Development of cyclooxygenase and lipoxygenase metabolites of arachidonic acid after transient cerebral ischemia

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Vasoactive arachidonic acid metabolites are postulated to play a role in the pathogenesis of cerebral ischemia. In order to characterize the local generation of cyclooxygenase and lipoxygenase metabolites of arachidonic acid in transient ischemia with reperfusion, Mongolian gerbils were studied for regional cerebral blood flow (CBF), using the hydrogen clearance technique, and for cerebral levels of the thromboxane metabolite TXB2, and prostaglandins 6-keto-PGF1α and PGE2, as well as the leukotriene LTB4. The gerbils were anesthetized with pentobarbital, and half of the animals were pretreated with the cyclooxygenase inhibitor indomethacin. All received 10 or 20 minutes of dense forebrain ischemia followed by reperfusion of 10 minutes, 50 minutes, or 100 minutes. A separate control group received no ischemic lesion.

Regional CBF decreased significantly from 23.7 ± 2.6 to 4.3 ± 1.7 cc/100 gm/min during ischemia (p < 0.01). Reperfusion resulted in initially normal flows (22.5 ± 5.1 cc/100 gm/min) followed by a progressive hypoperfusion (11.3 ± 2.7 cc/100 gm/min). All metabolites showed parallel significant (p < 0.05) increases after transient ischemia and reperfusion compared to baseline levels (values (in pg/mg protein) were: TXB2 45.5 ± 7.1 vs 23.3 ± 3.6; 6-keto-PGF1α 262.8 ± 47.9 vs 175.8 ± 26.8; PGE2 256.5 ± 35.6 vs 112.5 ± 11.2; and LTB4 37.8 ± 4.6 vs 24.6 ± 6). These levels were all significantly decreased (p < 0.05) by pretreatment with indomethacin except for the leukotriene LTB4, which was increased. Transient cerebral ischemia results in a reperfusion abnormality and the local generation of cyclooxygenase products, which are reduced by pretreatment with indomethacin; however, cyclooxygenase inhibition may result in increased substrate availability for the lipoxygenase system. Studies of such an interaction may lead to new understandings of the pharmacological modification of detrimental vascular changes after transient cerebral ischemia.

Key Words • prostaglandins • thromboxanes • leukotrienes • transient cerebral ischemia • cerebral blood flow • gerbil

Arachidonic acid metabolites derived from both the cyclooxygenase and lipoxygenase pathways are of theoretical importance in the development of infarction after cerebral ischemia. Ischemia stimulates the release of arachidonic acid from membrane phospholipids. The resulting arachidonate can be metabolized by either cyclooxygenase or lipoxygenase enzyme systems to substances of theoretical vasoactive activity.

Cyclooxygenase enzymes catalyze the formation of the unstable cyclic endoperoxides prostaglandin (PG) G2 and PGH2, leading to the generation of prostanoids including the vasoactive thromboxane (TX) A2 and prostacyclin. Thromboxane A2 is a potent vasoconstrictor and platelet aggregator that may limit reperfusion after ischemia and lead to further infarction. Various agents, including aspirin and indomethacin, are thought to exert beneficial effects in situations of cerebral ischemia, presumably as a result of their action on the cyclooxygenase pathway of arachidonic metabolism.

The lipoxygenase pathway of arachidonate metabolism results in the formation of the leukotrienes. These have vasoactive roles in other organs; specifically, leukotriene (LT) B is known to inhibit formation of the vasodilator prostacyclin, and LTC promotes capillary permeability and edema formation in the lung. In light of their theoretical effects, we chose to examine ischemic brain for these metabolites as well as their possible interaction with cyclooxygenase products.
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Arachidonic acid metabolism is stimulated by ischemia, but in both cyclooxygenase and lipoxygenase pathways oxygen is required, and activated blood components (primarily platelets) play a role. It could be predicted that in complete ischemia, therefore, such metabolites are not formed, but with reperfusion an increase in vasoactive metabolites may be seen. The appearance of these metabolites, especially leukotrienes, after specifically cerebral ischemia has not been documented. Similarly, the conditions leading to the stimulation of each pathway as well as the temporal relationship between the generation of cyclooxygenase and lipoxygenase products has not been studied.

This study was undertaken to test the hypotheses that the generation of both vasoactive cyclooxygenase and lipoxygenase arachidonate metabolites is initiated by transient cerebral ischemia, and that the production of these metabolites would differ by enzyme system with the depth and duration of ischemia as well as the duration and completeness of reperfusion. The production of the cyclooxygenase products 6-keto-PGF₁₀, TXB₂, and PGE₂ was measured in the Mongolian gerbil after conditions of transient total forebrain ischemia followed by reperfusion. This was contrasted with the production of the immunoreactive leukotriene LTB₄ after similar ischemic insults. The effect of the cyclooxygenase inhibitor indomethacin on the generation of these two classes of metabolites was also studied.

Materials and Methods

Transient cerebral ischemia was produced by bilateral carotid artery occlusion in the Mongolian gerbil. It is a well characterized model of cerebral ischemia in an animal lacking a complete circle of Willis or significant anastomoses between the anterior and posterior cerebral circulation.

Cerebral Blood Flow

To confirm the presence of transient cerebral ischemia with bilateral carotid artery occlusion, regional cerebral blood flow (rCBF) was measured by the H₂ clearance technique. The areas rendered ischemic were studied for arachidonate metabolite levels. Animals were anesthetized with 40 mg/kg intraperitoneal pentobarbital. They were placed in a Kopf stereotaxic frame and, with the aid of a Zeiss surgical microscope, four microcraniectomies large enough to accept an H₂ clearance electrode were made using a high-speed drill. These were placed bilaterally in reference to the bregma: 3 mm anterior, 2 mm lateral, and 3 mm posterior, 3 mm lateral. With the aid of a micromanipulator, a 25-μ platinum/iridium (90/10) electrode was placed at each site and secured in place with Coeflex dental impression material which sealed the microcraniectomy. Each electrode was insulated with Teflon except for the terminal 0.5 mm, which was placed within the cortex.

After the electrodes were secured, a tracheostomy was made, and both common carotid arteries were isolated in the neck. The left femoral artery was cannulated for measurement of blood pressure and arterial blood gases. An Ag/AgCl reference electrode was placed subcutaneously.

Electrodes were polarized to +200 mV and H₂ saturation was accomplished by spontaneous respiration of room air, through which a stream of H₂ gas was passed. Electrode outputs were amplified using a device similar to that described by Pastzor, et al. Following the abrupt cessation of H₂ administration, the amplified outputs were monitored during desaturation using a computer-directed scanning voltmeter.* Blood flows were calculated from the curves relating the log of the H₂ concentration and time using linear regression.

Data from an individual electrode were accepted if the electrode output changed with H₂ saturation and if initial rCBF was greater than 15 cc/100 gm prior to any manipulation.

In 10 animals, two baseline rCBF measurements were taken then both carotid arteries were occluded for 10 minutes, during which time rCBF was measured to confirm the presence of profound ischemia within the area of brain measured. This was followed by release of the carotid artery clamps and 50 minutes of reperfusion, with rCBF measured at 20 and 50 minutes to confirm reperfusion. Blood flow during carotid artery occlusion was compared to initial flows at the same site by analysis of variance. At the end of this period, arterial blood gases were measured and the animals were sacrificed.

Arachidonic Acid Metabolites

In a separate protocol, 88 animals were studied for changes in tissue levels of arachidonic acid metabolites in areas of the brain shown to be rendered transiently ischemic by the previous rCBF studies. This avoided potential stimulation of arachidonic metabolism by the insertion of H₂ electrodes.

Short Ischemia Period. The first 48 animals were studied for results of a short duration of ischemia. These animals were divided into three groups. The first group was anesthetized with pentobarbital (40 mg/kg) and maintained as a control group with no carotid artery occlusion. The second group was anesthetized with pentobarbital. After a tracheostomy and bilateral isolation of the common carotid arteries, the vessels were clamped for 10 minutes and reperfused for 50 minutes. The third group was anesthetized with pentobarbital and pretreated with intraperitoneal indomethacin (4 mg/kg) prior to bilateral carotid artery occlusion for 10 minutes and reperfusion for 50 minutes.

At the conclusion of each experiment, the animal was sacrificed by decapitation and the brain was rapidly removed. The forebrain area corresponding to the re-

* Data acquisition control unit, Model 3497A, manufactured by Hewlett-Packard Co., 1501 Page Mill Road, Palo Alto, California.
region confirmed to be ischemic by the earlier rCBF studies of bilateral carotid occlusion was excised and rapidly frozen at −80°C.

The rapidly frozen brain specimens were homogenized in citrate buffer, extracted with ether, and dried under N₂. The residue was dissolved in ethylacetate and benzene and applied to a silica acid column packed in 40% ethylacetate in benzene. It was eluted with ethylacetate/benzene/methanol and dried under a stream of N₂. This eluent was studied by radioimmunoassay for the prostaglandin PGE₂, the prostacyclin derivative 6-keto-PGF₁α, the thromboxane TXB₂, and the leukotriene derivative LTB₄. Arachidonate metabolite levels were compared statistically using an analysis of variance with significance accepted at p < 0.05.

**Longer Ischemia Period.** The remaining 40 gerbils were studied for tissue levels of immunoreactive arachidonate metabolites after a longer duration of ischemia. All animals were prepared and anesthetized as in the short ischemia groups. In this protocol, however, bilateral carotid artery occlusion was maintained for 20 minutes, followed by reperfusion of 10 minutes or 100 minutes. Animals were sacrificed at the end of these reperfusion times and brain was sampled for arachidonate metabolites as detailed above. Equal groups of indomethacin-pretreated animals (4 mg/kg intraperitoneally) were studied after identical reperfusion times.

**Control Group.** A separate control group of animals was anesthetized and underwent tracheostomy and neck dissection with anesthesia maintained for the full period of the experiment without carotid artery occlusion. As the protocols differed in duration of anesthesia, which could affect metabolite formation, separate control groups were therefore studied for each protocol.

**Results**

**Regional Cerebral Blood Flow**

In the 10 gerbils in which baseline rCBF was recorded bilaterally, initial rCBF's were very similar at both anterior and posterior electrode positions (23.7 ± 2.6 vs 24.9 ± 1.7 cc/100 gm/min: no significant difference) (Fig. 1). Bilateral carotid artery occlusion reliably created a condition of forebrain ischemia at both anterior (4.3 ± 1.4 cc/100 gm/min) and posterior (4.6 ± 0.9 cc/100 gm/min) electrode positions. Occlusion caused a significant (p < 0.01) decrease in all zones measured. This cerebral ischemia was relieved by release of both carotid artery clamps after 10 minutes of ischemia. An early increase in flow was seen at 20 minutes of reperfusion (22.5 ± 5.1 cc/100 gm/min), followed by an eventual significant compromise of reperfusion (11.3 ± 2.7 cc/100 gm/min at 50 minutes of reperfusion: p < 0.01) (Fig. 1). Mean pCO₂ at the end of the experiment was 34.2 ± 3.4 mm Hg.

**Prostaglandin Tissue Levels**

Anterior cerebral hemisphere tissue samples were taken from the 88 gerbils studied for arachidonic acid metabolite levels. Tissue was taken at the level where the anterior rCBF recordings were made in the above study. The rCBF recordings confirmed that tissue samples were taken from regions subjected to dense ischemia, and not from marginally ischemic “penumbra” areas. Tissue prostaglandin levels of the prostanooids TXB₂, PGE₂, 6-keto-PGF₁α, and the leukotriene LTB₄ were studied in two protocols.

In the short-ischemia protocol, 10 minutes of ischemia followed by 50 minutes of reperfusion (in animals receiving no indomethacin) elicited significant increases in all three prostanoid metabolites as compared to control levels (p < 0.05) (Fig. 2). Prostacyclin metabolite 6-keto-PGF₁α increased 220% over control values after ischemia and reperfusion to 486.9 ± 59 pg/mg protein; PGE₂ increased 320% over control levels to 227.6 ± 13.1 pg/mg protein. The thromboxane metabolite TXB₂ increased 420% over control values for a maximum value of 21.4 ± 1.7 pg/mg protein. After a short period of ischemia and reperfusion, the levels of all three prostanoid metabolites were significantly decreased by pretreatment with indomethacin as compared to the untreated animals that underwent ischemia, as follows (in pg/mg protein): TXB₂ 11.8 ± 2.4 (p < 0.05), 6-keto-PGF₁α 343.5 ± 84.8 (p < 0.05), PGE₂ 128.6 ± 28.3 (p < 0.05) (Fig. 2).

The leukotriene metabolite LTB₄ was increased in these animals after 10 minutes of ischemia and 50 minutes of reperfusion (46.7 ± 10.9 vs control 35 ± 7.82 pg/mg protein), and was further increased by the cyclooxygenase inhibitor indomethacin, but this difference was not statistically significant (Fig. 2).

This difference between prostanoid and leukotriene metabolites could be secondary to either an insufficient ischemic insult or a transient peak of the metabolite that would be missed in this protocol. The second protocol, therefore, sampled tissue from the same brain region, but after a longer ischemic insult (20 minutes)
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and with two periods of reperfusion (10 and 100 minutes). In this protocol all metabolites were markedly increased as compared to non-occluded controls after 10 minutes of reperfusion (Fig. 3): 6-keto-PGF_{1α} increased 149% to 262.8 ± 47.9 pg/mg protein; PGE_{2} increased 228% to 256.5 ± 35.6 pg/mg protein; the thromboxane TXB_{2} increased 195% to 45.9 ± 7.1 pg/mg protein; and the leukotriene derivative LTB_{4} increased 153% to 37.8 ± 4.6 pg/mg protein. All changes were significant to p < 0.05. Further reperfusion resulted in the return of all metabolites to control levels by 100 minutes of reperfusion.

Indomethacin pretreatment had a profound effect on the generation of arachidonate metabolites within this protocol. The prostanoid products of the cyclooxygenase system were all significantly (p < 0.05) decreased at each reperfusion time point after 20 minutes of ischemia when compared to animals rendered ischemic without indomethacin pretreatment (Fig. 3). In this study, reduction below control levels was noted, suggesting that an endogenous level of production of prostanoids may exist in cerebral tissue which can be decreased by indomethacin and increased by transient ischemia.

Indomethacin inhibition of the cyclooxygenase metabolism of arachidonate would theoretically allow greater substrate availability to the lipoxygenase enzyme system. In this protocol, indomethacin pretreatment resulted in a further increase in LTB_{4} after 10 minutes
of reperfusion to 42.2 ± 6 pg/mg protein, but this increase did not reach significance (Fig. 3D).

Discussion

In this study, a controlled method of producing cerebral hemisphere ischemia was used to render an area of cerebral hemorrhage densely ischemic (mean rCBF less than 5 cc/100 gm/min) and then permit reperfusion. Tissue samples taken from the area of ischemia after 10 and 50 minutes of reperfusion showed a marked increase in the levels of the cyclooxygenase products of arachidonic acid metabolism: 6-keto-PGF₁α, PGE₂, and TXB₂ as compared with control. Leukotriene LTB₄ was also increased after 10 minutes of reperfusion.

After release of bilateral carotid artery occlusion, CBF returned to near baseline levels, but, with time after transient ischemia, a reperfusion abnormality with decreased flow was noted (Fig. 1). This is consistent with a postulated detrimental effect of vasoactive arachidonate products. In transient dense ischemia, these metabolites would appear after resumption of flow, and delivery of oxygen to the damaged area allowed enzymatic metabolism of arachidonate.10,16

The process by which cerebral ischemia proceeds to infarction remains a problem of clinical and experimental importance. Dietary free fatty acids, membrane arachidonic acid, and their derivatives have been implicated in the vascular events after cerebral ischemia and are thought to have a possible role in protection from infarction.3,47 Dietary manipulation of free fatty acids affects the synthesis of certain arachidonic acid metabolites.8,20,39 Cerebral vasospasm after subarachnoid hemorrhage is also modified by arachidonic acid metabolites.4,12,43 The exact contribution of arachidonic metabolites to the pathophysiology of cerebral infarction remains unclear. Thromboxane A₂, a vasoconstrictor, is thought to play a role, but its interaction with other factors or opposite effect is not well established.38 Increases in its stable metabolite TXB₂ have been noted by Gaudet, et al.,13,15 after carotid occlusion, but the degree of regional cerebral ischemia was not measured in these studies. Interestingly, Gaudet, et al., noted an increase in the metabolites only if the ischemic area was reperfused prior to sacrifice. This correlates with the findings of Crockard, et al.,7 that more ischemic edema was seen after partial ischemia below a threshold level than after total ischemia.

The present studies agree with previous observations which indicate that oxygen availability (reperfusion) is essential for the reported increases in arachidonic acid metabolites.3,40 Our findings also expand on previous observations by demonstrating the presence of leukotrienes after ischemia with reperfusion. Similarly, platelets and activated blood components are critical for the production of increased TXA₂ and LTB₄ after ischemia, further emphasizing that the contribution of arachidonate products to infarction may best be seen after incomplete or transient ischemia.29 Measuring the depth and duration of cerebral ischemia at the brain region sampled for arachidonic metabolites would then be of importance in determining their contribution to the development of infarction. In this study the increase in LTB₄ was noted only after early reperfusion in the long-ischemia protocol. The threshold duration of ischemia for the development of prostanoid metabolites appears to be shorter than that of immunoreactive LTB₄.

Hydrogen clearance studies of rCBF were used in this study to confirm the presence of dense ischemia (less than 5 cc/100 gm/min) in the area sampled. Furthermore, comparison of data from the anterior and posterior electrodes (Fig. 1) shows that the densely ischemic areas extended at least as far as 3 mm posterior to the bregma without significant posterior collateral circulation. As the prostaglandin tissue samples were all taken anterior to this electrode site, no area of "penumbra" or partial ischemia would be included.

In this study, indomethacin significantly decreased the production of 6-keto-PGF₁α, PGE₂, and TXB₂ after transient ischemia (Figs. 2 and 3). Indomethacin has been the subject of other investigations attempting to modify the pathophysiology of both focal and experimental ischemia.18,44 Effects on edema formation and adenylyl cyclase activity have shown indirect evidence for the activity of prostaglandins after brain ischemia.18,31,44 Other studies, however, did not confirm that indomethacin affected either peripheral edema after trauma or central nervous system edema after ischemia. These observations suggest either the involvement of other factors or that sufficient ischemia to initiate the metabolism of arachidonate by cyclooxygenase had not been reached in those experimental models.5,14

In this experiment, the tendency for indomethacin to increase the early reperfusion peak in LTB₄ suggests that blockage of the cyclooxygenase system may allow greater substrate availability to the lipoxygenase system. The action of leukotrienes could then partially explain the limitation in the clinical usefulness of indomethacin after ischemia.

The leukotriene derivatives of lipoxygenase metabolism of arachidonate have vasoactive components and a possible inhibitory effect on the cyclooxygenase system.1,11,12,32,37 Leukotrienes are also affected by calcium and glucocorticoids, suggesting a possible explanation of how dexamethasone helps improve gerbil survival after partial ischemia.19,24,42,45

Other factors may interact with arachidonate metabolism and affect outcome after transient cerebral ischemia. Polyamines, due to their effect on cerebral edema, may be another mediator of cerebral dysfunction after injury.26 Further studies of these as well as the kallikrein-kinin system, opiate antagonists, and the effect of hyperglycemia may increase our understanding of the complex pathophysiologic process involved in the progression of cerebral ischemia to infarction.9,28,35,36,41,46

The present study demonstrates a strong link between transient dense ischemia and the local generation of
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both cyclooxygenase and lipoygenase products. The enzyme systems leading to these metabolites are linked by a common substrate, but differ in the degree of ischemia needed to stimulate each and their response to indomethacin. As described above, this appears to be but one factor in the progression of reperfusion abnormalities leading to eventual infarction. It is hoped that further investigations of the interaction of these various systems that are stimulated by partial ischemia will lead to effective methods of limiting the adverse outcome of transient or incomplete cerebral ischemia.

References


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