Brain Eicosanoid Levels in Spontaneously Hypertensive Rats After Ischemia With Reperfusion: Leukotriene C₄ as a Possible Cause of Cerebral Edema

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The relation of brain eicosanoids to progression of cerebral edema was studied in stroke-resistant spontaneously hypertensive rats subjected to incomplete global brain ischemia induced by bilateral occlusion of the common carotid arteries. Thromboxane B₂ and 6-keto prostaglandin F₁α levels were significantly elevated 5 minutes after reperfusion but returned to control levels by 30 minutes. In contrast, leukotriene C₄ levels increased 2 hours after bilateral common carotid artery occlusion and peaked 30 minutes after reperfusion, with higher levels persisting until 60 minutes after reperfusion. Cerebral ischemia was accompanied by cerebral edema early after reperfusion. The edema correlated with increased leukotriene C₄ levels. That the increased brain water content was causally related to an increase in leukotriene C₄ was supported by results obtained following administration of the 5-lipoxygenase inhibitors ONO-LP-016 and AA-861. Both inhibitors suppressed the increased leukotriene C₄ and brain water contents after reperfusion. Our results indicate that leukotriene C₄ is closely associated with an induction of ischemic cerebral edema. (Stroke 1988;19:372-377)

Fatty acids are liberated from the plasma membranes of ischemic tissue through various pathways. It is believed that these free fatty acids, particularly arachidonic acid, aggravate cell damage imposed on ischemic brain. Arachidonic acid has been the focus of repeated investigation as it relates to brain edema and endothelial cell damage of cerebral arteries.

When the brain is subjected to severe ischemia, the amount of free arachidonic acid increases abruptly and then decreases gradually when circulation is restored. Since increased levels of arachidonic acid persist when the oxygen supply is restored, conditions exist for enhanced oxidation of arachidonic acid. For example, recirculation is accompanied by a prominent increase in prostaglandins, which are formed from arachidonic acid by cyclooxygenase. Among these prostaglandins, thromboxane A₂ (TXA₂), which is derived primarily from platelets, has potent platelet aggregating and vasoconstrictor effects. By contrast, prostaglandin I₃ (PGI₃), which is derived predominantly from vascular endothelial cells, acts to prevent platelet aggregation and vasoconstriction. TXA₂ and PGI₃ are rapidly converted to the stable metabolites TXB₂ and 6-keto-PGF₁α.

In addition, a lipoxygenase pathway exists in which arachidonic acid is converted by 5-lipoxygenase. From the Second Department of Internal Medicine, (H.M., A.T., Ya.K., J.S., K.I.) and Second Department of Biochemistry (Yo.K., T.S., H.K., Y.Y.), Nippon Medical School, Tokyo, Japan.

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5-lipoxygenase dehydrase to leukotrienes. Of these substances, leukotriene C₄ (LTC₄) has particularly strong vasoconstrictor activity and promotes increased vascular permeability, perhaps by damage to the blood–brain barrier.

We examined the effect of incomplete global cerebral ischemia on the levels of TXB₂, 6-keto-PGF₁α, and LTC₄ in the brain of stroke-resistant spontaneously hypertensive rats (SHRSR) and the relation between LTC₄ and the progression of cerebral edema. (2-Amino-4-tert-butyl-6-(α-hydroxy-4-chlorobenzyl)phenylhydrochloride (ONO-LP-016) and 2,3,5-trimethyl-6-(12-hydroxy-5,10-dodecanyl)-1,4-benzoquinone (AA-861) inhibit 5-lipoxygenase in vitro. We also studied the effects of these compounds on LTC₄ production and on cerebral edema to assess their relation.

Materials and Methods

A total of 95 SHRSR were investigated. Bilateral common carotid artery occlusion followed by reperfusion was performed in 74 male SHRSR (300 g) aged 12–16 weeks. In 20 rats the brain was analyzed for 6-keto-PGF₁α and TXB₂ contents. In 53 rats the relation between LTC₄ and water content was studied. In 11 rats AA-861 was administered, and seven rats were given ONO-LP-016. Four of the 11 rats receiving AA-861 were used for analysis of brain 6-keto-PGF₁α and TXB₂ contents, and the seven rats given ONO-LP-016 were used to study the relation between LTC₄ and tissue water content.

All rats were anesthetized with 2% halothane by inhalation. Their carotid arteries were exposed through a frontal midline incision of the neck and then separated...
from the vagal nerves. The bilateral common carotid artery was occluded by clipping the carotid arteries for 2 hours; circulation was then reestablished by removing the clips. At different intervals after reperfusion rats were killed by microwave application to the head (Toshiba microwave applicator, Tokyo, Japan) output of 5 kW for 1.5 seconds), which also immediately inactivated enzymes in the brain. Rats serving as controls were similarly anesthetized, and their carotid arteries were exposed; the rats were then killed by microwave irradiation 4 hours after sham operation without carotid artery occlusion. The brain, including the brainstem, was removed in a gloved chamber (temperature 37°C, relative humidity 95%), weighed, and frozen at −80°C until biochemicals were assayed.

The brains of the 20 rats in which 6-keto-PGF₁α and TXB₂ were assayed were homogenized in 4 ml 0.05 M Tris·HCl buffer (pH 7.4) with a Teflon homogenizer at 0°C and then centrifuged at 10,000g for 10 minutes. [³H]6-keto-PGF₁α or [³H]TXB₂, each with an activity of 1,000 cpm, was added to each supernatant for calculation of recovery. The supernatant extraction developed by Powell was modified as follows. The sample was diluted with 40 ml 7.5 mM acetate buffer (pH 5.9) and adjusted to pH 3.0 with 1.0N HCl and passed through a Sep-Pak C18 cartridge (Waters Assoc., Milford, Massachusetts) that was prewashed with 20 ml ethanol and 20 ml distilled water. The whole sample was passed through the cartridge, followed in turn by 20 ml distilled water, 20 ml ethanol:water (15:85), and 20 ml petroleum ether. Finally, the prostaglandins were eluted with 4 ml methanol. The prostaglandin fraction was divided into two aliquots; one was used for TXB₂ radioimmunoassay (RIA) and the other for 6-keto-PGF₁α RIA. Each aliquot was concentrated under nitrogen gas and resuspended with 200 μl assay buffer of the RIA kit.

³H kits were used for RIA of TXB₂ (cross-reactivity: TXB₂ 100%, PGD₂ 2.0%, PGE₂ 0.1%) and 6-keto-PGF₁α (cross-reactivity: 6-keto-PGF₁α 100%, PGF₂α 0.3%, PGE₂ 0.2%). For recovery calculation, 50 μl sample solution was used. Recovery of [³H]TXB₂ and [³H]6-keto-PGF₁α was 65−75% and 70−80%, respectively. Addition of a standard amount (0.2 ng) of LTC₄ to the aliquot yielded correct recovery according to the standard curve, and reliability of the RIA kits was thus confirmed. Radioactivity was counted in duplicate samples in a Hewlett-Packard Tricarb 4640 liquid scintillation counter (Palo Alto, California) with 3 ml Atomlight (New England Nuclear) counted in duplicate samples in a Hewlett-Packard Tricarb 4640 liquid scintillation counter (Palo Alto, California) with 3 ml Atomlight (New England Nuclear) that was prewashed with 20 ml ethanol, 10 ml 7.5 mM acetate buffer (pH 5.9), and 10 ml distilled water; the supernatant fluid was subsequently applied to elute hydrophilic compounds and neutral fats. LTC₄ was eluted with 4 ml methanol. The fraction was condensed under nitrogen gas and suspended with 200 μl assay buffer of the RIA kit.

After ischemia and reperfusion, the brain was removed from the skull of each of the 53 rats in which LTC₄ and LTD₄ were assayed. The rats were killed by microwave application to the head (Toshiba microwave applicator, Tokyo, Japan) output of 5 kW for 1.5 seconds), which also immediately inactivated enzymes in the brain. Rats serving as controls were similarly anesthetized, and their carotid arteries were exposed; the rats were then killed by microwave irradiation 4 hours after sham operation without carotid artery occlusion. The brain, including the brainstem, was removed in a gloved chamber (temperature 37°C, relative humidity 95%), weighed, and frozen at −80°C until biochemicals were assayed.

The brains of the 20 rats in which 6-keto-PGF₁α and TXB₂ were assayed were homogenized in 4 ml 0.05 M Tris·HCl buffer (pH 7.4) with a Teflon homogenizer at 0°C and then centrifuged at 10,000g for 10 minutes. [³H]6-keto-PGF₁α or [³H]TXB₂, each with an activity of 1,000 cpm, was added to each supernatant for calculation of recovery. The supernatant extraction developed by Powell was modified as follows. The sample was diluted with 40 ml 7.5 mM acetate buffer (pH 5.9) and adjusted to pH 3.0 with 1.0N HCl and passed through a Sep-Pak C18 cartridge (Waters Assoc., Milford, Massachusetts) that was prewashed with 20 ml ethanol, 20 ml 7.5 mM acetate buffer (pH 5.9), 10 ml ethanol:water (15:85), and 10 ml distilled water; the supernatant fluid was subsequently applied to elute hydrophilic compounds and neutral fats. LTC₄ was eluted with 4 ml methanol. The fraction was condensed under nitrogen gas and suspended with 200 μl assay buffer of the RIA kit.

³H kits were used for RIA of TXB₂ (cross-reactivity: TXB₂ 100%, PGD₂ 2.0%, PGE₂ 0.1%) and 6-keto-PGF₁α (cross-reactivity: 6-keto-PGF₁α 100%, PGF₂α 0.3%, PGE₂ 0.2%). For recovery calculation, 50 μl sample solution was used. Recovery of [³H]TXB₂ and [³H]6-keto-PGF₁α was 65−75% and 70−80%, respectively. Addition of a standard amount (0.2 ng) of LTC₄ to the aliquot yielded correct recovery according to the standard curve, and reliability of the RIA kits was thus confirmed. Radioactivity was counted in duplicate samples in a Hewlett-Packard Tricarb 4640 liquid scintillation counter (Palo Alto, California) with 3 ml Atomlight (New England Nuclear) that was prewashed with 20 ml ethanol, 10 ml 7.5 mM acetate buffer (pH 5.9), and 10 ml distilled water; the supernatant fluid was subsequently applied to elute hydrophilic compounds and neutral fats. LTC₄ was eluted with 4 ml methanol. The fraction was condensed under nitrogen gas and suspended with 150 μl buffer of the RIA kit. For RIA, 100 μl of each sample was analyzed using a RIA kit ([³H]-LTC₄, New England Nuclear). A 50-μl sample was used for recovery calculation. The recovery of [³H]LTC₄ was 65−75%. Addition of a standard amount (0.2 ng) of LTC₄ to the aliquot yielded the correct recovery according to the standard curve, thus confirming the reliability of the RIA kit. Radioactivity was counted similarly for TXB₂ and 6-keto-PGF₁α.

Content of TXB₂ and 6-keto-PGF₁α in Stroke-Resistant Spontaneously Hypertensive Rats

**FIGURE 1.** Effect of 2-hour bilateral common carotid artery occlusion on level of thromboxane B₂ (TXB₂) (●) and the prostaglandin 6-keto-PGF₁α (○) in stroke-resistant spontaneously hypertensive rats. *Significantly different (p<0.01) from sham-operated control. Data are mean ± SD.
in two doses of 1.0 ml each following the same time schedule as for ONO-LP-016. The total dose given was 200 mg/kg. In seven rats, ONO-LP-016 suspended in 0.4% Tween 80 saline and adjusted to 42 mg/ml was injected in two doses of 0.5 ml each; one dose was injected intraperitoneally just before carotid artery occlusion and the other 1 hour after the occlusion. Total dose was 70 mg/kg. After 30 minutes of reperfusion the seven rats were killed by microwave application to the head, and the brains were used to assay LTC₄ and brain water content. AA-861 and ONO-LP-016 were donated by Takeda Chemical Co., Osaka, Japan, and Ono Pharmaceutical Co., Osaka, Japan, respectively.

All results are reported as mean ± SD. Statistical analysis was assessed by analysis of variance, Ryan’s method.

Results

The TXB₂ content in brains of the control group was 111 ± 10 pg/g brain (n = 4), and the content in the 0-minute reperfusion group (at 0 minutes of reperfusion after 2 hours of bilateral common carotid artery occlusion) was 175 ± 21 pg/g (n = 3). After 5 minutes of reperfusion, the TXB₂ content was elevated significantly to 1,243 ± 251 pg/g (n = 4, p < 0.01). After 30, 60, and 120 minutes of reperfusion, the levels of TXB₂ were 132 ± 20 (n = 3), 124 ± 19 (n = 3), and 131 ± 32 pg/g (n = 3), thus showing a return to control levels (Figure 1).

The 6-keto-PGF₁α contents of the brains were 80 ± 12 pg/g brain in the control group (n = 4) and 91 ± 12 pg/g (n = 3) at the end of ischemia (0 minutes of reperfusion). The content was significantly elevated to 441 ± 120 pg/g (n = 4, p < 0.01) at 5 minutes of reperfusion and fell to 67 ± 14 pg/g (n = 3) at 30 minutes, 84 ± 5 pg/g (n = 3) at 60 minutes, and 58 ± 6 pg/g (n = 3) at 120 minutes of reperfusion (Figure 1).

The LTC₄ content in brains of the control group was 0.40 ± 0.14 ng/g (n = 17), and it was elevated to 0.90 ± 0.64 ng/g (n = 9) at the end of 2 hours of ischemia. The LTC₄ levels in the reperfused groups were 0.92 ± 0.35 ng/g (n = 4) at 5 minutes, 1.57 ± 0.63 ng/g (n = 14) at 30 minutes, and 1.26 ± 0.70 ng/g at 60 minutes (n = 6). At the end of ischemia and after 30 minutes of reperfusion LTC₄ levels were significantly elevated (p < 0.05) compared with the control. After 120 minutes of reperfusion, the LTC₄ content had decreased to 0.48 ± 0.23 ng/g (n = 7), that is, returned to normal. Obviously, peak levels of LTC₄ were observed after 30 minutes of reperfusion (Figure 2).

The brain water content in the control group was 77.16 ± 0.53% (n = 17). At the end of ischemia, the content was 77.41 ± 0.40% (n = 9). The brain water content in the reperfused group at 30 minutes (n = 14)
increased to 78.20 ± 0.90%; this increase is significant (p < 0.05) compared with the control group. The brain water content at 60 minutes was 78.00 ± 1.20% (n = 6) and at 120 minutes was 77.45 ± 0.24% (n = 7), that is, similar to the control level (Figure 3). Brain water content was not measured after 5 minutes of reperfusion.

Administration of 200 mg/kg AA-861 did not significantly suppress the rise in TXB2 and 6-keto-PGF1α contents after 5 minutes of reperfusion (Figure 4). However, 70 mg/kg ONO-LP-016 and 200 mg/kg AA-861 significantly suppressed the production of LTC4 (p < 0.05) and were associated with reduced brain water content after 30 minutes of reperfusion (not significant) (Figures 5 and 6).

Discussion

We investigated the cerebral eicosanoid levels in incomplete global cerebral ischemia. In our model of bilateral common carotid artery occlusion in SHRSR, cerebral blood flow during ischemia ranged between 0 and 40% of control.21-22 TXB2 and 6-keto-PGF1α levels were not increased during ischemia but were elevated significantly after 5 minutes and declined again after 30 minutes of reperfusion. LTC4 levels in the brain increased significantly during the 2 hours of ischemia and were further elevated after 30 minutes of reperfusion, when they reached their highest value. The levels decreased thereafter toward control levels. The water content was not significantly increased during ischemia but was increased after 30 minutes of reperfusion and decreased thereafter. The lipoxygenase inhibitors AA-861 and ONO-LP-016 significantly suppressed the LTC4 increase after reperfusion and reduced the brain water content, but the change was not significant.

It has been reported that the cyclooxygenase products TXA2 and 6-keto-PGF1α are produced endogenously in the brain.23 Furthermore, it has been reported that these cyclooxygenase products increase during reperfusion after an ischemic insult.10-12 Our results are in agreement with those results. Thus, these cyclooxygenase products accumulated after reperfusion but not during ischemia of 2 hours duration (Figure 1). Cyclooxygenase may not act under circumstances of depleted tissue oxygen. After reperfusion, abundant oxygen is presumably available in the ischemic brain and this, in conjunction with raised levels of arachidonic acid, may then accelerate cyclooxygenase activity, with resultant rapid, transient elevation of TXB2 (Figure 1). We speculate that TXA2 (as reflected by TXB2 production) is produced where platelets adhere to and aggregate on damaged endothelial cells of the cerebral vessels.

The 6-keto-PGF1α content also increased immediately and significantly after reperfusion and then decreased to control levels (Figure 1). The production of PGI2 (as measured by 6-keto-PGF1α content) may be

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**Figure 4.** Effect of AA-861 on brain levels of thromboxane B2 (TXB2) and the prostaglandin 6-keto-PGF1α in stroke-resistant spontaneously hypertensive rats at 5 minutes' reperfusion after 2-hour bilateral common carotid artery occlusion. Data are mean ± SD. n.s., not significant.

**Figure 5.** Effects of AA-861 and ONO-LP-016 on brain levels of leukotriene LTC4 in stroke-resistant spontaneously hypertensive rats at 30 minutes' reperfusion after 2-hour bilateral common carotid artery occlusion compared with sham-operated control. Data are mean ± SD. i.p., intraperitoneal injection; p.o., per os.
related to the formation of TXA₂, to prevent interruption of circulation and platelet aggregation. These ideas are consistent with the hypothesis that vascular endothelium is damaged by ischemia as a result of accumulation of arachidonic acid⁶ and that when reperfusion supplies oxygen, an acceleration of prostaglandin synthesis occurs.¹⁰⁻¹² Ischemia produced morphologic changes, such as appearance of microvilli and bulging of the endothelium,⁴⁴ on the luminal vessel surface. Reperfusion of the tissue may produce platelet adhesion and secondary aggregation. It is tempting to speculate that this and platelet adhesion and aggregation and the resultant damage to the endothelium explain the proportionally larger increase in TXB₂ than 6-keto-PGF₁α. TXA₂ may affect the deterioration of cerebral blood flow in experimental cerebral ischemia.²⁵⁻²⁶

In addition, endogenous lipoxygenase activity²⁷⁻²⁸ and leukotriene levels in the brain²⁹ have been studied. When LTC₄ is administered intravenously or on the brain surface, it does not appear in the parenchyma and produces only modest cerebral vasoconstriction and minimal disruption of the blood–brain barrier.³⁰⁻³¹ These results indicate that exogenous LTC₄ may not penetrate the blood–brain barrier or the pia-arachnoid. Leukotrienes injected into brain parenchyma induce blood–brain barrier breakdown³₂ as occurs in vasogenic cerebral edema.³³⁻³⁵ It has been reported that leukotriene levels rise in brain tissue after ischemic insults, subarachnoid hemorrhage, and concussive injury.³⁴⁻³⁵ Therefore, it is reasonable to consider leukotrienes as being causally related to the occurrence of ischemic cerebral edema.

Five minutes after reperfusion LTC₄ levels were not yet increased, and they did not rise until the cyclooxygenase products had become significantly elevated. These findings may be due to the enhanced synthesis of cyclooxygenase compounds immediately after reperfusion, which consumes arachidonic acid, thereby reducing the supply of substrate required for production of lipoxygenase compounds, such as LTC₄. Thirty minutes after reperfusion, the level of LTC₄ in the brain reached its highest value. LTC₄ concentration in the brain was elevated during the first 60 minutes of reperfusion, but at 120 minutes the LTC₄ concentration returned to control levels (Figure 2). TXB₂ levels returned to normal at 30 minutes after reperfusion, a time when brain edema was at its peak, indicating that TXA₂ may not directly participate in the induction of brain edema.

The decrease in LTC₄ concentration at 120 minutes may reflect the depletion of substrate or the washout of leukotrienes by reperfusion. Eicosanoids are believed to be catabolized at the site of production immediately after synthesis. However, blood flow may contribute to the disappearance of prostaglandins and leukotrienes. It has been shown by intraventricular injection that LTC₄ is transferred from cerebrospinal fluid to blood by an efficient transport system in the choroid plexus. The central nervous system may have similar mechanisms to prevent the accumulation of leukotrienes after an ischemic insult.³* The decrease of LTC₄ following reperfusion was more rapid in our rats than in Mongolian gerbils as reported by Moskowitz et al.³⁴ (Figure 2). This may be due to the lack of a fully functional circle of Willis and/or to a less efficient transport system for leukotrienes in the brain of Mongolian gerbils.

The brain water content increased significantly in the brain of SHRSR after 30 minutes of reperfusion. The increase in LTC₄ levels corresponded to this progression of cerebral edema (Figures 2 and 3). To confirm further the relation between LTC₄ and cerebral edema, we evaluated the effect of the 5-lipoxygenase inhibitors AA-861 and ONO-LP-016. It has been shown previously that AA-861 inhibits 5-lipoxygenase activity in peritoneal polymorphonuclear leukocytes and inhibits formation of the slow-reacting substance of anaphylaxis in guinea pigs without having any effect on cyclooxygenase activities in vitro.³⁷ AA-861 has no effect on the cardiovascular system (unpublished data). As predicted, AA-861 and ONO-LP-016 suppressed LTC₄ production (Figure 5) and also seemed to reduce cerebral edema, but not significantly (Figure 6). Our results support the likelihood that increased endogenous brain LTC₄ after carotid occlusion and reperfusion is the immediate stimulus to provoke ischemic cerebral edema. However, it cannot be excluded that AA-861 also slightly suppressed the synthesis of the cyclooxygenase products TXB₂ and 6-keto-PGF₁α after reperfusion (Figure 4). Our results suggest that the reduction of LTC₄ and brain edema with AA-861 administration may have been primarily through amelioration of the
effects on cerebral blood flow, with secondary reduction of TXB₂, and 6-keto-PGF₁α.

We speculate that endogenous production of LTC₄ in the brain after an ischemic insult correlates more closely with brain edema formation than with production of prostaglandins such as TXA₂.

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References


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