Identification and Quantitative Analysis of Hydroxy-Eicosatetraenoic Acids in Rat Brains Exposed to Regional Ischemia

Masaaki Usui, Takao Asano, and Kintomo Takakura

To clarify possible roles in the pathogenesis of ischemic brain edema, identification and quantitative analysis of hydroxy-eicosatetraenoic acids (HETEs) in rat brains exposed to middle cerebral artery occlusion were carried out using high-performance liquid chromatography. Rat brain sampling was done by in situ freezing 24 and 72 hours after occlusion. Only a small amount of 15-HETE was found in control rat brains. Twenty-four hours after ischemia, 11-HETE appeared, and the amount of 15-HETE tended to increase. Seventy-two hours after ischemia, when brain edema reached its maximum, 5-, 8-, 9-, 11-, 12-, and 15-HETEs were identified, and the amounts of all HETEs except 8- and 12-HETE were significantly increased. The detection of 5-HETE in ischemic rat brain indicates the simultaneous production of leukotrienes in the same brain area. The above results support the view that lipoxygenase products may play significant roles in the formation of ischemic brain edema.

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ISCHEMIC cerebral edema is a serious and sometimes fatal complication of stroke, but the precise mechanism of edema formation is not fully understood yet. In recent study of the arachidonate cascade, the possible roles of potent bioactive eicosanoids in various pathologic conditions have been attracting more attention. In the brain, membrane-bound fatty acids were released following ischemic insult or seizure activity. Among these fatty acids, arachidonic acid was shown to cause brain edema and destruction of capillary endothelial membranes when injected directly into the brain. Arachidonic acid is a substrate for two oxygenases, and its conversion to various eicosanoids seems to be responsible for these pathologic states. The administration of the cyclooxygenase inhibitor indomethacin did not alleviate ischemic brain edema, or rather aggravated it, which led us to speculate that lipoxygenase metabolites rather than cyclooxygenase products might play key roles in the pathogenesis of ischemic brain edema, if eicosanoids were involved at all. However, little is known about lipoxygenase metabolism and the pathophysiology of its metabolites in the brain. The following experiments were undertaken to identify the lipoxygenase metabolites and to clarify their relation to ischemic brain edema.

Materials and Methods

Occlusion Model and Method of Brain Sampling

Eight male Sprague-Dawley rats weighing about 200 g were anesthetized with 2% halothane, and electrocauterized and severed at the proximal portion just medial to the olfactory tract. In 4 sham-operated rats, the surgical procedure was done in a similar manner except for the MCA occlusion. After surgery, the rats were returned to their cages and fed ad libitum. These rats were anesthetized again with 2% halothane 24 and 72 hours after MCA occlusion. After tracheostomy was performed, they were given intraperitoneal (i.p.) injections of pancuronium bromide (0.08 mg/kg) and put on a respirator (Model 131, Princeton Medical Instruments, Inc.); halothane concentration was reduced to 0.5% at that time. A midline incision was made on the rat scalp. The skin and temporal muscles were disected from the skull to expose the upper half of the calvaria. The rat brain was frozen in situ with liquid nitrogen. While the whole brains were still frozen, the left hemispheres were chiseled out. During this procedure, liquid nitrogen was poured repeatedly to maintain the frozen state. The thalamus and hypothalamus were trimmed off as much as possible. Each sample was weighed (Mettler HL 52, Mettler Instrument AG.) and stored at -80°C for later analysis.

HPLC Analysis

The left hemisphere was homogenized with cold 99.5% ethanol and centrifuged at 2,000 rpm for 10 minutes. The supernatant was evaporated to dryness under reduced pressure, and the residue was again dissolved in ethanol. It was centrifuged a second time in the same way. Methanol was added to the supernatant, and the mixture was centrifuged a third time. Part of the supernatant was used for reversed-phase (RP) high-performance liquid chromatography (HPLC) and the rest for straight-phase (SP) HPLC.

RP-HPLC was carried out using a column of YMC-Pack A-302-ODS (4.6 × 150 mm, Yamamura Ka-
The samples for SP-HPLC were concentrated under reduced pressure, and 50% methanol was added. Samples were passed through a column of TSK-GEL-120 T-ODS (8 × 300 mm, Toyo Soda, Tokyo) and eluted with acetonitrile:methanol:water:acetic acid (3000:1000:2000:6 by vol) at a flow rate of 2 ml/min. The fraction between 34 and 56 minutes retention time was gathered and evaporated to dryness in vacuo. The residue was dissolved in methanol. After being concentrated under reduced pressure, the sample was dissolved in hexane and used for SP-HPLC analysis on a column of Micro-Porasil (3.9 × 300 mm, Waters Assoc.) with n-hexane:isopropanol:acetic acid (495:6:0.5 by vol) at a flow rate of 2 ml/min. The effluent was monitored at 238 nm on a UV detector.

Six authentic hydroxy-eicosatetraenoic acids (HETEs), namely, 5-, 8-, 9-, 11-, 12-, and 15-HETE, were used as standards for identification and quantitative analysis by retention time and peak height, respectively. The retention times of 8- and 12-HETE are the same on RP-HPLC and cannot be separated by the present method. They will be designated as (8- and 12-)HETE.

**Recovery Rate of HETE**

One hemisphere of a normal rat brain was removed by in situ freezing as described above and homogenized with cold 99.5% ethanol to which authentic 11-HETE at 5 doses, namely, 5, 10, 15, 25, and 30 ng, was added. These samples were pretreated by the method for RP-HPLC described above. The supernatants were subjected to RP-HPLC under the conditions above for determination of the quantity of 11-HETE. The same doses of authentic 11-HETE dissolved in a mixture of methanol and distilled water were also subjected to RP-HPLC as controls. The recovery rate of 11-HETE contained in brain samples was then determined.

**Results**

**Recovery rate of 11-HETE.** The recovery rates of exogenous 5, 10, 15, 20, and 30 ng doses of 11-HETE were 95, 92, 94, 95, and 94%, respectively.

**Identification of HETE in rat brains.** On RP-HPLC, a very small peak corresponding to 15-HETE was observed in the sham-operated control brains. No peaks suggestive of other HETEs were found (Figure 1). On the other hand, peaks corresponding to 5-, 9-, (8- and 12-), 11-, and 15-HETE were found in the brains rendered ischemic for 72 hours (Figure 2). On SP-HPLC of the rat brains 72 hours after ischemia, 6 peaks corresponding to all 6 authentic HETEs were found (Figure 3). The results of both RP- and SP-HPLC showed that 5-, 8-, 9-, 11-, 12-, and 15-HETE were contained in rat brains 72 hours after ischemia.

**Quantitative changes of HETEs during ischemia.** In the sham-operated control rat brains, only 15-HETE was found, at 2.1 ± 2.1 (mean ± SEM) ng/g wet wt. Twenty-four hours after MCA occlusion, 11- and 15-HETE were found, at 1.6 ± 1.6 and 9.1 ± 3.8 ng/g wet wt, respectively. Seventy-two hours after MCA occlusion, 5-, 9-, (8- and 12-), 11-, and 15-HETE were found, at 18.3 ± 2.8, 16.6 ± 3.0, 23.7 ± 14.9, 14.0 ± 3.4, and 19.8 ± 5.5 ng/g wet wt, respectively. The value for (8- and 12-)HETE represented the sum of the values for these HETEs. The amounts of all HETEs except (8- and 12-)HETE were significantly increased 72 hours after MCA occlusion. The increases in 11- and 15-HETE 24 hours after MCA occlusion did not reach significance (Table 1).

**Discussion**

Recent progress in the study of lipoxigenase metabolism of arachidonic acid has thrown more light on the
Column: YMC-Pack A-302-ODS
Mobile phase: CH,CN : CH,OH : H,0 : CH,COOH
(3000 : 1000 : 2000 : 6)
Flow rate: 1ml/min
Detector: UV238nm

FIGURE 2. Reversed-phase HPLC of rat brains 72 hours after ischemia. The numbers represent the positions of authentic 15-, 11-, 8- and/or 12-, 9-, and 5-hydroxy-eicosatetraenoic acid (HETE) in numerical order. Five peaks corresponding to the authentic HETEs were found in rat brains 72 hours after middle cerebral artery (MCA) occlusion.

physiologic and pathologic roles of the eicosanoids. It is well known that lipoxygenase products increase vascular permeability and vasoconstriction in skin, lung, and other tissues and that they are involved in various pathologic conditions such as asthma and inflammation. The inhibition of lipoxygenase reduced the size of cardiac infarction. Therefore, it may be expected that lipoxygenase products are involved in the pathogenesis of cerebral edema following head trauma or cerebral ischemia. Concerning lipoxygenase metabolism in the brain, however, scant data are available. Sautebin, Spagnuolo, and others reported the presence of hydroxy acids in rat and gerbil brains, but the identification of these hydroxy acids was not mentioned. The immunoreactive leukotrienes C4 and D4 and 12-HETE were detected by Moskowitz, Kiwak, and others in gerbil brains after ischemia and concussive injury.

In this study, a small amount of only 15-HETE was detected in the sham-operated control rat brains. The presence of 12-HETE in the hypothalamus of normal rat brains was reported elsewhere. The difference between these findings was attributed to differences in the models used. The thalamus and hypothalamus were removed from our rat brains before HPLC because these structures were not directly affected by MCA occlusion. Even if some of the hypothalamus was left, the amount of 12-HETE would be far below the detection level.

We used the rat MCA occlusion model to see the relation between ischemic cerebral edema and lipoxygenase metabolism. This model is known to provide a reproducible and consistent lesion assessed by regional cerebral blood flow and the extent of the ischemic area. We analyzed the time course of edema formation in the ischemic hemisphere of this model, and we measured HETEs 24 hours after MCA occlusion, when the edema was progressing most rapidly, and at 72 hours, when the edema reached its maximum. In the brain rendered ischemic for 72 hours, the amounts of all 6 HETEs were increased, and the increases in 5-, 9-, 11-, and 15-HETE were significant. This result is considered to be important evidence linking lipoxygenase products with ischemic brain edema. Twenty-four hours after ischemia, only 11- and 15-HETE were increased, though not significantly. According to our study of the eicosanoid synthesizing capacity of rat brain microvessels, the lipoxygenase activity was enhanced at 24 hours after MCA occlusion by as much as, or more than, at 72 hours. Because enzyme activity in the isolated condition does not always reflect in situ activity, the discrepancy between lipoxygenase activity and the amounts of lipoxygenase products in the brain 24 hours after ischemia could be reasonable, but the cause of this discrepancy should be cleared up in the future.

Some of the HETEs and hydroperoxy-eicosatetraenoic acids (HPETEs) have strong chemotactic and chemokinetic potency for human neutrophils, or mucus-releasing action on human airways. However, most of the physiologic roles of HETEs and HPETEs are still unknown. Some lipid hydroperoxides are potent activators of cyclooxygenase, while some can be in-

<table>
<thead>
<tr>
<th>Duration of ischemia</th>
<th>5-HETE</th>
<th>9-HETE</th>
<th>8- and/or 12-HETE</th>
<th>11-HETE</th>
<th>15-HETE</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control) (n = 4)</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>2.1 ± 2.1</td>
</tr>
<tr>
<td>24 hours (n = 4)</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>1.6 ± 1.6</td>
<td>9.1 ± 3.8</td>
</tr>
<tr>
<td>72 hours (n = 4)</td>
<td>18.3 ± 2.8*</td>
<td>16.6 ± 3.0†</td>
<td>23.7 ± 14.9</td>
<td>14.0 ± 3.4†</td>
<td>19.8 ± 5.5†</td>
</tr>
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</table>

HETE, hydroxy-eicosatetraenoic acid; MCA, middle cerebral artery. Each value is the mean ± SEM ng/g wet wt. Significantly different from the control at *p < 0.01; †p < 0.05.
FIGURE 3. Straight-phase HPLCs of A: 6 authentic hydroxy-eicosatetraenoic acids (HETEs) and B: rat brains 72 hours after ischemia. The numbers represent the positions of authentic 12- 15-, 11-, 9-, 8-, and 5-HETE in numerical order. The rat brain 72 hours after middle cerebral artery (MCA) occlusion contains substances corresponding to all 6 authentic HETEs.

Column: Microporasil (Waters Associates)
Mobile phase: n-C$_6$H$_{14}$ : (CH$_3$)$_2$CHOH : CH$_2$COOH (45 : 6 : 0.5)
Flow rate: 2 ml/min
Detector: UV238 nm

Hibitors of the same enzyme. Prostaglandin synthesis is inhibited irreversibly by HPETEs but reversibly by the corresponding HETEs. These HPETEs and HETEs also inhibit the 5-lipoxygenase pathway reversibly. These bioactivities of lipoxygenase products indicate that they may provide a fine regulatory mechanism for activities of the cyclooxygenase and lipoxygenase pathways. The present study shows that an ischemic insult to the rat brain leads to excessive production of lipoxygenase metabolites in the affected hemisphere. Previously, we showed that a lipid hydroperoxide enhanced the lipoxygenase activity of rat brain microvessels. This seems to imply that the activation of lipoxygenase is facilitated by its own products, leading to a breakdown of the regulation and an accelerated production of hydroperoxides in some pathologic conditions. Lipid hydroperoxides such as 12- and 15-HPETE are potent bioactive substances causing marked vasoconstriction and inhibition of PGI$_2$ synthesis. Excessively accumulated hydroperoxides may exert significant influences on the cerebral circulation or biomembrane function, which might result in edema formation. Further studies will be necessary to elucidate the precise roles of HETEs in the pathophysiology of cerebral edema formation. Our study did not aim at detecting leukotrienes, but the presence of 5-HETE in the ischemic rat brain disclosed by this study suggests the involvement of leukotrienes in the same area because 5-HETE and leukotrienes are derived from the same precursor, 5-HPETE.

According to the study of the metabolism of exogenous arachidonic acid by rat brain microvessels, HETE production was not inhibited by indomethacin but by lipoxygenase inhibitors such as eicosatetraenoic acid or caffeic acid. This seems to imply the existence of lipoxygenases in rat brain microvessels and the possibility of enzymatic biosynthesis of HETEs in the ischemic rat brain. The antioxidation of arachidonic acid released after ischemia to HETEs is another possible pathway, but it remains to be seen where and how these HETEs were produced.

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References


**KEY WORDS**  • brain edema  • cerebral ischemia  • lipoxigenase  • HETE  • lipid hydroperoxide
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