Expression of Cyclooxygenase-2 mRNA After Global Ischemia Is Regulated by AMPA Receptors and Glucocorticoids

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Background and Purpose—Cyclooxygenase-2 (COX-2) is implicated in ischemic neuronal death. In focal ischemia, its mRNA induction is mediated through N-methyl-D-aspartic acid (NMDA) receptors and phospholipase A2. Because mechanisms of neuronal death involving COX-2 in global ischemia are unclear, we studied the time course and regulation of COX-2 expression in rat brain global ischemia.

Methods—Global ischemia was induced by a 4-vessel occlusion method. COX-2 mRNA levels were demonstrated with in situ hybridization and COX-2 protein with immunocytochemistry. Several animals were pretreated with MK-801, an NMDA receptor antagonist; 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline (NBQX), an α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonist; and dexamethasone.

Results—In the cortex, the CA3 hippocampal region and dentate gyrus expression of COX-2 mRNA peaked at 4 to 8 hours, while in the CA1 region COX-2 mRNA levels were high at 4 to 24 hours. COX-2 protein was induced in the corresponding regions at 12 to 24 hours, but in the CA1 neurons the protein was still seen at 3 days. COX-2 mRNA induction in the cortex was inhibited by NBQX and dexamethasone and in CA1 neurons was inhibited by NBQX. MK-801 did not suppress COX-2 induction.

Conclusions—COX-2 is differentially induced in the cortex and hippocampal structures after global ischemia. The prolonged COX-2 expression in the vulnerable CA1 neurons is regulated by AMPA receptors, suggesting that COX-2 expression is likely to be associated with AMPA receptor–mediated neuronal death in global ischemia. Glucocorticoids may not be efficiently used to inhibit ischemia-induced COX-2 expression in the hippocampus. (Stroke. 1999;30:1900-1906.)

Key Words: free radicals • gene expression • hippocampus • prostaglandins • rats

Cyclooxygenase-2 (COX-2) is the inducible form of the 2 prostaglandin-synthesizing enzymes and is exclusively present in excitatory neurons, such as glutamatergic cells in the normal brain.1–4 Previous studies indicate that prostaglandins have several functions that are potentially protective in brain ischemia. For example, prostanoids produce dilatation of cerebral arteries,5–7 which could increase cerebral blood flow in ischemic tissue. In cultured microglia, prostaglandin E2 has been reported to inhibit expression of inducible nitric oxide synthase (iNOS)8 and production of interleukin-1β,9 2 key mediators of inflammation. In addition, prostaglandins protect cultured cortical neurons against glutamate toxicity.10 However, COX-2 is believed to play a negative role in brain injury, including ischemia, because inflammatory cytokines cause a rapid induction of COX-2,11,12 COX enzymes generate superoxide and cause inflammation,1,2 and, most importantly, COX inhibitors reduce experimental brain edema13,14 and reduce brain damage after global15–17 and focal brain ischemia.18,19 Recently, COX-2 gene expression has been shown to be induced in spreading depression and focal brain ischemia by activation of N-methyl-D-aspartic acid (NMDA) receptors and phospholipase A2 (PLA2).20 In focal ischemia COX-2 expression is long lasting, and specific COX-2 inhibitors still reduce ischemic damage when administered 6 hours after the insult.19 Interestingly, nitric oxide produced by iNOS has been found to positively regulate COX-2 activity in focal brain ischemia.21 Because both the time course and regulation of COX-2 expression are important for pharmacological interventions designed to inhibit COX-2, we decided to determine the expression of COX-2 mRNA and protein at different time points in the rat model of global brain ischemia. We also studied whether antagonists of NMDA and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and glucocorticoids suppress COX-2 expression in this model.

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Materials and Methods

Animals

Global ischemia was induced by 4-vessel occlusion according to the method of Pulsinelli and Brierley (1979). Male Wistar rats were prepared for global ischemia under 1% halothane anesthesia in a 30% O₂/70% N₂O mixture by electrocauterizing the vertebral arteries bilaterally. On the following day, the rats were reanesthetized and the common carotid arteries were exposed bilaterally. The anesthesia was discontinued, and forebrain ischemia was induced by occluding the common carotid arteries with microvascular clips for 20 minutes. Arterial blood pressure and blood gases were recorded from the femoral artery. Body temperature was maintained at 37°C with a heating blanket. Five animals were not subjected to ischemia and served as controls. Four and 12 hours and 1, 3, and 7 days after ischemia, the animals were killed by decapitation or by paraformaldehyde (4%) perfusion, and the tissues were processed for in situ hybridization or immunocytochemistry.

Drug Treatments

MK-801 hydrogen maleate (RBI; 3 mg/kg IP) was given 30 minutes before ischemia; dexamethasone sodium phosphate (MSD; 3×3 mg/kg IP) was given 30 minutes before and 1 and 4 hours after ischemia; and 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline (NBQX) (Tocris Cookson; 2×30 mg/kg IP) was given 20 minutes before and 90 minutes after ischemia. The doses selected were previously described as efficient.3,20,24,26,39

In Situ Hybridization

An oligonucleotide complementary to the rat coding sequence 5' TCATCACA-CTGCCCTAATTCAGCCTCTCATCTGCAAATTA-3' was 3'-end-labeled with ³⁵S-dATP and used for an overnight hybridization at 42°C. The specificity of the oligonucleotide probe has been shown in Northern blotting in rat brain ischemia. An oligonucleotide with same length and GC ratio similar to the antisense oligonucleotide but without homology to any known gene sequences was used as a control. A digital image analysis system (MCID4, Imaging Research) was used to obtain optical density measurements over the sections. The gray levels corresponding to the ¹⁴C plastic standards (Amersham) lying within the exposure range of the film were determined and used as a fourth-degree polynomial approximation to construct a gray level to activity transfer. Densitometric measurements were done from 3 to 5 sections (at −2.8±0.2; −3.1±0.2; −3.3±0.2; −3.6±0.2; 3.8±0.2 mm from bregma) per animal (n = 5 in control/sham group, n = 3 for different time points, n = 4 for MK-801 and dexamethasone groups, and n = 3 for NBQX group for animals processed for in situ hybridization).

Immunocytochemistry

Free-floating (50 μm) sections were reacted with the primary antibody (Transduction Laboratories; 1:100). After incubation with figure 2. Time course of COX-2 mRNA expression measured on x-ray film in situ hybridization autoradiographs. A, Cortex; B, CA1 pyramidal cell layer; C, CA3 pyramidal cell layer; D, dentate gyrus, granule cell layer. Values represent the mean ± SEM from 3 to 5 animals. *Expression significantly higher compared with control (Co) value (P<0.05, 2-tailed Mann-Whitney U/Wilcoxon rank sum W test).
Results

The physiological variables of the nontreated and drug-pretreated ischemic animals were within the normal range and did not show significant differences between the groups.

Figure 1 shows in situ hybridization autoradiographs of COX-2 expression at 4, 8, and 24 hours and 3 and 7 days after global ischemia compared with sham-operated animals. The mRNA levels were clearly increased in the hippocampus and cortex as early as 4 hours after ischemia, peaking at 8 hours. Increased levels of COX-2 mRNA were still detected in the CA1 region at 1 day and in the dentate gyrus at 3 days after ischemia. Quantitative analysis (Figure 2) confirmed the observations and showed that whereas COX-2 mRNA levels in the cortex, dentate gyrus, and CA3 region peaked at 4 and 8 hours after ischemia, the mRNA levels were statistically significantly increased at 4 to 24 hours in the CA1 region, indicating prolonged COX-2 induction in the vulnerable CA1 neurons. In addition, the expression of COX-2 mRNA remained slightly but significantly upregulated in the dentate gyrus at 1 and 3 days after ischemia. Hybridization with the control oligonucleotide did not result in any detectable signal (not shown).

Figure 3 shows the time course of COX-2 immunoreactivity in the hippocampus. A low basal expression of COX-2 protein was present in the dentate gyrus and CA3 section in sham-operated animals. Twelve hours after ischemia, COX-2 immunoreactivity was seen in the granular cells of the dentate gyrus and in CA1 to CA3 pyramidal neurons. The immunoreactivity was exclusively neuronal and was the strongest in the CA1 pyramidal and dentate granular cells. The immunoreactivity was further increased at 24 hours. At 3 days the immunoreactivity was back to control levels in the CA3 and dentate gyrus but remained at a higher level in CA1 pyramidal neurons. In the cortex COX-2 immunoreactivity was increased at 12 hours, was slightly decreased at 1 day, and was back to the control level 3 days after ischemia (not shown).

Figure 4 shows in situ hybridization autoradiographs of COX-2 mRNA from pretreated animals 8 hours after the insult. The quantitative data of the pretreatments are shown in Figure 5. Pretreatment with NBQX, an AMPA/kainate receptor antagonist, decreased the ischemia-induced COX-2 mRNA expression in the cortex and CA1 region. Pretreatment with MK-801 did not block the induction of COX-2 in any region studied, but it increased significantly the expression in the CA3 region. Finally, pretreatment with dexamethasone decreased COX-2 expression in the cortex but was without effect in the hippocampus.

Discussion

Previous studies have shown that while both NMDA and AMPA receptors contribute to focal brain infarction,23 only the AMPA type of glutamate receptors may contribute to hippocampal neuronal damage in global ischemia models,24–26 possibly because of inactivation of NMDA recep-

![Figure 3. Photomicrographs show COX-2 immunoreactivity in the hippocampus in a sham-operated brain (control) and 12 hours, 1 day, and 3 days after global ischemia. The COX-2 protein is exclusively neuronal and expressed in all hippocampal regions in response to ischemia. Arrows point to COX-2 immunoreactivity in the CA1 pyramidal cell layer 3 days after the ischemia (D) and to immunoreactive CA1 pyramidal neurons 1 day after ischemia (E). Bar=500 μm (A through D), bar=50 μm (E).]
tors early in the onset of global ischemia. Induction of COX-2 mRNA and protein, mainly through activation of NMDA receptors and PLA₂ in focal ischemia but also through activation of AMPA receptors in global ischemia, suggests that COX-2 is a mediator of glutamate toxicity in both focal and global ischemia. The results also indicate that the induction pathway of COX-2 expression is different in global and focal ischemia. This observation may have implications for the therapeutic strategies aimed at inhibition of COX-2 in different models of brain diseases.

While several genes are induced in the vulnerable CA1 pyramidal cells after global ischemia, very few of the induced genes reach expression at the protein level. The induced COX-2 mRNA is converted to protein in global ischemia, and the protein still remains upregulated in the CA1 pyramidal cells 3 days after the insult, thus overlapping with the period of delayed death of these neurons. Previous studies have suggested that inhibitors of COX enzymes are neuroprotective in global ischemia models when administered before the onset of ischemia. Our results show that COX-2 expression takes place directly in the neurons destined to die. This is in agreement with a recent study by Nakayma et al, which showed COX-2 induction in the same neurons after global brain ischemia. In the same study, COX-2 inhibitors were protective when administered 30 minutes after ischemia. The delayed and long-lasting COX-2 expression in CA1 pyramidal cells suggests further that inhibition of COX-2 might still be beneficial when administered days after ischemic insult. Because iNOS is induced in astrocytes of the CA1 subfield 3 days after global ischemia and nitric oxide produced by iNOS has been shown to contribute to COX-2 activity (possibly without altering COX-2 expression), inhibition of iNOS could also serve as neuroprotection through COX-2 inhibition just before the start of the delayed death of CA1 neurons.

The result that pretreatment with MK-801 increased COX-2 expression in the CA3 region is surprising. In the posterior cingulate and retrosplenial cortex, MK-801 causes neuronal degeneration and induction of heat shock proteins and immediate early genes, including COX-2. Low doses of MK-801 are known to induce c-fos and alter expression of NMDA receptor subunits in the entorhinal cortex, which gives rise to the afferent innervation of the dentate gyrus and CA1 pyramidal cells. This effect of the NMDA receptor antagonists is thought to occur by disinhibition when NMDA receptors stimulating inhibitory neurons are blocked, resulting in reduced function of inhibitory interneurons regulating excitatory cortical neurons. Our results show a tendency of MK-801–induced COX-2 expression in the cortex as well. Even though MK-801 or other NMDA receptor antagonists are not known to induce any damage of the hippocampal neurons,
it is possible that a slight reduction in the inhibitory input to CA3 pyramidal cells results in COX-2 expression.

Dexamethasone is well known to prevent hypoxic-ischemic brain damage in neonatal rats,33 but its role in brain ischemia of adult rodents is less clear. Several studies have demonstrated that dexamethasone aggravates neuronal death in the hippocampus.34–36 However, dexamethasone reduces brain edema and mortality in rat global ischemia37 and reduces edema in gerbil global ischemia;38 long-term posts ischemic dexamethasone treatment reduces the damage inflicted in the caudate nucleus but not in the hippocampus in the rat model of global ischemia.35 Since COX-2 is one of the enzymes mediating ischemic damage, dexamethasone may be inefficient in protecting hippocampal neurons against ischemia, partially because it does not block COX-2 expression in the hippocampus. Interestingly, Weidenfeld et al39 showed in 1987 that 40 μm dexamethasone in vitro reduces prostaglandin E2 release from the cortex but not from the hippocampus, thus supporting our conclusion.

Administration of dexamethasone has been reported to increase plasma glucose from 5 to 6 mmol/L to 10 to 11 mmol/L,40 but studies reporting no alterations in plasma glucose after dexamethasone pretreatment have also been published.34,35 We have previously shown that glucose levels of 22 mmol/L increase spreading depression–induced COX-2 expression by 50%, whereas 10 mmol/L plasma glucose had no effect.41 In the present study plasma glucose levels were not controlled. Because dexamethasone may induce only a modest increase in plasma glucose and because dexamethasone in the present study reduced COX-2 expression, it is unlikely that the effect of dexamethasone is due to altered plasma glucose. Another physiological factor that could affect the results is body temperature, which in the present study was not altered by any treatment during ischemia. However, the posts ischemic temperatures were not measured. Because both MK-801 and NBQX reduce body temperature after global ischemia according to some reports,23,42 but we observed decreased COX-2 expression only after NBQX treatment, it is unlikely that body temperature significantly affects the present results.

In addition to the role of iNOS in COX-2 activation, a possible explanation for differential regulation of COX-2 expression in global and focal ischemia and in the cortex and hippocampus is distinct activation of transcription factors in response to ischemia. COX-2 has several regulatory elements in the 5′ flanking region, such as an activator protein-1 binding site, cAMP-responsive element, and binding sites for nuclear factor-κB, an oxidative stress–responsive transcription factor.43 Whether these transcription factors are differentially activated in the cortex and hippocampus after global ischemia is not known. The possibility that glutamate receptor–independent expression of COX-2 is more predominant in the hippocampus than in the cortex also remains to be studied.

Altogether, the results show that COX-2 expression is strong and lasting in the most vulnerable neurons after global ischemia. Whereas antagonists of AMPA glutamate receptors are potentially protective when given immediately before or within hours after global ischemic insult, COX-2 inhibitors still have a target in the dying CA1 neurons when delayed neuronal death starts 3 to 4 days after stroke.

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References

Cyclooxygenase catalyzes the formation of prostaglandins and thromboxanes. Two forms of the cyclooxygenase enzyme have been identified. The constitutively expressed cyclooxygenase-1 (COX-1) is localized in a number of cell types, including platelets, renal cells, and gastric mucosa. The inducible cyclooxygenase (COX-2) is mainly found in leukocytes and brain. Prostaglandins and thromboxanes have long been implicated in the pathogenesis of ischemic brain injury. The preferential distribution of COX-2 in the brain has led to extensive studies exploring its pathogenetic role in ischemic brain injury. COX-2 is expressed following both focal and global ischemia, and COX-2 inhibitors are effective in reducing ischemic injury in both focal and global ischemia models. The preceding article by Koistinaho and associates examined the regulatory mechanism of ischemia-induced expression of COX-2 mRNA in a 4-vessel global ischemia model. The main thrust of this study is the observation that NBQX, an AMPA receptor antagonist, is effective in blocking postischemic COX-2 expression. This novel finding raises the possibility that the neuroprotective action of AMPA blockade in global ischemia models may be due in part to the inhibition of COX-2 expression. Lack of inhibitory effects of MK-801, an NMDA receptor antagonist, suggests that the glutamate receptor mechanism in the inhibition of COX-2 expression is selective and restricted to the AMPA receptor.

The present study also explored the effects of dexamethasone on postischemic COX-2 expression. Dexamethasone is a synthetic glucocorticoid that is known to repress COX-2 expression. Unlike other COX-2 inhibitors that have been shown to reduce ischemic brain injury, glucocorticoids exacerbate hippocampal neuronal degeneration following global ischemia. In the present study,
dexamethasone inhibits COX-2 expression in the cerebral cortex but not the hippocampal CA1 region. Whether the site-selective effects of dexamethasone are related to the detrimental glucocorticoid action in forebrain global ischemia is not known. It should be noted that studies on the regulation of COX-2 expression by Koistinaho and colleagues were limited to the mRNA level. It is not clear whether protein expression follows the same pattern. More importantly, it would be interesting to know whether changes in enzyme activity and prostaglandin and thromboxane contents are in accordance with the extent of COX-2 expression noted in various experimental paradigms. Conflicting glucocorticoid actions have been reported in a number of global ischemia models. Correlating dexamethasone effects on COX-2 expression with the pathological outcomes in this model will serve to clarify the role of COX-2 in the pathogenesis of ischemic injury.

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