Maturational Regulation and Regional Induction of Cyclooxygenase-2 in Rat Brain: Implications for Alzheimer’s Disease

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We explored the constitutive expression, maturational regulation, and relation to kainic-acid-induced apoptosis of cyclooxygenase (COX)-2 mRNA in rat brain. In adult rats, COX-2 mRNA was expressed primarily in limbic structures. Constitutive COX-2 mRNA expression increased markedly between Postnatal Day 7 (P7) and P14, reaching adult levels by P21. Despite intense KA-induced seizures, no COX-2 mRNA induction was found before P14 in any brain region examined. During response to KA-induced seizures in adult brain, COX-2 mRNA induction paralleled temporally and overlapped anatomically the appearance of cellular morphological features of apoptosis in subsets of cells of the pyramidal neuron layer of the hippocampal formation, amygdaloid complex, and pyriform cortex. While COX-2 mRNA showed KA-induced elevation in the granule cell layer of the dentate gyrus, no detectable morphological features of apoptosis were found in this region. Finally, monotypic culture of rat cortico-hippocampal neurons confirmed the neuronal expression of COX-2 in vitro and revealed that COX-2 is induced during response to glutamate treatment, leading to neuron death. These studies may provide novel insights into the role of COX-2 and mechanisms of action of nonsteroidal anti-inflammatory drugs in Alzheimer’s disease. © 1997 Academic Press

INTRODUCTION

The role of inflammation in Alzheimer’s disease (AD) is incompletely understood, but is now receiving considerable attention in part due to evidence from epidemiological (3, 7, 8, 21, 24, 25, 32) and clinical (33) studies showing that nonsteroidal anti-inflammatory drugs (NSAIDs) may favorably influence the clinical expression of AD. The mechanisms and site of action by which NSAIDs influence manifestations of AD are unknown, but may involve inhibition of cyclooxygenases (COXs).

COXs are enzymes that catalyze the formation of prostaglandin (PG)-H₂ from arachidonic acid (AA); PG-H₂ is further metabolized to physiologically active PGs (PG-D₂, PG-E₂, and PG-F₂α), prostacyclin (PG-I₂), and thromboxanes. Specific PGs have diverse, often antagonistic, effects on different tissues. For example, PG-I₂ and PG-E₂ are potent vasodilators that may contribute to the inflammatory response, while PG-F₂α is a vasoconstrictor.

There are two known COX isoforms, COX-1 and COX-2, which are similar in amino acid sequence and enzymatic functions although they are physiologically distinct. COX-1 is constitutively expressed at different levels in different cell types. COX-2 is mitogen-inducible but is mostly undetectable in peripheral tissues under control conditions (19, 26). COX-2 mRNA rises rapidly in response to inflammatory stimuli such as IL-1β and lipopolysaccharide, and it decreases in response to glucocorticoids, while COX-1 mRNA is unaffected, suggesting that COX-2 is the isoform that mediates inflammation (11, 26). Recent evidence suggests that COX-2 may mediate mechanisms of cell survival and cell adhesion in peripheral cells under defined conditions (20, 43).

The role of COX-1, COX-2, and PG synthesis in normal and AD brain has not been fully characterized. The importance of PGs in brain function may be independent of inflammatory mechanisms. PG receptors are found in the hypothalamus and thalamus as well as in the limbic system (45). PGs are involved in hypothalamic–pituitary hormone secretion (18), regulation of temperature (44), and the sleep–wake cycle (14). There is recent evidence that COX-2 mRNA is regulated in rat brain by synaptic activity and glucocorticoids (2, 16, 46).

In peripheral tissues such as synovium, the efficacy of NSAIDs is attributed to suppression of neutrophil activation and inhibition of the synthesis of inflamma-
tory PGs (1). Since there is scant evidence that neutrophils or PGs are important in the pathophysiology of AD, the mechanism by which NSAIDs might influence the clinical expression of AD has been unclear (discussed in Ref. 28). Investigation of the role of COX-2 in neurodegenerative mechanisms is likely to clarify this issue.

The goal of this study was to explore the expression and maturational regulation of COX-2 mRNA in rat brain during the response to kainic acid (KA)-induced seizures, a model of hippocampal neurodegeneration.

MATERIALS AND METHODS

Animals and Excitotoxic Lesions

Male adult Sprague-Dawley rats of different ages were maintained in a controlled light and temperature environment, with food and water ad libitum. Because in both adult and maturing rats the response to KA is variable, only animals with seizures characteristic of "limbic status epilepticus" were used for these studies, as previously described (5, 39). In adult rats (200–300 g), hippocampal excitotoxic lesions were induced by subcutaneous injection of KA (10 mg/kg, Sigma). Because KA uptake is higher in young rats than in adults (6), KA doses were adjusted to produce optimal seizures without reaching lethal doses [from 2 mg/kg at Postnatal Day 7 (P7) to 6 mg/kg at P25]. Saline-injected rats were used as control (0 h time point). Both adult and maturing rats were sacrificed at each indicated time after onset of seizures.

In Situ Hybridization

At various intervals after the onset of seizures, the rats were sacrificed, and the brains were quickly removed, rinsed in cold phosphate buffer (PBS, 10 mM, pH 7.4), and immersed in methylbutane at −25°C for 3 min. Brains were sliced coronally (10 µm) and frozen sections were mounted on polylysine-coated slides and stored at −70°C. For immunocytochemistry (ICC) or in situ hybridization (ISH), frozen sections were postfixed in PBS containing 4% paraformaldehyde (30 min, room temperature) and rinsed in PBS. For ISH, tissue sections were rinsed in 0.1 M triethanolamine(TEA), pH 8.0, incubated in acetic anhydride (AA, 0.25% v/v in TEA, 10 min), and rinsed in TEA and PBS. Following AA treatment, tissue sections were hybridized with 35S-cRNA probes (0.3 µg/ml, 2 × 10^9 dpm µg^-1) made from COX-2 linearized cDNA transcription vectors (13). Following hybridization (3 h, 50°C), stringency washes...
**FIG. 3.** Maturational influence on COX-2 mRNA expression during response to KA-induced seizures. Micrographs were generated from autoradiographic images. For anatomical distribution of changes refer to Fig. 1. Control, unlesioned vehicle injected rats; KA 8 H, KA-treated rats sacrificed 8 h after seizures onset; postnatal day, P-7, P-14, P-21.

**FIG. 4.** Time course of COX-2 mRNA changes in rat brain during responses to KA treatment: maturational influences and regional distribution of changes. Optical densities were quantified from autoradiographic images. Statistics were calculated by ANOVA followed by additional post hoc analysis. Data are expressed as means \(\pm\) SEM, \(n = 4\)–6 per group, \(*P < 0.01\) vs 0 h group (saline injected group); 4, 8, 16, 30, and 120 h, time in hours after onset of KA induced seizures. DG, dentate gyrus; CA1, CA2, and CA3, subregions of the neuronal pyramidal layer of the hippocampal formation; PAC, parietal cortex; PYC, pyriform; AC amygdaloid complex; THAL, ventroposterior thalamic nuclei.
FIG. 5. Maturational influence on the distribution of COX-2 mRNA expression and induction in rat hippocampus. COX-2 mRNA in P21 (A and B) and adult (C and D) hippocampal formation as assessed by in situ hybridization assay and visualized by emulsion autoradiography using dark-field illumination. In A and C, COX-2 mRNA expression in control rats (vehicle injected); in B and D, COX-2 mRNA 8 h after the onset of KA-induced seizures. Abbreviations: DG, dentate gyrus; CA3, subregions of the pyramidal layer of the hippocampal formation. Arrows point toward the superficial layer of DG (stratum granulosum). Bar, 200 µm.
FIG. 7. KA-induced COX-2 and apoptosis in adult rat. In A and B, COX-2 mRNA expression in CA3 hippocampal pyramidal neurons of control and KA-treated rat, respectively; in C, CA3 subregion of the hippocampal formation of KA lesioned rats showing neurons with apoptotic features (arrows). In D and E, COX-2 mRNA expression in pyriform cortex of control and KA-treated rat respectively; in F arrows point toward apoptotic cells of pyriform cortex of KA-lesioned rats. In G and H COX-2 mRNA expression in cells of the amygdaloid complex of control and KA-treated rats, respectively; in I arrows point toward apoptotic cells of the amygdaloid complex of KA-lesioned rats. COX-2 mRNA was assayed by in situ hybridization and visualized by emulsion autoradiography under dark-field illumination. In situ 3’ end-labeling was used to assess apoptotic features following KA-induced seizures. Bar: (A, B, D, E, G, and H) 200 µm; (C, F, and I) 40 µm.
(0.1× SSC, 60°C), and dehydration, slides were exposed to X-ray film for 7 days for quantification. Optical densities were integrated by computerized video densitometry from film autoradiograms and analyzed quantitatively using an image analysis system with software from Drexel University (41). Slides were then exposed to NTB-2 emulsion (Eastman Kodak, Rochester, NY) for microscopic analysis of COX-2 mRNA distribution as previously described (28); tissue sections were counterstained with cresyl violet. Hybridization of brain tissue sections with sense 35S-cRNA strand probes controlled for specificity and gave negative results (not shown).

In Situ End-Labeling

In parallel studies, paraformaldehyde-fixed brain tissue sections were dehydrated, air dried, and incubated with dATP, dCTP, dGTP, and dUTP (13 µM) digoxigenin-11-dUTP, and DNA polymerase I (Boehringer Mannheim) at 10 units/100 µl at 37°C for 2 h. The reaction was stopped by addition of 20 mM EDTA (pH 8.0). Sections were incubated at room temperature overnight with an alkaline phosphatase-conjugated digoxigenin antibody (Genius System, Boehringer Mannheim) diluted 1:200 in 5% sheep serum diluted in PBS, pH 7.5. Colorimetric detection with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate was performed with the Genius System by the manufacturer’s protocol (34).

COX-1 and COX-2 cDNA Probes

Bluescript plasmid (Stratagene) containing the full-length rat COX-1 cDNA (2.7 kb) was linearized by digestion with CiaI, while a PCRII plasmid (Invitrogen) containing the coding sequence for rat COX-2 (1.8 kb) was linearized by PstI (13). Linearized plasmids were purified using Elu-Quick (Schleicher & Schuell) after agarose gel electrophoresis.

Northern Blot Hybridization Assay

For RNA extraction, following sacrifice rat brain was dissected and stored at −75°C for subsequent RNA extractions. Total RNA was extracted from pools of hippocampal tissue (28, 35). Briefly, tissues were homogenized for 1 min in 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.5), 0.5% sarcosyl, and 0.1 M β-mercaptoethanol, final volume 0.5 ml. After acidified phenol–chloroform extraction and ethanol precipitation, the RNA pellet was rinsed consecutively with 70 and 100% ethanol. The purified RNA was then dissolved in 0.5% sodium dodecyl sulfate (SDS) and stored at −75°C. Total RNA was quantified in a UV spectrophotometer. Total RNA (5 µg) from tissue was electrophoresed on denaturing (0.2 M formaldehyde) agarose gels and transferred to nylon membrane (Nyland 66 plus; Hoeffer, San Francisco CA) in 2× SSC (35).

Blot hybridization was carried out with 106 cpm/ml of antisense COX-1 or COX-2 [32P]cRNA probes in 50% formamide, 1.5× SSPE, 1% SDS, 0.5% dry milk, 100 µg/ml yeast total RNA, and 500 µg/ml salmon sperm DNA at 53°C for about 15 h. Blots were washed to a final stringency of 0.2× SSC, 0.2% SDS at 72°C. Blots were exposed to Kodak X-ray film (XAR-5, Eastman Kodak) with intensifying screens at −70°C.

Cell Cultures

Cortico-hippocampal neuron cultures were derived from embryonic rat brain. E16-E18 embryos were dissected in Hank’s balanced salt solution and cultured as described (28, 30). Quality of primary neuronal cultures was confirmed immunocytochemically and revealed >95% purity based on neuron-specific enolase (NSE) immunoreactivity and cell type morphology assessment (not shown) (28). Culture medium was changed every 3 days. For glutamate neurotoxicity studies, 8-day-old cultures were treated with glutamate (250 µM, Sigma) in the presence of 2.4 mM Ca2+ and 0.8 mM Mg2+. After 6 h of glutamate exposure, culture medium was replaced with fresh medium; COX-2-like immunoreactivity was assessed in neurons 12 h later.

Immunocytochemical Detection of COX-2 in Monotypic Cultures of Primary Rat Neurons

Control and glutamate-treated cultures were post-fixed in PBS containing 4% paraformaldehyde (30 min, room temperature), rinsed in PBS, pretreated with normal serum, and incubated overnight at 4°C with primary antibodies. COX-2 antiserum (rabbit IgG) was raised against a synthetic peptide (CNASASHSRLD-DINPT) encompassing the C-terminal region of the murine COX-2. The antiserum reacts with human and rat COX-2 but not with COX-1 as assessed by Western blot analysis (15). Vectastain ABC kit (Vector, Burlingame, CA) was used in subsequent steps to complete the diaminobenzidine (DAB) staining (28). Immunoadsorption of COX-2 antisera with synthetic COX-2 peptides controlled for specificity; adsorption was carried out overnight at 4°C with synthetic COX-2 peptides at 30 µg/ml.

Statistical Analysis

Analysis utilized statistical analysis software (CSS from Statsoft). ANOVA was performed followed by post hoc analysis. P values < 0.05 were considered to indicate statistically significant differences. In situ hybridization studies used n = 4-6 rats per group; data were computed from two tissue sections (bilaterally) encom-
passing the entire hippocampal formation of each individual rat.

RESULTS

COX-2 Expression in Adult Rat Brain

Figure 1 shows by ISH the regional distribution of COX-2 mRNA expression in brain regions of the adult rat. COX-2 mRNA was most notable in limbic structures, but was also present in neocortex, consistent with previous reports (16, 46). In the hippocampal formation, COX-2 mRNA was selectively expressed in cells of the granule and pyramidal neuron layers. COX-2 mRNA expression was also found in the outer layer of the parietal cortex, the pyriform cortex, and cells of the amygdaloid complex.

Maturational Regulation of Hippocampal COX-2 mRNA Expression

Using ISH we found that during maturation COX-2 mRNA expression is differentially regulated in subsets of cells of neuronal layers of the hippocampal formation (Fig. 2 and Fig. 3, top). From P7 to P14, COX-2 mRNA showed a greater than twofold increase in the granule cell layer of the dentate gyrus and in the CA3 subdivision of the pyramidal cell layer (P < 0.001, Fig. 2). Though the expression of COX-2 mRNA was lower in the other brain regions examined, the pattern of maturational expression was similar (Fig. 2). By P21, COX-2 mRNA expression approximated adult levels in all subregions examined (Fig. 2 and Fig. 3, top).

Response to KA-Induced Seizures

To further explore the maturational regulation of COX-2 in brain, we examined COX-2 mRNA expression during responses to KA-induced seizures postnatally. Despite intense seizure activity after KA treatment, no detectable change in COX-2 mRNA expression was found in any brain region examined at P7 (Figs. 3 and 4A); changes in COX-2 mRNA expression at 120 h post-KA treatment in the P7 group indicate developmental maturation rather than response to KA excitotoxicity (Fig. 4A). In contrast to the P7 group, at P14 and P21 COX-2 mRNA increased within 4–8 h after onset of KA-induced seizures in all the hippocampal subregions examined (Figs. 4B and 4C). The level of COX-2 mRNA expression returned toward control levels within 120 h (5 days) after treatment in P14, P21, and adult rat brain.

Within the dentate gyrus, control COX-2 mRNA expression in P21 rats (and in P14 rats, not shown) was asymmetric, selectively localized to the more superficial neurons of the stratum granulosum rather than the deeper granule cells of the dentate gyrus blade (Fig. 5A). In response to KA treatment, COX-2 mRNA induction showed similar asymmetry of expression (Fig. 5B). In contrast, the asymmetry within the dentate gyrus was less notable in control animals and after KA induction of the adult group (Figs. 5C and 5D).

In parallel studies, Northern blot hybridization of total RNA from hippocampus of adult rats 12 h after KA-induced seizures confirmed COX-2 mRNA induction (Fig. 6). No detectable induction of COX-1 mRNA was found in the same rat brain (Fig. 6).

KA-Induced COX-2 and Apoptosis in Adult Rat

By 8 h after the onset of KA-induced seizures, COX-2 mRNA induction in cells of the CA3 region of the hippocampal formation (Fig. 7B), pyriform cortex (Fig. 7E), and amygdaloid complex (Fig. 7H) of the adult brain paralleled temporally and overlapped anatomically the onset of apoptosis as assessed by in situ end-labeling in the same brain regions (Fig. 7C, CA3 regions of the hippocampus; Fig. 7F, pyriform cortex; Fig. 7I, amygdaloid complex). Cellular COX-2 mRNA expression in adult, control (Figs. 7A, 7D, and 7G) and KA-treated (Figs. 7B, 7E, and 7H) rats was identified by emulsion autoradiography using ISH assays.

Immunocytochemical Evidence of Neuronal COX-2 Expression/Regulation in Response to Glutamate in Vitro

To further explore the regulation of COX-2 in mechanisms of excitotoxicity, primary cultures of rat hippocampal neurons were exposed to glutamate in vitro. At baseline, constitutive COX-2 expression is demonstrated by immunocytochemistry (Fig. 8A). Twelve hours after exposure to glutamate an apparent qualitative induction of COX-2 immunoreactivity coincided with a marked reduction in the number of neurons (Fig. 8B). The apparent elevation of COX-2 immunoreactivity in glutamate-treated neurons may reflect in part...
FIG. 8. Immunocytochemical evidence of neuronal COX-2 expression/regulation in response to glutamate in vitro. COX-2-like immunoreactivity in monotypic cultures of rat primary hippocampal neurons in (A) control and (B) after glutamate exposure (12 h). In C and D control and treated cultures immunoreacted with immunoadsorbed COX-2 antibody, respectively. COX-2 immunoadsorption was carried out overnight at 4°C with synthetic COX-2 peptides at 30 µg/ml. Bar: 50 µm.
accumulation rather than induction of COX-2 expression, since some degree of perikaryal atrophy and regression of neuronal processes was evident in the neurons by 12 h after exposure to glutamate (Fig. 8B, arrows). Immunoadsorption of COX-2 antiserum with COX-2 peptide abolished COX-2 immunostaining in both control and glutamate-treated neurons (Figs. 8C and 8D).

DISCUSSION

These studies explored the regional distribution and maturational regulation of COX-2 mRNA expression in rat brain, including the response to excitotoxic insults that model aspects of neuron death in AD. COX-2 mRNA expression in control and KA-treated animals was evident mostly in the pyramidal and granule cell layers of the hippocampal formation, in the amygdaloid complex, and in subregions of cerebral cortex. These results generally conform to the regional distribution and seizure induction of COX-2 recently reported (2, 16, 46). No detectable induction of hippocampal COX-1 mRNA expression was found at any time postlesioning using in situ hybridization assay (not shown) and by Northern blot assay 12 h after the onset of KA-induced seizures. Monotypic culture of rat hippocampal neurons confirmed neuronal expression of COX-2 in vitro and revealed that COX-2-like immunoreactivity is induced during glutamate-induced neurotoxicity.

In adult rats the induction of COX-2 mRNA overlapped the onset of apoptosis in subsets of hippocampal pyramidal neurons and also in pyriform cortex and amygdaloid complex regions, as assessed by the end-labeling technique. This finding, along with the in vitro COX-2 induction after glutamate exposure, supports the hypothesis that COX-2 overexpression may be related to mechanisms of neuronal death. However, the correspondence between COX-2 induction and neuronal death was not complete. There was induction of COX-2 mRNA in the granule neuron layer of the dentate gyrus following KA treatment, but there was no evident neuronal damage in this layer. In addition, within brain regions susceptible to KA excitotoxicity, such as the hippocampal pyramidal layer CA3, only a fraction of neurons with high levels of COX-2 mRNA show morphological features of apoptosis by 8 h after KA in adult rats.

The role of COX-2 in neurodegeneration is unknown. It is possible that COX-2 induction may be deleterious to neuronal survival, which could explain the apparent benefit of NSAIDs in AD (cited above). This hypothesis is supported by studies showing that COX-2 is induced by transient brain ischemia (31) and that NSAIDs diminish hippocampal ischemic damage (36). However, the induction of neuronal COX-2 mRNA after KA treatment in vivo or glutamate in vitro might also be consistent with the hypothesis that COX-2 is involved in rescue mechanisms of surviving neurons. For example, there is evidence that intestinal epithelial cells genetically engineered to overexpress COX-2 mRNA have high levels of BCL2 protein expression and are more resistant to conditions leading to apoptotic death (42).

One possible mechanism by which COX-2 and neuronal death might be associated involves the control of transcription factor DNA-binding activity that could potentially produce long-lasting changes in gene expression. For example, both rat and mouse COX-2 promoters share several regulatory elements, including SP-1, that might potentially be involved in COX-2 induction in brain (40). The SP-1 consensus sequence binds the zinc finger transcription factors, such as zif268, which also show rapid induction in response to KA excitotoxicity (Schreiber, unpublished observation). Identifying the regulatory factors responsible for the differential expression of COX-2 may provide a better understanding of the mechanisms responsible for select neuronal vulnerability. Recent evidence suggests that the intracellular platelet activating factor (PAF)-inhibition, which limits the induction of the transcription factor immediate-early gene zif268 mRNA, also attenuates COX-2 expression (4, 22, 23).

We found that COX-2 but not COX-1 mRNA is regulated during the response to KA-induced seizures. Interestingly, COX-1 Northern hybridization analysis of total RNA from rat hippocampus revealed two major mRNA species of approximately 7.0 and 2.7 kb, which, we infer, may represent alternative splice variants of COX-1 mRNA. Expression of a 7.0-kb COX-1 mRNA species in brain tissue has been reported (17), and differential splicing of COX-1 mRNA has been reported in peripheral tissue (12).

There is evidence that KA may control COX-2 mRNA expression through KA or NMDA receptor activation in rat brain. For example, elevation of COX-2 mRNA in rat cortex following intracerebral infusion of KA is attenuated by treatment with the NMDA antagonist MK-801; glucocorticoids also inhibit this COX-2 induction, as expected (2). The effects of NMDA antagonists and glucocorticoids on COX-2 mRNA expression during the response to experimental lesions leading to neuronal death are currently under investigation in our laboratory.

The subregional distribution of COX-2 mRNA signal in dentate gyrus of developing rats (p14/p21) may represent maturational processes. The dentate gyrus of the adult rat contains 5 x 10^6 granule cells that migrate from the hilus (37). During maturation, the cells in the more superficial stratum granulosum (external layer of the dentate gyrus as shown in Fig. 5) are formed earlier (9, 37) and appear to express higher levels of COX-2 mRNA in comparison to the deeper...
granule cells. It is interesting to note that neurogenesis in the dentate gyrus during maturation and in adult life is regulated by adrenal steroids (10); glucocorticoids regulate COX-2 expression (26).

Our studies confirm recent reports that COX-2 in the rat brain is maturationally regulated during response to excitotoxicity in a pattern consistent with that of the transcription factor immediate-early gene c-fos mRNA (16, 38, 46). Interestingly, COX-2 mRNA induction was dissociated from KA-induced seizure activity during the early maturational stage as also found for c-fos mRNA. While rats at P7 developed intense seizures, no detectable COX-2 mRNA induction was found following KA treatment. Ongoing studies aim at the identification of potential mechanisms controlling COX-2 induction with respect to maturation and susceptibility to KA-induced seizures.

To establish a direct causal relationship between COX-2 activation and mechanisms of death/survival in neurons, future studies in our laboratory will aim at the selective inhibition of COX-2 gene expression and/or COX-2 protein function during the response to conditions leading to neuronal death. These studies may provide important insights into the potential utility of anti-inflammatory drugs in the treatment of AD.

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