Biosynthesis of Leukotrienes in Canine Cerebral Vasospasm

Masayuki Yokota, MD, Eiichi Tani, MD, and Yukio Maeda, MD

We produced cerebral vasospasm in 29 dogs by the “two-hemorrhage” method of intracisternal injections, 2 days apart, of autogenous arterial blood. Leukotriene (LT) C4, LTD4, and LTE4 were purified from incubated basilar artery, medulla oblongata, hypothalamus, median eminence, and blood clot from around the basilar artery using reverse-phase high-performance liquid chromatography, and the amount of each LT was quantified separately by bioassay with guinea pig ileum. The biosynthetic capacity for total LTs was approximately three times higher in the hypothalamus and median eminence than in the basilar artery and medulla oblongata in the eight normal dogs. In the dogs with subarachnoid hemorrhage, the biosynthetic capacity was increased significantly both before and 2 hours after the second injection of blood on Day 2 and was normal on Day 7 in the basilar artery and medulla oblongata, whereas the biosynthetic capacity was decreased significantly 2 hours after the first and second injections of blood and was increased significantly on Day 7 in the hypothalamus and median eminence. In blood clot the biosynthetic capacity was increased continuously after the first injection of blood. Thus, the biosynthetic capacity for total LTs showed a time- and tissue-specific change after subarachnoid hemorrhage. (Stroke 1989;20:527-533)

Recently, the vascular tissues including the cerebral arteries have been shown to produce leukotriene (LT) C4, LTD4, and LTE4 (5-lipoxygenase products of arachidonic acid) in addition to cyclooxygenase products. Although reports of the effects of LTs on the cerebral circulation are conflicting, the cerebral arteries as well as the pial arterioles have been shown to contract after local application of LTC4 or LTD4. Therefore, the lipoxygenase products of arachidonic acid might play an important homeostatic role in the cerebrovascular system, either alone or in conjunction with other vasoactive substances, as physiologic antagonists of vasodilators such as prostacyclin (PGI2).

The synthesis of LTs has been reported to increase in gerbil brain after subarachnoid hemorrhage (SAH), and 5-hydroxyeicosatetraenoic acid has been found in the cerebrospinal fluid of patients with SAH, suggesting an activation of 5-lipoxygenase. In a previous study, we showed that the angiographic evidence of canine cerebral delayed vasospasm was reduced significantly by daily treatment with 2-(12-hydroxydodeca-5,10-diynyl)-3,5,6-trimethyl-1,4-benzoquinone (AA-861), a selective inhibitor of 5-lipoxygenase, after SAH, suggesting that LTs are responsible for the development of cerebral vasospasm. In our present study, we examine sequential changes of the biosynthetic capacities for LTs after induction of SAH in incubated canine basilar artery, medulla oblongata, hypothalamus, median eminence, and blood clot and discuss the relation of the biosynthetic capacity for LTs and the development of cerebral vasospasm.

Materials and Methods

Thirty-seven adult mongrel dogs weighing 8-16 kg were sedated with 10 mg/kg i.m. ketamine hydrochloride and then 15 mg/kg i.v. pentobarbital sodium. Experimental SAH was produced in 29 dogs by two successive injections, 2 days apart, of 5 ml fresh autogenous arterial blood each into the cisterna magna, as reported by Varsos et al. Vertebral angiography was carried out via the femoral artery before the first injection of blood and before the dogs were killed by perfusion with 1,000 ml oxygenated cold Tyrode’s solution following anesthesia with ketamine hydrochloride and pentobarbital sodium. The calibers of the middle third of the basilar and vertebral arteries were measured at their narrowest points on the angiograms with the use of a microdensitometer and expressed as a percent of the control calibers of those segments before the first injection of blood. The dogs were

From the Department of Neurosurgery, Hyogo College of Medicine, Nishinomiya, Japan.

Address for reprints: Masayuki Yokota, MD, Department of Neurosurgery, Hyogo College of Medicine, Mukogawa-cho 1-1, Nishinomiya, Hyogo, Japan 663.

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killed 2 hours (Day 0, n=7) or 2 days (Day 2, n=6) after the first injection of blood or 2 hours (Day 2, n=6) or 5 days (Day 7, n=10) after the second injection of blood to measure the sequential change in the biosynthetic capacity for LTs.

The dogs were killed by perfusion with 1,000 ml oxygenated cold Tyrode's solution after angiography. The brains were removed rapidly, and frontal sections approximately 0.5 mm thick were sliced from the medulla oblongata, hypothalamus, and median eminence. The basilar arteries were quickly cleaned of blood clot and connective tissue and then chopped into slices approximately 1.0 mm thick with a McIlwain tissue chopper. The blood clot was homogenized with a cold stirrer (Homogenizer 1080, Ikemoto Scientific Technology Co., Ltd., Tokyo, Japan). Slices of the basilar artery, medulla oblongata, hypothalamus, and median eminence as well as the homogenized blood clot were preincubated in Tyrode's solution containing 2.8 μM indomethacin at 37° C for 5 minutes before the addition of 5 or 7.5 μM calcium ionophore A23187 and 18.3 or 75 μM arachidonic acid. The basilar artery was incubated 5 minutes, the brain 10 minutes, and blood clot 20 minutes. After the addition of two to four volumes of ethanol, the incubation mixture was centrifuged at 2,000 rpm for 10 minutes; the supernatant was kept at -80° C.

The supernatant was evaporated to dryness under reduced pressure. The residue was dissolved in 100-200 μl methanol:water (1:1 by vol); 100 μl of the solution was subjected to reverse-phase high-performance liquid chromatography (RP-HPLC) with a TSK Gel-ODS-120T precolumn (4.0 x 10 mm, Toyo Soda Co., Ltd., Tokyo, Japan) and a YMC-A302-ODS column (4.6 x 150 mm, Yamamura Chemical Laboratories Co., Ltd., Kyoto, Japan) using a developing solvent system of acetonitrile:water:acetic acid (900:300:1300:3 by vol, buffered to pH 5.6 with ammonium hydroxide) at a flow rate of 1 ml/min. The optical density of the column effluent was monitored continuously with an ultraviolet detector at 280 nm.

Segments of guinea pig ileum, 20 mm long and 5 mm wide, were suspended on rigid parallel prongs in an organ bath filled with Tyrode's solution gassed with 5% CO2 in O2 and kept at 37° C; the segments were allowed to stabilize at a resting tension of 1 g for 2 hours before the start of the bioassay. Their isometric tensions in response to authentic LTs and HPLC-purified samples were measured with a Nihon-Koden FD transducer (Nihon-Koden Kogyo Co., Tokyo, Japan). Cumulative dose-response curves of authentic LTC4, LTD4, and LTE4 were prepared separately in the guinea pig ileum segments, and the activities of LTC4, LTD4, and LTE4 in HPLC-purified samples were thereafter assayed separately in the presence of 3.5 μM mepyramine and 0.69 μM atropine by plotting the contractile responses of guinea pig ileum segments on the dose-response curves. Finally, 1.0 μM FPL 55712 was infused over the guinea pig ileum segments to examine the abolition of contraction induced by authentic LTs and HPLC-purified samples.

Data are expressed as mean±standard deviation. Differences between values were assessed using the two-tailed t test for uncorrelated pairs.

Results

Calibers of the basilar and vertebral arteries were measured before the dogs were sacrificed for the bioassay. The calibers as mean percentage sizes in the dogs with SAH are shown in Figure 1, demonstrating a progressive vasoconstriction following the induction of SAH, particularly after the second intracisternal injection of blood.

Figure 2 shows an ultraviolet absorbance profile of products eluted using RP-HPLC from the medulla oblongata of a normal dog. Peaks I, II, and III of the sample curve have elution times corresponding to those of authentic LTC4, LTD4, and LTE4, respectively. In addition, the materials in peaks I, II, and III caused contractions of guinea pig ileum segments that had time courses and relative potencies consistent with the presence of LTC4, LTD4, and LTE4, respectively, as shown in Figure 3. The contractions were unaffected by indomethacin or by receptor antagonists for histamine and acetylcholine, but the contractions were susceptible to antagonism by FPL 55712, indicating the presence of LTs. Similar evidence was found for the eluted products of the basilar artery, hypothalamus, median eminence, and blood clot.

The biosynthetic capacities of the basilar artery, medulla oblongata, hypothalamus, median eminence, and blood clot for LTC4, LTD4, and LTE4, as well as total LTs (LTC4+LTD4+LTE4) in the normal dogs and those with SAH are shown in Table 1 and Figure 4. The ratios of the amounts of LTC4, LTD4, and LTE4 generated in the incubated tissue of the dogs with SAH was different from that of the normal dogs, suggesting different rates of metabolism of LTE4 from LTC4.
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FIGURE 2. Reverse-phase high-performance liquid chromatograms of mixture of authentic leukotriene (LT) C4, LTD4, and LTE4 (upper curve) and sample of medulla oblongata from normal dog (lower curve). Peaks I, II, and III have elution times corresponding to authentic LTC4, LTD4, and LTE4, respectively. Eluted compounds were monitored with ultraviolet detector at 280 nm.

The amount of total LTs produced in the hypothalamus and median eminence of the normal dogs was approximately three times that in the medulla oblongata and basilar artery (Table 1). The amount of total LTs produced in the basilar artery and medulla oblongata in the dogs with SAH was significantly increased on Day 2 before (p<0.001, p<0.02, respectively) and after (p<0.001, p<0.01, respectively) the second injection of blood and was not different from normal on Day 7. The amount of total LTs on Day 0 2 hours after the first injection was not significantly different from normal in the basilar artery and medulla oblongata, suggesting no early effect of blood in these tissues on the biosynthetic capacity for total LTs. The amount of total LTs generated in the hypothalamus and median eminence in the dogs with SAH was significantly decreased 2 hours after the first (p<0.01, p<0.001, respectively) and 2 hours after the second (p<0.02, p<0.01, respectively) injections of blood and was significantly increased 5 days after the second injection (p<0.05 for both). The significant decrease of total LTs in the hypothalamus and median eminence 2 hours after both injections suggests an early effect of blood in these tissues on the biosynthetic capacity for total LTs. The amount of total LTs produced in blood clot was significantly increased 2 days after the first (p<0.01) and 5 days after the second (p<0.001) injections of blood compared with that 2 hours after the first injection.

Discussion

SAH induces increased synthesis of LTs in gerbil brain and of 5-hydroxyeicosatetraenoic acid in human cerebrospinal fluid, suggesting activation of 5-lipoxygenase. The sources of LTs following SAH may be brain tissue, cerebral artery, and blood clot because all three generate LTs. The medulla oblongata is a perfusion territory of a spastic vertebrobasilar system, and the greatest amount of LTC4 in rat brain is detected in the hypothalamus and median eminence. Consequently, we selected the medulla oblongata, hypothalamus, and median eminence, in addition to the basilar artery and blood clot, as tissues in which to measure LT biosynthetic capacities in normal dogs and in those with SAH after in vitro stimulation with calcium ionophore A23187.

The formation of LTs from arachidonic acid is initiated by 5-lipoxygenase; arachidonic acid is converted to 5(5)-hydroperoxyeicosatetraenoic acid, which is further converted to unstable LTA4; LTA4 is transformed by the addition of glutathione at C-6 into LTC4, and then stepwise enzymatic elimination of glutamic acid and glycine in the peptide moiety leads to formation of LTD4 and LTE4, respectively. The metabolic rate of transformation of LTC4 to LTE4 in the incubated tissue was different in the normal dogs and those with SAH, as shown in Table 1. In addition, the contractile activity of each LT for the intracranial artery was different. Consequently, we examined the change of LT biosynthetic capacity after the induction of SAH by measuring the total amounts of LTC4, LTD4, and LTE4 generated in each incubated tissue.

LTs have potent vasoconstrictor activities in peripheral vessels, but reports of the effects of LTs on the cerebral circulation are equivocal. Human cerebral artery taken at autopsy has been reported to neither contract nor relax after the addition of 10^-7 to 10^-10 M LTC4 or LTD4. However, Tagari et al showed that 2x10^-9 to 2x10^-7 M LTD4 constricted human intracranial artery in vitro.
### Table 1. Amount of LTs Generated in Dogs After Intracisternal Injection of 5 ml Autogenous Arterial Blood

<table>
<thead>
<tr>
<th>LT</th>
<th>Normal</th>
<th>First injection</th>
<th>Second injection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=8)</td>
<td>Day 0 (n=7)</td>
<td>Day 2 (n=6)</td>
</tr>
<tr>
<td>Basilar artery</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LTC₄</td>
<td>31.9±16.5</td>
<td>27.9±8.1</td>
<td>50.0±13.4</td>
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<tr>
<td>LTD₉</td>
<td>25.4±12.4</td>
<td>16.0±7.5</td>
<td>40.2±11.6</td>
</tr>
<tr>
<td>LTE₄</td>
<td>32.0±9.7</td>
<td>42.0±16.2</td>
<td>68.2±29.1†</td>
</tr>
<tr>
<td>Total</td>
<td>89.3±15.1</td>
<td>85.9±16.9</td>
<td>158.4±31.4‡</td>
</tr>
<tr>
<td>Medulla oblongata</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LTC₄</td>
<td>28.9±18.3</td>
<td>22.4±14.2</td>
<td>49.0±32.9</td>
</tr>
<tr>
<td>LTD₉</td>
<td>32.4±21.7</td>
<td>16.7±7.5</td>
<td>39.2±26.7</td>
</tr>
<tr>
<td>LTE₄</td>
<td>27.9±11.3</td>
<td>47.6±28.1</td>
<td>70.7±18.6‡</td>
</tr>
<tr>
<td>Total</td>
<td>89.1±42.8</td>
<td>86.7±28.7</td>
<td>158.8±46.7‡</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>LTC₄</td>
<td>84.1±41.8</td>
<td>42.0±19.3‡</td>
<td>83.2±48.2</td>
</tr>
<tr>
<td>LTD₉</td>
<td>63.9±27.7</td>
<td>37.6±23.1</td>
<td>56.0±16.7</td>
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<tr>
<td>LTE₄</td>
<td>106.6±42.2</td>
<td>62.7±20.0†</td>
<td>130.3±33.4</td>
</tr>
<tr>
<td>Total</td>
<td>254.6±79.0</td>
<td>142.3±38.0*</td>
<td>269.5±60.1</td>
</tr>
<tr>
<td>Median eminence</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LTC₄</td>
<td>89.8±45.2</td>
<td>51.1±26.4</td>
<td>85.3±50.7</td>
</tr>
<tr>
<td>LTD₉</td>
<td>93.8±36.0</td>
<td>29.0±10.4*</td>
<td>49.3±14.3†</td>
</tr>
<tr>
<td>LTE₄</td>
<td>98.3±17.6</td>
<td>83.6±31.6</td>
<td>127.2±23.8†</td>
</tr>
<tr>
<td>Total</td>
<td>282.0±61.9</td>
<td>163.7±26.8§</td>
<td>245.2±57.0</td>
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<tr>
<td>Blood clot</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LTC₄</td>
<td>3.9±2.9</td>
<td>10.0±5.3†</td>
<td>8.5±2.8§</td>
</tr>
<tr>
<td>LTD₉</td>
<td>4.0±2.8</td>
<td>6.2±2.0</td>
<td>4.0±1.0</td>
</tr>
<tr>
<td>LTE₄</td>
<td>6.4±3.7</td>
<td>12.2±4.0†</td>
<td>9.3±4.1</td>
</tr>
<tr>
<td>Total</td>
<td>14.3±8.1</td>
<td>28.3±5.0*</td>
<td>21.8±6.1</td>
</tr>
</tbody>
</table>

Data are mean±SD pmol/g wet wt. Day after first injection of blood; LT, leukotriene.

*†‡§p<0.01, 0.05, 0.001, 0.02, respectively, different from normal (for basilar artery, medulla oblongata, hypothalamus, and median eminence) or Day 0 (for blood clot).

||p<0.01, 0.05, respectively, different from before second injection.

and that the injection of $1 \times 10^{-6}$ to $15 \times 10^{-6}$ M/kg LTD₄ into the rat carotid artery induced constriction of the internal and external carotid arteries. In addition, the infusion of 1, 3, and $9 \times 10^{-8}$ M/min LTC₄ or LTD₉ into the porcine internal carotid artery induced the rapid onset of a dose-related reduction in cerebral blood flow that lasted for up to 25 minutes.5 Rosenblum10 found that $3 \times 10^{-8}$ to $4 \times 10^{-7}$ M LTB₄, LTC₄, or LTD₄ caused a dose-related constriction of the mouse pial arteriole when applied topically, and Mayhan et al9 reported that topical application of $3 \times 10^{-10}$ to $3 \times 10^{-8}$ M LTC₄ induced modest constriction of the hamster pial arteriole. In contrast, Kamitani et al32 found that local application of $1.6 \times 10^{-9}$ to $3.1 \times 10^{-6}$ M LTC₄ or LTD₄ neither constricted nor dilated the rabbit pial arteriole. The reason for the above discrepancies is uncertain but may include factors such as drug stability,30 species differences, or the condition of the isolated arterial segments.

In our previous study, angiographic evidence of cerebral delayed vasospasm in dogs was reduced significantly by daily treatment with AA-861, a selective inhibitor of 5-lipoxygenase, after the induction of SAH, and the contractile property of excised basilar arteries in response to vasoconstrictor agents was improved significantly.14 Similar evidence was reported after treatment with ibuprofen or high-dose methylprednisolone, two anti-inflammatory agents.33 LTs are considered to be chemical mediators in inflammatory reactions,23 and their synthesis was decreased by glucocorticoids.34 It is suggested, therefore, that LTs are etiologic factors responsible for the development of delayed vasospasm.

Our results show that SAH induces a time- and tissue-specific change of LT biosynthetic capacity. That 2 hours after the first blood injection on Day 0 was not changed significantly in the basilar artery or medulla oblongata but was decreased significantly in the hypothalamus and median eminence. In addition, there was no evidence of any change of biosynthetic capacity of canine basilar artery for prostaglandin (PG) on Day 0.173 Consequently, the decrease in the caliber of the vertebral and basilar arteries on Day 0, although a small LT biosynthetic capacity was found in blood clot, might not be related to arachidonic acid metabolites.
The decrease in the caliber of the vertebral and basilar arteries was not severe before the second injection of blood on Day 2, but the LT biosynthetic capacities at that time had increased significantly in the basilar artery and medulla oblongata as well as in blood clot and had returned to normal in the hypothalamus and median eminence. In a "single-hemorrhage" canine model, the biosynthetic capacity of the basilar artery for PGE$_2$ was increased significantly on Day 2. Consequently, the arachidonic acid metabolites responsible for the development of vasospasm on Day 2 could be the increased amounts of LTs from the basilar artery and medulla oblongata as well as blood clot and the increased amounts of PGE$_2$ from the basilar artery.

Angiographic evidence of vasospasm became more severe 2 hours after the second injection of blood on Day 2. The LT biosynthetic capacities of the basilar artery and medulla oblongata at that time were still significantly higher than normal and had not changed significantly from that before the second injection of blood, whereas the biosynthetic capacities of the hypothalamus and median eminence were again decreased significantly. It is unknown why the LT biosynthetic capacities of the hypothalamus and median eminence are more vulnerable to injection of blood than those of the medulla oblongata and basilar artery. The slight decrease in LT biosynthetic capacity of blood clot might be caused by a diluting effect of injected blood. The mean percent decreases in the caliber of the vertebral or basilar artery 2 hours after the first and second injections of blood were similar if calculated from the calibers before each injection, but the vasocontractile conditions before each injection of blood were different. The severe vasospasm 2 hours after the second injection might be caused by denervation supersensitivity of both arteries to injected blood, as well as by the injected blood itself.

The angiographic evidence of vasospasm was still severe on Day 7, but the LT biosynthetic capacities of the basilar artery and medulla oblongata were normal. Consequently, there was no correlation between delayed vasospasm and LT synthesis in the basilar artery or medulla oblongata on Day 7. The clinical and experimental results suggest that delayed vasospasm is reduced only after the early removal of subarachnoid blood. In addition, the differential effects of AA-861 were shown in delayed vasospasm in dogs: intravenous infusion of AA-861 at 6.5 x 10$^{-4}$ mg/kg/min for 2 hours had no effect on the angiographic evidence of delayed vasospasm once vasospasm had developed, whereas oral doses at 100 mg/kg/day for 7 days following the induction of SAH reduced delayed vasospasm. It is suggested, therefore, that irreversible events occur in vasospasm at a certain time following SAH (Day 2), leading to delayed vasospasm. Inflammatory cells were scattered in the wall of spastic canine basilar artery on Day 7, but Faleiro et al found a marked decrease in the caliber of the vertebral and basilar artery 2 hours after the second injection of blood.
increase in the number of mast cells in the tunica media of human cerebral artery after SAH. In addition, Hughes and Schianchi reported the infiltration of inflammatory cells into the adventitia and of many macrophage-like cells into the tunica media of human cerebral artery in vasospasm. Since leukocytes, mast cells, and macrophages have been shown to produce LTs, cerebral artery infiltrated by such cells could release more LTs than noninfiltrated cerebral artery. The gradual increase in LT biosynthetic capacity of blood clot after the induction of SAH might parallel the increase in the number of inflammatory cells in blood clot after the injection of blood.

The LT biosynthetic capacity of the hypothalamus and median eminence was approximately three times that of the medulla oblongata in normal dogs (as shown in rats and) and increased significantly on Day 7. The binding capacity of LTC4 was lower in the hypothalamus than in the brainstem of rats, suggesting an easier release of LTs from the hypothalamus. The transport system for LTC4 in the choroid plexus may protect the brain from excessive amounts of LTC4, but some LTs produced in the hypothalamus and median eminence might gain access to the subarachnoid space. Nevertheless, the LT biosynthetic capacities of the hypothalamus and median eminence were rather decreased before Day 7. Much more work needs to be done to determine the pathophysiologic significance of increased LT biosynthetic capacities of the hypothalamus and median eminence on Day 7.

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