectively. Postischemic CBF in animals treated with both U-63557A and EPA was 30.0 ml/100 gm/min, significantly higher than in saline-infused gerbils. Brain levels of 6-keto prostaglandin (PG)F_1alpha (the metabolite of PGF_2alpha) were significantly higher in gerbils treated with U-63557A and EPA compared to gerbils given EPA alone. This study indicates that U-63557A potentiates the effects of EPA on postischemic CBF. This is probably due to the ability of U-63557A to increase prostacyclin formation in the vessel wall.

KEY WORDS • cerebral ischemia • cerebral edema • prostaglandins • cerebral blood flow
burr-holes were made bilaterally over the frontal and parietal cortex, taking care to preserve the integrity of the dura and cortex beneath. Four Teflon-coated platinum electrodes (125 μm in diameter) were inserted stereotaxically 0.5 mm into the cerebral cortex. Exposed cortex around the electrodes was covered with moist Gelfoam, and the electrodes were fixed in place with acrylic cement. A silver/silver chloride reference electrode was placed subcutaneously in the right leg.

**Measurements**

Regional CBF was measured by the hydrogen clearance technique. Brain specific gravity measurements were made as described by Nelson, using a bromobenzene-kerosene density gradient column. At the conclusion of the experiment, four 2-cm samples of gray matter surrounding each electrode (but excluding the area perforated by the electrode and white matter) were measured for specific gravity. The dissection was carried out under kerosene with the aid of magnification, and samples were transferred immediately to the gradient column. Before each series of measurements, the column was calibrated with K2SO4 solutions of known specific gravity. A column was used only if the correlation coefficient of linearity was greater than 0.995.

In gerbils that were used for prostaglandin determinations, the brains were removed in less than 1 minute after death, and the left cerebral hemisphere was placed in pentane, cooled in dry ice. The frozen brains were weighed and placed in a 30-ml tissue-grinding tube kept on ice. Aspirin-formic acid solution was prepared by dissolving 0.207 gm of acetylsalicylic acid in 100 ml of 0.75% saline. Aliquots (4.5 ml) were frozen in plastic vials and stored. Prior to use, 0.5 ml of 88% formic acid was added to a vial containing the frozen aspirin solution, and the contents were allowed to thaw slowly on ice. Aspirin-formic acid solution (1 ml) was added to the frozen tissue. The tissue was then slowly homogenized using a serrated-tip Teflon pestle. The homogenized tissue was poured into a test tube. The glass-grinding tube was rinsed with 4 ml of ethyl acetate, which was then added to the homogenized brain tissue in the test tube. The homogenized tissue and ethyl acetate were mixed for 30 seconds with a vortexer and centrifuged for 5 minutes at 2500 rpm. The top layer was removed and dried under an air stream. Three milliliters of 0.1 M phosphate-buffered saline was added to the sample, followed by 3 ml of petroleum ether at 30° to 60° C. The sample was mixed for 60 seconds and centrifuged as before. The sample was then stored at -20° C until prostaglandin assays were performed.

Prostaglandin (PG)E2, PGF2α, 6-keto PGF1α, and TXB2 levels were determined by radioimmunoassay using hydrogen (3H)-labeled compounds.* Dextran-coated charcoal was used to separate bound from free ligand. The limits of sensitivity were 2 pg/tube (PGE2), 1.3 pg/tube (PGF2α), 4.5 pg/tube (keto PGF1α), and 0.7 pg/tube (TXB2). The cross-reactivity of the antibodies to the triene prostaglandins TXB2 and Δ6-keto PGF1α has not been determined. Cross-reactivity to the antibody for PGE2 to PGE3 was 7.8%.

**Preparation of Fatty Acids for Injection**

The EPA (all cis-5,8,11,14,17-eicosapentaenoic acid, 90% pure) was obtained as the ethyl ester. The contaminants included 11% 18:4 (n-3), 12% 20:4 (n-6), 0.7% 20:4 (n-3), and 1.0% 22:6 (n-3). The ester was saponified under nitrogen, using a Firestone valve‡ to remove air. About 250 mg was refluxed for 1 hour in a one-piece condenser flask with 7 ml of 0.3 M NaOH in methanol and water. The reaction mixture was extracted with 5-ml portions of hexane, which removed about 2 mg of oil, then acidified with HCl and extracted with three 5-ml portions of hexane. The pooled extracts were washed with water, dried with sodium sulfate, and evaporated to dryness with a stream of nitrogen in a Thunberg tube. To the acid was added enough 0.3 M NaOH to bring the pH to about 8.3, and enough isotonic saline to bring the concentration of the acid to 10 mg/ml. The air in the tube was removed with the Firestone valve, and the mixture was warmed and sonicated to produce a cloudy suspension.

Portions of the suspension (0.4 ml) were dispensed into test tubes made from Pyrex tubing 0.6 mm in outside diameter. These were frozen in dry ice, evacuated with a high-vacuum pump, and sealed. The tubes were stored at -20° C until use, when they were opened and diluted with an equal volume of alkaline saline (10 ml saline + 0.2 ml of 0.3 M NaOH) to yield a clear solution, pH about 8.5. Sodium 5-(3′-pyridinylmethyl)benzofuran-2-carboxylate (U-63557A)§ was dissolved in saline to a final concentration of 1 mg/ml.

**Experimental Protocol**

In all ischemic animals, the carotid arteries were occluded for 15 minutes. In the 35 gerbils that had CBF and brain water determinations, the clips were removed after 15 minutes of ischemia, and cerebral circulation was restored for 2 hours. Measurements of CBF were taken prior to occlusion, during occlusion, and at 5, 30, 60, 90, and 120 minutes after reperfusion. The brains were then immediately removed for specific gravity determinations.

In 10 gerbils, 0.0334 ml/min of 0.9% saline was infused, starting 5 minutes prior to carotid artery occlusion.
Protection of cerebral blood flow in gerbils

The infusion was continued during occlusion and reperfusion at 0.2 ml/hr for 135 minutes. In five gerbils, 0.167 mg of EPA was infused over 1 minute starting immediately after carotid artery occlusion, and was continued as a constant infusion at a rate of 1 mg/hr for 135 minutes. After carotid artery occlusion, 10 gerbils were given U-63557A, 10 mg/kg intraperitoneally with the EPA infusion, and 10 gerbils were given U-63557A, 10 mg/kg intraperitoneally without EPA infusion.

Brain prostaglandin levels were determined in 35 additional gerbils that received EPA treatment. In sham-operated gerbils, 0.167 mg/min of EPA was infused for 5 minutes followed by constant infusion of 1 mg/hr in 10 animals, or U-63557A (10 mg/kg intraperitoneally) was given 5 minutes after starting the EPA infusion in six animals. After 65 minutes of infusion, the brains were removed and assayed for prostaglandin levels. In ischemic gerbils, 0.167 mg/min of EPA was infused for 5 minutes followed by a constant infusion of 1 mg/hr in 10 animals, or U-63557A (10 mg/kg) was given 5 minutes after starting the EPA infusion in nine animals. After 30 minutes of infusion, both carotid arteries were occluded for 15 minutes followed by 15 minutes of reperfusion, after which the brains were removed for analysis. The infusion of EPA (1 mg/hr) continued during occlusion and reperfusion.

Statistical Analysis

The unpaired t-test was used for statistical analysis between groups to determine significant differences between CBF, edema, and prostaglandin levels.

Results

Prior to carotid artery occlusion, regional CBF in saline-treated control gerbils was 27.4 ml/100 gm/min. During bilateral carotid artery occlusion, CBF was less than 5 ml/100 gm/min. There was a short period of hyperemia 5 minutes after reperfusion. After 30 minutes of reperfusion, CBF fell to 17.7 ml/100 gm/min and remained depressed at 19.2 ml/100 gm/min after 2 hours of reperfusion. In gerbils treated with EPA and U-63557A, CBF decreased from 29.5 to 23.6 ml/100 gm/min, but improved after 2 hours of reperfusion to 30.0 ml/100 gm/min, significantly higher than control saline-treated gerbils at p < 0.001 (Fig. 1). In animals treated with U-63557A or EPA alone, postischemic CBF's were higher than controls, but this difference was not significant at the p < 0.05 level (Fig. 1). The differences in brain edema were not significant among the four groups (Fig. 2).

The increased level of 6-keto PGF1α in brain was significantly higher after ischemia in gerbils infused with EPA and U-63557A compared to gerbils given EPA alone (Table 1). Blood pressure and arterial blood gas measurements were similar in all groups. There was no decrease in systemic blood pressure after treatment with EPA alone or with EPA and U-63557A (Table 2).

Discussion

We previously reported that intravenous infusions of EPA, if given prior to ischemia and in high doses, significantly improved postischemic CBF.3 It was concluded that infusions of EPA resulted in a selective increase in PGI2 in areas of ischemia. Moncada and Vane11,12 noted that platelets adhering to the vessel wall could feed the vessel enzyme, prostacyclin synthetase, with endoperoxides, which are subsequently converted to prostacyclin. During infusions of EPA, platelets adhering to the vessel wall could convert EPA to cyclic endoperoxides. These endoperoxides could either be converted to TXA2 (which is inactive) in the platelets or be transferred to the vessel wall to form PGI3, which is a vasodilator and inhibitor of platelet aggregation. Theoretically, a platelet thromboxane synthetase inhibitor, which could prevent the conversion of endoperoxides to thromboxanes, could increase the quantity of
TABLE 1
Brain prostaglandin levels in sham-operated and ischemic gerbils*

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>6-Keto PGF1α</th>
<th>TXB2</th>
<th>PGF2α</th>
<th>PGE2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>4.32 ± 1.99</td>
<td>2.06 ± 0.79</td>
<td>14.38 ± 8.43</td>
<td>40.84 ± 14.36</td>
</tr>
<tr>
<td>Ischemic</td>
<td>8.03 ± 2.57</td>
<td>3.08 ± 0.57</td>
<td>44.71 ± 9.99</td>
<td>46.39 ± 11.30</td>
</tr>
<tr>
<td>Sham + EPA</td>
<td>5.79 ± 2.35</td>
<td>1.36 ± 0.45</td>
<td>8.71 ± 3.34</td>
<td>36.79 ± 14.44</td>
</tr>
<tr>
<td>Ischemic + EPA</td>
<td>10.73 ± 1.81</td>
<td>2.89 ± 0.65</td>
<td>33.94 ± 8.79t</td>
<td>46.58 ± 16.00</td>
</tr>
</tbody>
</table>

*Prostaglandin (PG) levels are means ± standard deviation (pg/mg). EPA = eicosapentaenoic acid; TX = thromboxane.
†Significance: p < 0.02.
‡Significance: p < 0.05.

TABLE 2
Systemic blood pressure (BP), arterial blood gases, and amount of irrigation through the arterial catheter*

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>BP (mm Hg)</th>
<th>PaO2 (torr)</th>
<th>PaCO2 (torr)</th>
<th>pH</th>
<th>Irrigation by Heparinized Saline (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-Ischemia</td>
<td>51.6 ± 5.7</td>
<td>48.5 ± 8.9</td>
<td>101.1 ± 16.11</td>
<td>7.18 ± 0.08</td>
<td>0.36 ± 0.19</td>
</tr>
<tr>
<td>2 Hrs Post-Reperfusion</td>
<td>54.6 ± 5.8</td>
<td>54.6 ± 11.3</td>
<td>75.2 ± 7.7</td>
<td>4.14 ± 7.0</td>
<td>0.36 ± 0.08</td>
</tr>
<tr>
<td>control saline</td>
<td>52.0 ± 5.9</td>
<td>49.3 ± 5.0</td>
<td>79.8 ± 22.4</td>
<td>4.11 ± 8.5</td>
<td>0.42 ± 0.22</td>
</tr>
<tr>
<td>EPA</td>
<td>55.5 ± 4.4</td>
<td>52.8 ± 9.3</td>
<td>79.9 ± 14.2</td>
<td>4.02 ± 4.9</td>
<td>0.34 ± 0.27</td>
</tr>
<tr>
<td>U-63557A</td>
<td>54.6 ± 5.6</td>
<td>54.6 ± 11.3</td>
<td>75.2 ± 7.7</td>
<td>4.14 ± 7.0</td>
<td>0.36 ± 0.08</td>
</tr>
<tr>
<td>EPA + U-63557A</td>
<td>52.0 ± 5.9</td>
<td>49.3 ± 5.0</td>
<td>79.8 ± 22.4</td>
<td>4.11 ± 8.5</td>
<td>0.42 ± 0.22</td>
</tr>
</tbody>
</table>

*Values are means ± standard deviation. EPA = eicosapentaenoic acid.

endoperoxides available for transfer to the vessel wall and thus further increase prostacyclin formation.

In the present study, U-63557A—a selective platelet thromboxane synthetase inhibitor8—potentiates the beneficial effects of EPA on postischemic CBF. The metabolite of PGI2, 6-keto PGF1α, was higher in EPA animals treated with U-63557A, which is consistent with our hypothesis that platelet thromboxane synthetase inhibition increases prostacyclin production. The increase in 6-keto PGF1α must, however, be interpreted cautiously. First, EPA is converted to A17 6-keto PGF1α, and we have not determined the cross-reactivity of our antibody for 6-keto PGF1α with A17 6-keto PGF1α. A large increase in A17 6-keto PGF1α may not be detected in our assay or may only be reflected as a slight increase in 6-keto PGF1α. Second, our infused EPA contained 1% arachidonate, which could also account for the increase in 6-keto PGF1α.

The advantage of EPA and U-63557A therapy over infusions of pure prostacyclin is that it avoids the systemic hypotension which occurs with pure prostacyclin infusions.1,17 Culp, et al.,18 reported that EPA is a poor substrate for cyclo-oxygenase at peroxide levels which occur in non-ischemic tissue. Increasing peroxide tone in an incubate containing cyclo-oxygenase increased the conversion of EPA considerably. Accordingly, in areas of ischemia where peroxide levels are higher, the formation in platelets of cyclic endoperoxides from EPA may be increased. This could produce an increased transfer of these endoperoxides to the vessel wall and, subsequently, increased formation of PGI3. In non-ischemic areas, without platelet adhesion to the vessel wall and with lower peroxide levels, the formation of PGI3 may be less. Prostacyclin has a very short half-life. Although levels of PGI3 may be high in ischemic areas, the levels of circulating PGI3, which could affect systemic resistance vessels and produce hypotension, may be low.

The failure of our study to show that U-63557A does not prevent the increase in brain TXB2 after ischemia does not exclude the possibility that U-63557A prevents thromboxane production in platelets. Our assay most likely is measuring thromboxane formed from the vessel wall.14 This source of thromboxane is not inhibited by aspirin at doses that prevent platelet thromboxane formation.15

U-63557A potentiates the beneficial effects of intravenous EPA on postischemic CBF. This effect we believe is due to the ability of U-63557A to increase PGI3 production in the vessel wall.

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