Ischemic Brain Edema Following Occlusion of the Middle Cerebral Artery in the Rat. II: Alteration of the Eicosanoid Synthesis Profile of Brain Microvessels


SUMMARY Using the rat middle cerebral artery occlusion model, alterations in the eicosanoid synthetic capacity of brain microvessels following ischemia were studied by radiochromatography. Brain microvessels of normal rats predominantly produced hydroxyacids with relatively small amounts of PGD2 and PGE2 from exogenous arachidonic acid. Confirmation that hydroxyacids and prostaglandins were products respectively of lipoxygenase(s) and cyclooxygenase was obtained by experiments using indomethacin and eicosatetraynoic acid. The eicosanoid synthetic capacity of the brain microvessel, especially of hydroxyacids, was significantly enhanced 24 and 72 hours after the onset of ischemia. Because this is the phase of maximum edema in the present model, enhanced eicosanoid production in the brain microvessel may be involved in the mechanisms that underly ischemic brain edema.

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BREAKDOWN of the blood-brain barrier (BBB) to plasma solutes such as proteins and electrolytes constitutes an essential mechanism underlying ischemic brain edema. In agreement with previous reports,1-4 our studies of the rat middle cerebral artery (MCA) occlusion model revealed that there was a close correlation between the increases in the brain water and sodium contents throughout the development of ischemic brain edema.5 Therefore, from a therapeutically important point of view, it seems of utmost importance to identify those chemical factors that might affect the BBB permeability following ischemia.

Several compounds, such as cyclic AMP, biogenic amines, and the kallikrein-kinogen-kinin system, have been suggested to be possible candidates that might cause BBB permeability changes.6 7 However, with each of these compounds, inconsistent data have been reported.7 8 In this respect, the hypothesis suggested by Demopoulos et al9 seems of interest. They assumed that in the brain rendered ischemic, active oxygens are generated and these cause membrane disruption due to propagation of free radical reactions. This represents a form of lipid peroxidation due to non-enzymatic reactions. On the other hand, it was recently shown that another form of lipid peroxidation due to enzymatic reactions, namely, the arachidonate cascade, exists in the brain. Free arachidonic acid accumulates following ischemia,10-12 and at least part of it is peroxidized via the arachidonate cascade.13-15 Cyclooxygenase and lipooxygenase products, such as prostaglandins and leukotrienes, are known to possess multiple biological actions, which may be related to the occurrence of brain edema.16 17 In addition to these products, an oxygen radical, currently deemed as hydroxyl radical, is liberated in the conversion of PGG2 to PGH2.18 19 Hydroperoxides (HPETEs) which can act as free radicals or may release more potent oxidants through the action of the hydroperoxidase associated with PG synthetase20 are produced by lipoxygenases. Therefore, when the role of free radicals in ischemic brain edema is considered, it seems indispensable to investigate the activity of the arachidonate cascade following ischemia.

In the present study, the eicosanoid synthetic capacity of the cerebral microvessel obtained from hemispheres of normal or MCA-occluded rats was examined. Brain microvessels were specifically studied because they are reportedly rich in the enzymes comprising the arachidonate cascade,20-22 and, mostly being parenchymal small vessels, it constitutes essential parts of the cerebral microcirculation as well as being the site of the BBB. The unilateral MCA occlusion in the rat was used as the model of cerebral ischemia, because it was shown to provide a well-reproducible pattern of edema development.5

Materials and Methods

Preparation of the Rat Brain Microvessel

Male Sprague-Dawley rats, weighing 250-300 g were subjected to unilateral MCA occlusion by the method described elsewhere.5 Sixteen rats were successively operated on in a day, and they were sacrificed 24 and 72 hours later. Following brain perfusion with 100 ml of physiological saline from the heart at a pressure of 120 mmHg, the brain was rapidly removed and divided into its two hemispheres. The 16 hemispheres of the MCA-occluded or sham-operated side were assembled and used to prepare the brain microvessel. Such a procedure was repeated three to four times for each experimental group.

Preparation of the brain microvessel was carried out according to the method of Gesce et al22 with minor modifications. All steps in the procedure were carried out in the cold (0-4°C). Briefly, each hemisphere was chopped with scissors in 10 volumes of 0.25 M-sucrose chopped with scissors in 10 volumes of 0.25 M-sucrose...
crose, 5 mM-EDTA, and 50 mM-Tris HCl (pH 7.3). Then, the suspension was serially passed through nylon meshes having pore sizes of 670 µm and 335 µm, respectively. The filtrate was centrifuged at 3,500 g for 10 minutes, and the resultant precipitate was resuspended into the same buffer. Each 2.5 ml were layered on a discontinuous density gradient consisting of 1.0 M-, 1.3 M-, and 1.5 M-sucrose (3 ml each). Then the gradient was centrifuged at 58,000 g for 60 minutes. The subfraction at the bottom of the tube were collected and resuspended in the above-described buffer. Light and electron microscopic study revealed that the fraction mainly consisted of microvessels with a diameter of 10-50 µm.

Metabolism of [14C]-arachidonic Acid by the Rat Brain Microvessel

The metabolism of [14C]-arachidonic acid by the rat brain microvessel was studied within the day of preparation as follows.23 The microvessel (40-50 µg protein) was preincubated for 10 minutes at 37°C in 0.1 ml of 50 mM-Tris HCl (pH 7.4) containing 10.0 mM-glucose with or without drugs. After preincubation, 50 µl of [14C]-arachidonic acid (0.2 µCi; final concentration of 6.5 µM) were added to the incubation mixture, which was incubated for an additional 60 minutes at 37°C in the dark without agitation. The reaction was terminated by chilling, followed by quickly adding an appropriate amount of 1 N-HCl to bring the pH of the reaction mixture to 3.0. Then the mixture was extracted two times with 8 ml of ethyl acetate. The resulting organic phase was evaporated to dryness with a stream of nitrogen. The residue was then dissolved in a small amount of ethanol and applied to a precoated silica gel thin-layer plate (60F 254, Merck). Prostaglandins D₂, E₂, 6-keto-PGF₁α (Ran Biochem), arachidonic acid (Sigma), and [14C]-TXB₂ (New England Nuclear, Boston, Mass.) were also applied to the plate. The plate was developed in ethyl acetate-isooctane-acetic acid-water (11:5:2:10, organic phase). The plate was dried and scanned for its radioactivity by a radiochromatogram scanner (TRM-1B, Aloka). Nonradioactive arachidonic acid and prostaglandin markers were visualized by spraying with phosphomolybdic acid in ethanol. The areas corresponding to authentic standard arachidonic acid and prostaglandin markers were clearly visible compared with the radioactivity scanned for the plate. A typical thin-layer chromatography pattern of eicosanoid production of the microvessels obtained from the normal and MCA-occluded rats (24 hours postoperatively) is shown in figure 1. The percentage conversion of [14C]-arachidonic acid is summarized in table 1.

From these results, it became apparent that normal rat microvessels predominantly produced hydroxy-acids, whereas ETYA significantly reduced lipoxygenase products (hydroxyacids), but ETYA did not significantly reduce lipoxygenase products (hydroxyacids), but ETYA did. The addition of indomethacin to the incubation mixture caused a significant reduction of only the cyclooxygenase products, whereas ETYA significantly reduced all the products (table 2). Thus indomethacin did not significantly reduce lipoxgenase production of hydroxyacids, but ETYA did.

Discussion

Fatty acids comprising membrane lipids are liberated and accumulate in the brain following ischemia.10-12 Although most of these free fatty acids are considered to be washed out of the brain or reacylated, conversion of arachidonic acid to prostaglandins via the arachidonate cascade13-15 and/or to unidentified peroxides16 has been shown to occur in various models. Because of the potent biological effects of prostaglandins, it has been suspected that the arachidonate cascade is involved in the genesis of ischemic brain edema.

The role of prostaglandins in brain edema has been
studied in experiments in which indomethacin was used as an inhibitor of cyclooxygenase. These studies consistently failed to find evidence of a beneficial effect,26-28 suggesting that cyclooxygenase products are not major factors in the production of edema. However, as indicated by a previous report28 and the present result (table 2), indomethacin in the dose range used in those experiments probably does not inhibit lipoxygenases. In addition, it is known that indomethacin increases the availability of arachidonic acid to lipoxygenases.29 Therefore, even if the cyclooxygenase pathway may not be involved in the occurrence of ischemic brain edema, the role of the lipoxygenase pathway needs to be clarified.

The present study revealed that the cerebral microvessel of normal rats has the capacity to convert exogenous arachidonic acid to various prostaglandins and hydroxy (hydroperoxy) acids. The percent conversion of $^{14}$C-arachidonic acid to each product was in good agreement with the result of Gecse et al.32 Further, the eicosanoid synthetic capacity of the brain microvessel was shown to be significantly enhanced following MCA occlusion. The study using ETYA and indomethacin (table 2) indicates that prostaglandins and hydroxyacids detected by the present method are products of cyclooxygenase and lipoxygenase, respectively. Therefore, the present study suggests that the activities of both enzymes in the brain microvessel were enhanced following MCA occlusion. As this occurred during the period of 24-72 hours after MCA occlusion when the edema development was most rapid in the present model,24 it is tempting to speculate that the enhanced eicosanoid synthesis in the brain microvessel is causally related to edema formation.

As with the results obtained with other models, the administration of indomethacin in the present model did not effect beneficially brain edema (unpublished data). Considering these results, cyclooxygenase products may not play a major role in the occurrence of brain edema also in the present model, although the measurement of each prostaglandin in situ is required before a conclusion is reached.

On the other hand, little has been known about the lipoxygenase activity or the kinds and actions of lipoxygenase products in the brain or the brain microvessel. In organs other than the brain, however, there is evidence that lipoxygenase products increase the vessel permeability. Leukotrienes C$_{4}$ and D$_{4}$, which are lipoxygenase products, were shown to increase plasma exudation when injected into the skin. In the perfused lung, lipoxygenase products enhanced transalveolar exudation. Also, the inhibition of lipoxygenase activity by BW755C was shown to mitigate the inflammatory response of the ischemic myocardium. These results together with the present data showing that the lipoxygenase activity of the microvessel was significantly enhanced concomitant to the edema development raise the possibility that the products of lipoxygenase rather than of cyclooxygenase are relevant to the pathogenetic mechanism underlying ischemic brain edema. In support of this view, we have previously observed that the intrathecal injection of 15-hydroperoxy-arachidonic acid (15-HPAA), a plant lipoxygenase product, caused prolonged vasospasm of the basilar artery in dogs associated with pronounced degenerative changes in the arterial endothelium.

Insomuch as the present study revealed that the enhancement of the eicosanoid synthetic capacity of the brain microvessel paralleled the development of brain edema following MCA occlusion in rats, their relationship obviously requires further elucidation. Among

![Graph showing eicosanoid profiles of the rat brain microvessel](image)

**Figure 1.** Representative radiochromatographic tracings of normal rats (solid line) and MCA-occluded rats (24 hours after the MCA occlusion: broken line) are shown. The RF value of each peak is indicated at the bottom of the figure.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Hydroxyacids</th>
<th>PGD$_{2}$</th>
<th>PGE$_{2}$</th>
<th>TxB$_{2}$</th>
<th>PGF$_{1a}$</th>
<th>6-keto-PGF$_{1a}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (24)</td>
<td>3.24±0.22</td>
<td>1.21±0.18</td>
<td>0.98±0.26</td>
<td>0.38±0.04</td>
<td>0.73±0.21</td>
<td>0.50±0.06</td>
</tr>
<tr>
<td>24 hr-sham (9)</td>
<td>3.15±0.37</td>
<td>1.28±0.20</td>
<td>0.93±0.16</td>
<td>0.44±0.08</td>
<td>0.64±0.04</td>
<td>0.44±0.07</td>
</tr>
<tr>
<td>24 hr-MCA (44)</td>
<td>8.83±0.88†</td>
<td>2.63±0.31*</td>
<td>2.08±0.26†</td>
<td>1.59±0.22*</td>
<td>1.68±0.27*</td>
<td>1.44±0.26*</td>
</tr>
<tr>
<td>72 hr-MCA (24)</td>
<td>6.43±0.43†</td>
<td>4.72±0.66†</td>
<td>2.35±0.31†</td>
<td>1.14±0.18†</td>
<td>1.88±0.20†</td>
<td>1.46±0.19‡</td>
</tr>
</tbody>
</table>

Numbers in parentheses indicate the number of experiments.

Normal vs MCA at †p < 0.05; ‡p < 0.01; ††p < 0.001. (Student's t-test.)

The eicosanoid profiles of the rat brain microvessel are shown as percentage conversion of $^{14}$C-arachidonic acid. Normal: Normal rats; 24 hr-sham: rats sacrificed 24 hours after the sham operation; 24 hr-MCA: rats sacrificed 24 hours after the MCA occlusion; 72 hr-MCA: rats sacrificed 72 hours after the MCA occlusion. The difference from the normal control values was statistically examined using Student's t-test.

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Table 1. Eicosanoid Profiles of The Rat Brain Microvessel
various eicosanoids, the role of lipoygenase products which were shown to be the major eicosanoid products of the brain microvessel in the present study, would merit further investigation.

Acknowledgments

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References


TABLE 2  Effects of Indomethacin and ETYA on the Eicosanoid Synthesis of the Brain Microvessel

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Hydroxyacids</th>
<th>PGD2</th>
<th>PGE2</th>
<th>TXB2</th>
<th>PGF2α</th>
<th>6-keto-PGF1α</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (24)</td>
<td>3.24 ± 0.22</td>
<td>1.21 ± 0.18</td>
<td>0.98 ± 0.26</td>
<td>0.38 ± 0.04</td>
<td>0.73 ± 0.21</td>
<td>0.50 ± 0.07</td>
</tr>
<tr>
<td>Indomethacin (1 x 10^-5 M) (4)</td>
<td>4.22 ± 0.60</td>
<td>0.26 ± 0.07*</td>
<td>0.87 ± 0.08</td>
<td>0.17 ± 0.03</td>
<td>0.23 ± 0.05</td>
<td>0.25 ± 0.04</td>
</tr>
<tr>
<td>Indomethacin (1 x 10^-4 M) (4)</td>
<td>3.68 ± 0.47</td>
<td>0.20 ± 0.05*</td>
<td>0.18 ± 0.04†</td>
<td>0.06 ± 0.01†</td>
<td>0.11 ± 0.01*</td>
<td>0.13 ± 0.02*</td>
</tr>
<tr>
<td>ETYA (3 x 10^-4 M) (4)</td>
<td>1.99 ± 0.41*</td>
<td>0.23 ± 0.05*</td>
<td>0.32 ± 0.08</td>
<td>0.18 ± 0.03</td>
<td>0.19 ± 0.05</td>
<td>0.23 ± 0.02</td>
</tr>
<tr>
<td>ETYA (3 x 10^-4 M) (4)</td>
<td>1.40 ± 0.27†</td>
<td>0.10 ± 0.02*</td>
<td>0.17 ± 0.02*</td>
<td>0.06 ± 0.02†</td>
<td>0.13 ± 0.02</td>
<td>0.13 ± 0.00*</td>
</tr>
</tbody>
</table>

*p < 0.05, †p < 0.01.

Effects of indomethacin and ETYA on the eicosanoid synthesis of the brain microvessel obtained from normal rats are shown. Statistical analysis was undertaken using Student’s t-test.
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