Inducible Cyclooxygenase Expression in Canine Basilar Artery After Experimental Subarachnoid Hemorrhage

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Background and Purpose—Inducible cyclooxygenase (COX-2) has been found to play a pathological role in cerebral insult. We investigated the expression of COX-2 in the basilar artery after experimental subarachnoid hemorrhage (SAH).

Methods—In a canine ‘‘two-hemorrhage’’ model of SAH, the basilar arteries were obtained on day 2 after a cisternal injection of autologous blood or on days 4, 6, 7, or 9 after the second injection. Basilar arteries also were obtained 12 hours after intracisternal injection a cytokine: interleukin (IL)-1β (0.03 μg), IL-6 (3 μg), or IL-8 (10 μg). Western blotting with a polyclonal anti–COX-2 antibody was performed in these arteries.

Results—COX-2 protein was not demonstrated in the basilar artery in control animals without SAH. However, it was expressed in the basilar artery on days 2, 4, 6, and 7 after blood injection but not on day 9. Intracisternal injection of IL-1β, IL-6, or IL-8 also induced COX-2 in the basilar artery.

Conclusions—COX-2 expression was detected in basilar arterial tissue in both acute and chronic stages after SAH. Elevation of inflammatory cytokines after SAH may be involved in the induction of COX-2, which may produce sufficient quantities of eicosanoids to affect hemodynamics after SAH. (Stroke. 1998;29:1219-1222.)

Key Words: cytokines ■ prostaglandin-endoperoxide synthase ■ subarachnoid hemorrhage

Prostaglandins function diversely as autocrine and paracrine hormones, mediating many cellular and intercellular processes. COX is a rate-limiting enzyme in prostandoid synthesis. Two isoforms have been identified: COX-1, expressed constitutively in many tissues,1 and COX-2, which is induced in response to substances including inflammatory cytokines, endotoxin, and growth factors.2–4 SAH has been reported to elevate concentrations of inflammatory cytokines, which mediate intense inflammatory and immune responses.5–7 Excessive quantities of PGs induced by COX-2 may contribute to pathophysiological processes following SAH.

In the present study, we confirmed induction of COX-2 in the canine basilar artery in an experimental model of SAH and found that COX-2 is also induced by increased concentrations of inflammatory cytokines in the CSF.

Materials and Methods
Experiments were carried out in accordance with the guidelines for the care and use of animals in the physiological sciences as approved by the Physiological Society of Japan.

Mature mongrel dogs of either sex, weighing 8 to 14 kg, were used. All procedures were performed under general anesthesia with ketamine hydrochloride (10 mg/kg IM) and pentobarbital sodium (20 mg/kg IV). Respiration was spontaneous, via an endotracheal tube.

Experimental SAH Model
A two-hemorrhage model of SAH described in previous papers9,10 was used for this experiment. After a pre-SAH angiogram, the cisterna magna was punctured with a 22-gauge spinal needle, and 5 mL CSF was withdrawn. An equal volume of fresh autologous blood then was injected into the cisterna magna, and the animal was kept in a head-down position for 30 minutes to ensure that the blood had contact with the basilar artery. A second injection was given on day 3 following the first injection. After angiograms were performed on days 2, 4, 6, 7, and 9, the dogs were exsanguinated via the carotid artery in a head-down position for 30 minutes to ensure that the blood had contact with the basilar artery. A second injection was given on day 3 following the first injection. After angiograms were performed on days 2, 4, 6, 7, and 9, the dogs were exsanguinated via the carotid and femoral arteries and perfused with 500 mL normal saline. The basilar artery was removed carefully together with the brain. After the removal of clots, pieces of arachnoid membrane, and connective tissue, the basilar artery was frozen quickly in liquid nitrogen and stored at −80°C for later use. Tissues from 3 animals per time point were pooled.

Cytokine Injection Model
After the cisterna magna was punctured with a 22-gauge spinal needle, 1.5 mL CSF was withdrawn. IL-1β (0.03 μg), IL-6 (3 μg), or IL-8 (10 μg) was dissolved in a similar volume of normal saline just before use. The doses of each of the cytokines were determined by clinical observations that the concentrations of IL-1β, IL-6, and IL-8 in CSF about 6 hours after the onset of SAH were 4.3 ± 1.6, 1269 ± 421, and 7094 ± 2714 pg/mL, respectively (mean ± SE, n = 6). In preliminary testing, IL-1β (0.03 μg), IL-6 (3 μg), or IL-8 (10 μg) injected into the canine cisterna magna resulted in concentrations higher than the clinical ones even 12 hours after cytokine injection (data not shown). Therefore, this model overapproximated the circumstances of the acute stage of human SAH. Each cytokine was injected gently through a spinal needle. Canine basilar arteries were obtained 12 hours after intracisternal injection and stored frozen in
the manner of arteries after experimental SAH. Tissues from 3 animals were pooled for each cytokine.

**Western Blot Analysis**

The basilar arteries were minced and homogenized in electrophoresis sample buffer (30 µL/mg tissue; 2% SDS, 10% glycerol, 0.1% bromophenol blue, 2% mercaptoethanol, and 50 mmol/L Tris-HCl, pH 7.2). After sonication, solubilized proteins were subjected to SDS–polyacrylamide gel electrophoresis (10% acrylamide, 1.0-mm-thick slab gels). Proteins then were transferred to a polyvinylidene difluoride membrane that was incubated with rabbit polyclonal COX-2 antibody (1:500 dilution) for 45 minutes. After washing, the membrane was incubated for 30 minutes with donkey anti-rabbit IgG conjugated to horseradish peroxidase (1:1000). Peroxidase activity was visualized with an enhanced chemiluminescence Western blotting detection system (Amersham). Western blotting was performed twice on each pooled sample.

**Sources of Material**

IL-1β, IL-6, and IL-8 (recombinant human) were purchased from Genzyme. The rabbit polyclonal antibody against the C-terminal fragment of human COX-2 was obtained from Oxford Biomedical Research; the antibody does not cross-react with constitutive COX-1. All other chemicals were reagent grade or the best grade commercially available.

**Results**

**Angiography and Gross Inspection in the SAH Model**

Vertebral angiography demonstrated that the first injection of blood produced mild vasospasm (angiography on day 2), and a second injection of blood resulted in severe vasospasm in each case (angiography on days 4, 6, 7, and 9), as shown in a previous report.9,10 A typical angiogram on day 6 is shown in Figure 1. At basilar artery removal, all injected dogs demonstrated dense blood clots encasing the basilar artery and the ventral surface of the brain stem.

**Western Blot Analysis**

Western blot analysis using a specific antibody against the C-terminal fragment of human COX-2 showed that a distinct band corresponding in molecular size to the COX-2 protein was apparent in basilar artery tissue after SAH on days 2, 4, 6, and 7. Such a band was not detected in nontreated control basilar artery (Figure 2). On day 9 expression of COX-2 in the basilar artery was no longer observed.

Intracisternal injection of cytokines (0.03 µg IL-1β, 3 µg IL-6, or 10 µg IL-8) also induced COX-2–like protein in the basilar artery (Figure 3). COX-2–like protein showed doublet bands in which the protein corresponding to the lower weight is supposed to be a proteolytic digest of that of a higher one.11

**Discussion**

COX-2 is among the immediate early genes inducible by inflammation within the CNS. COX-2 mRNA and protein are induced to a remarkable extent by focal cerebral ischemia12,13 and are also induced by seizures or synaptic activity.14 Together with free radicals, the enzyme products, prostanooids, are believed to be involved in the deterioration of postischemic brain and signaling pathways after seizures. Recently, constitutive expression of COX-2 at low levels has been demonstrated, but its role in the brain has not totally been elucidated.14–17 COX-2 is detectable primarily in the cortex, hippocampus, and amygdala in normal brain and may be involved in polymodal sensory integration and in generation of the autonomic, endocrine, and behavioral responses.14–17 No constitutive expression of COX-2 is detected in...
normal glial or vascular endothelial cells in the CNS, the latter finding being consistent with our results.

Various physiological activities of PGs have been reported in the CNS, such as in wake-sleep cycles, febrile responses, and nociception. Following SAH, levels of arachidonic acid metabolites are elevated in the CNS. These prostanoids contribute to the control of cerebrovascular tone and regulation of cerebral blood flow in the normal physiological state. Pathological stimulation of the eicosanoid metabolite cascade may contribute to the development of vasospasm after SAH.

Inflammatory cytokines, such as IL-1, IL-6, and IL-8, have been reported to be induced in the CSF beginning in the acute stage after SAH. Many lines of evidence have confirmed that these cytokines have effects mediated via stimulation of the prostanoic cascade within the CNS. IL-6 induces fever through formation of prostanoids in rats and stimulates production of PGE2 in the rat hypothalamus and cerebral arteries. We also demonstrated that the concentration of PGE2 in CSF becomes elevated over preinjection concentrations by 4.5 hours after intracisternal injection of IL-6 (data not shown), in accordance with the findings of Dinarello et al. The effect of IL-1β on the production of eicosanoids by endothelial cells is more potent than that of IL-6. IL-1β dilates the canine basilar artery as a result of the COX-2 induction, but not via the formation of nitric oxide. IL-8 has been shown to stimulate human aortic smooth muscle cells to produce PGE2. Central administration of IL-8 induces fever in rabbits via COX products. From our data, IL-1β, IL-6, and IL-8 all induced a COX-2–like protein in canine basilar artery within 12 hours. COX-2 activates the COX cascade, resulting in production of all prostanoids (PGE2, prostacyclin, and thromboxane A2). The effects of increased COX activity appear to result from differential rates of synthesis of these products, which may be involved in many pathophysiological effects beginning in the acute stage after SAH.

In conclusion, we provide indirect evidence that inflammatory cytokines immediately induced by SAH may be responsible for expression of the COX-2 in the canine basilar artery from the acute stage. Expression of COX-2 may contribute to the elevation of eicosanoids in CSF, which participate in the development of pathological hemodynamics after SAH. Selective blockade of COX-2 recently has been accomplished and may represent a novel way to keep cerebral circulation intact after SAH.

References

COX-2 Expression After SAH

Cyclooxygenase is a membrane-bound, bifunctional enzyme (molecular weight, approximately 70 kDa) that catalyzes the conversion of arachidonic acid to PGG2 by cyclooxygenase action and to PGH2 by peroxidase activity. Thus, COX is an important rate-limiting step in the production of biologically active prostanooids. Two isoforms of COX (1 and 2) have been identified and cloned. COX-1 is constitutively expressed in most tissues and COX-2 is usually thought only to be the inducible form in most tissues, but recent evidence indicates that COX-2 is constitutively expressed in brain and cerebral blood vessels from a variety of species. Induction of COX-2 levels and prostanoid synthetic capacity in large cerebral arteries. Similarly, ischemia increases COX-2 levels and prostanoid synthetic capacity in large cerebral arteries.9

In the accompanying article, the authors report that COX-2 levels increased in dog basilar artery by 4 to 7 days after cisternal injection of autologous blood. Although the mechanism involved in increased COX-2 levels is not known with certainty, one potential candidate could be inflammatory cytokines, such as interleukins. However, many other agents and/or events associated with the presence of subarachnoid blood could also promote increased synthesis of COX-2. The role of increased basilar artery levels of COX-2 in vasospasm is unclear at this time. COX-derived prostanoids or superoxide anion have been reported to promote dilation or constriction in the cerebral circulation. Thus, increased COX-2 levels might counteract or promote vasospasm after subarachnoid hemorrhage. However, further research is needed in this area.

Editorial Comment

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