Pharmacology of Celecoxib in Rat Brain after Kainate Administration

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The cyclooxygenases (COXs) catalyze the committed step in the conversion of arachidonic acid to prostaglandins (PGs) and thromboxane (Smith et al., 2000). Two distinct COX enzymes are known, COX-1, which is present in most tissues, and a second isoform, COX-2 (Kujubu et al., 1991; Xie et al., 1991), absent in most normal tissues but the expression of which can be induced by many stimuli. COX-2 appears to be the isoenzyme responsible for prostaglandin formation at sites of inflammation (Masferrer et al., 1994; Seibert et al., 1994), and selective inhibitors of COX-2 such as celecoxib have been introduced into clinical practice for the treatment of osteoarthritis, rheumatoid arthritis and pain (Emery et al., 1999; Simon et al., 1999). Although the mechanism of the anti-inflammatory action of COX inhibitors is clear, the site of the analgesic effect is not known (Malmberg and Yaksh, 1992; Samad et al., 2001). Intrathecal administration of either a COX-2 inhibitor or nonsteroidal anti-inflammatory drug prevented hyperalgesia in a model of peripheral inflammatory pain, suggesting a role for central prostaglandins (Malmberg and Yaksh, 1992; Dirig et al., 1998). Prostaglandins are also produced in the brain after infection in response to pyrogenic cytokines, and are important in the febrile response (Elmquist et al., 1997). PGE_2 is the major prostaglandin produced in animal models of acute and chronic inflammation, both at the site of inflammation (Anderson et al., 1996; Portanova et al., 1996) and in the cerebrospinal fluid (Smith et al., 1998).

Recently, a membrane-bound enzyme that catalyzes formation of PGE_2 from PGH_2 was cloned, called microsomal PGE synthase (PGEs), which colocalizes with COX (Jakobsen et al., 1999; Murakami et al., 2000). The expression of PGEs is induced by inflammatory stimuli, e.g., in a rat model of lipopolysaccharide-induced pyresis and in the arthritic paw (Mancini et al., 2001). Recent data demonstrated that in a model of pyresis, COX-2 and PGEs are both centrally up-regulated and colocalize in endothelial cells (Ek et al., 2001; Yamagata et al., 2001).

COX-2 is constitutively expressed in neurons within the brain, particularly in pyramidal neurons of the hippocampus and cortex, and in the amygdala (Yamagata et al., 1993;
Beads, IGEPAL CA-630, benzamidine HCl, Na 3VO4, NaF, seaweed; subcutaneous injection in rats in-terneuronal cell death initiated by a variety of stimuli. Together, these data suggest that COX-2 may play a role in neuronal apoptosis (Andreasson et al., 2001). Taken to-gether, neurons display age-related cognitive defects and increased neuronal apoptosis (Andreasson et al., 2001). Interestingly, transgenic mice that over-express COX-2 in neurons are more susceptible to kainate-induced lethal seizures (Kelley et al., 1999). Conflicting results with COX-2 inhibitors have been obtained on kainate-induced seizures, with reports of enhancement and no effect or decreased seizures (Baran et al., 1994; Baik et al., 1999). Interestingly, transgenic mice that over-express COX-2 in neurons display age-related cognitive defects and increased neuronal apoptosis (Andreasson et al., 2001). Taken together, these data suggest that COX-2 may play a role in neuronal cell death initiated by a variety of stimuli.

Kainic acid (kainate; KA) is a minor amino acid found in Diginea simplex seaweed; subcutaneous injection in rats in-duces limbic seizures characterized by “wet dog” shakes, tremors, and salivation. KA treatment of rats results in de-layed neuronal necrosis in hippocampus and limbic cortex (Sperk, 1994) and marked cortical COX-2 expression (Chen et al., 1995). KA has pleiotropic actions including increases in extracellular glutamate and expression of a family of imme-diately early genes, including COX-2. (Faroqui and Horrocks, 1991; Sanz et al., 1997; Hashimoto et al., 1998). The kainate-induced seizures in the rat provide a model of centrally mediated, high level COX-2 induction. In the present study we used this model to assess the profile of PGs produced in brain and spinal cord, the expression of enzymes responsible for CNS prostaglandin biosynthesis, and the central action of celecoxib, a COX-2 inhibitor.

### Materials and Methods

**Materials.** Kainate, dexamethasone, methylcellulose, Protein G beads, IGEPAL CA-630, benzamidine HCl, Na,VO 4, NaF, β-glycerophosphate, and Tween 20 were purchased from Sigma-Aldrich (St. Louis, MO). ELISA reagents and COX-2 antibody were obtained from Cambay Chemical Co. (Ann Arbor, MI), peroxidase-conjugated anti-rabbit secondary antibody from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA), 8% and 10% 20S-polyacrylamide gel from Invitrogen (Carlsbad, CA), and an enhanced chemi-luminescence detection kit from Amersham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK). Complete protease inhibi-tor was obtained from Roche Diagnostics (Mannheim, Germany), Mycobacterium butyricum from Difco Laboratories Inc. (Detroit, MI), mineral oil from Mallinckrodt (Paris, KY), and a diaminobenzidine kit from DAKO (Carpinteria, CA). The selective COX-2 inhibitor celecoxib was synthesized by Pharmacia Chemistry.

**Treatment with Kainate.** Male Sprague-Dawley rats purchased from Charles River (250–300 g; Charles River Breeding Laboratories, Portage, MI) were housed and maintained in temperature- and humidity-controlled quarters with free access to water and food. Rats (five to eight per group) were fasted with free access to water >16 h before testing. KA was prepared in saline and at the dose of 10 mg/kg injected subcutaneously in a volume of 1 ml with a 25-gauge needle. KA-treated rats displayed a typical behavior syndrome (including wet-dog shakes, rearing, and tremor of the forepaws) for 30 min to 4 h after injection. KA-treated rats were studied for up to 24 h. All experiments were conducted under a protocol approved by the Institutional Animal Care and Use Committee.

**Induction of Arthritis.** Arthritis was induced in male Lewis rats (125–150 g; Harlan, Indianapolis, IN) by injection of 1 mg of M. butyricum in 50 μl of mineral oil into the right hind footpad (Bill-ingham, 1983). Fourteen days after injection of adjuvant, the con-tralateral left footpad volume was measured with a water displacement plethysmometer. Animals with paw volumes 0.37 ml greater than normal were then randomized into treatment groups. Drug administration was begun on day 15 postadjuvant injection and continued until final assessment on day 25. During this period contralateral paw volume measurements were taken intermittently. The typical increase in contralateral paw volume measured on day 25 ranged from 1.4 to 1.9 ml. Experiments were conducted under a protocol approved by the Institutional Animal Care and Use Committee.

**Compound Administration and Timing within Experi-ments.** Compounds were orally administered in 1 ml of 0.5% meth-ylocelulose + 0.025% Tween 20 either prophylactically 2 h before KA injection or therapeutically 20 h after KA injection. The amount of each compound dosed in each experiment is indicated in the figure legends.

**Measurement of COX Activity and Prostaglandin Content in CSF and CNS Tissue.** Whole brain was removed, weighed, and homogenized in different buffers. To measure the eicosanoid content, brain tissue was homogenized in 3 ml of 70% cold methanol in water. For COX activity assay, however, tissue was homogenized in 1.5 ml of Hank’s buffer with 30 μM arachidonic acid and left at room temperature for 5 min. Then, samples were brought to a concentra-tion of 70% methanol by addition of 3.5 ml of 100% methanol and were centrifuged for 20 min at 15,000g. The supernatant was evaporated under a nitrogen gas stream and resuspended in enzyme immunoassay buffer for TxB2, PGE2, 6-keto-PGF 1α, PGI2, and PGF2α, assays using ELISA kits. Data were calculated as either nanograms per gram of tissue or nanograms per milliliter of CSF.

**RNA Extraction and Design of Primers and Probes.** Total RNA was extracted using RNasy Mini Kit (QIAGEN, Valencia, CA) according to the manufacturer’s instructions. The concentration of RNA was measured using a UV spectrophotometer. Reverse transcrip-tion was performed with approximately 50 ng of RNA. Oligonu-cleotide primers and TaqMan probes were designed using Primer Express, version 1.0 (Applied Biosystems, Foster City, CA). We con-ducted BLAST searches to confirm the total gene specificity of the nucleotide sequences chosen for primers and probes. The sequences of primers and probe used were: for rat PGD synthase lipocalin-type primers, 5′-TGCTGAAAGCCGACTGATGATC3′ (sense), 5′-GGTGCCATGGCGAAGT-3′ (antisense) and 6-FAM-CGTTCTCGT-TCAAGACGGGACCA-TAMRA probe designed on sequence S67721. For rat PGEs primers 5′-TCGCAAGGACAGCACC-TAMRA probe designed on sequence J04488; for rat PGEs primers 5′-CTGCAAGGACAGGCCAAGT-3′ (sense), 5′-GGTGACCGCTTGCTTCTACAGA-3′ (antisense) and 6-FAM-CGTTCTCGT-TCAAGACGGGACCA-TAMRA probe designed on sequence S67721. For rat COX-2 primers 5′-GTCCTTTCACACCTTCCTACTACA-3′ (sense), 5′-CATGGGATTGTCGACGTC-3′ (antisense) and 6-FAM-AGGGCCCTTCTTCTGGTG-TAMRA probe designed on sequence S67721 for rat COX-1 primers 5′-AA-CACGCCTACTGAGAAACTG-3′ (sense) and 5′-CCGGATCGTC- TCAAGAATC-3′ (antisense), and 6-FAM-CCTCAACGGAAGAC-CTGGCCTTGT-TAMRA probe designed on sequence S67721.

**TaqMan PCR.** All TaqMan PCR was performed using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Each reaction mixture (25 μl) contained 5 μl of 5× TaqMan EZ buffer, 3 μl of Gene AmpEraser, 0.75 μl of dATP, dGTP, and dCTP, 1 μl of rTh DNA polymerase, 0.25 μl of AmpEraser from TaqMan EZ RT-PCR core reagents (Roche Applied Science, Branchburg, NJ) and 500
nM primers, 100 nM TaqMan probe, 5 μl of cDNA sample and water. The thermal cycling conditions comprised the initial steps at 50°C for 2 min, a second step at 60°C for 30 min, and a third step at 95°C for 5 min, followed by 40 cycles at 94°C for 20 s and at 62°C for 1 min. Amplification of the target genes was normalized to simultaneous amplification of an internal housekeeping gene, cyclophilin, and calibrated to a low expressing, normalized, target sample.

Western Blot Analysis. Brain and spinal cord samples were homogenized in ice-cold homogenization buffer (25 ml of 50 mM Tris, pH 8, with 0.5% IGEPEAL CA-630, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine HCl, 1 mM Na3VO4, 1 mM NaF, 10 mM β-glycerophosphate, and 1 tablet of Complete protease inhibitor) and probe sonicated five times for 20 s, at 40% power. The samples were then centrifuged at 16,000g for 25 min at 4°C. For COX-2 detection, supernatant protein content was assessed using a Bio-Rad protein assay. For PGEs detection, the supernatant was centrifuged at 100,000g for 1 h at 4°C, the pellet was resuspended, and the protein concentration was measured. Immunoprecipitation was performed by incubation for 30 min under rotation at room temperature with 10 μl concentration was measured. Immunoprecipitation was performed Tween 20 containing 5% milk powder before overnight 4°C blocking for 30 min in 100 mM Tris, pH 8, 150 mM NaCl, and 0.05% and transferred to nitrocellulose membranes. The membranes were formaldehyde. Paraffin-embedded brain blocks were cut into 5-
m of ice-cold phosphate-buffered saline followed by 200 ml of 10% sections, mounted onto Superfrost Plus slides, dewaxed in xylene, rehydrated in alcohol, and blocked for endogenous peroxidase (3% H2O2 in methanol) and avidin/biotin. Sections were treated with Tris-buffered saline-blocking agent (Tris-buffered saline, pH 7.4, 0.5% blocking agent, 0.3% Triton X-100) with 10% serum of the host of the secondary antibody used, and incubated with COX-2- or COX-1-specific antibody, 1:500 and 1:400 dilution, respectively. Specificity of the antibody was determined by using control sections that were incubated with isotype-matched IgG. Immunoreactive complexes were detected using biotinylated secondary antibody followed by streptavidin-horseradish peroxidase and visualized with the peroxidase substrate diaminobenzidine. Slides were counterstained with hematoxylin.

Statistical Analysis. Results are expressed as mean ± S.E.M. for each group of rats. The Student’s t test was used to determine the significant differences between group means. A p value <0.05 was considered to be statistically significant.

Results

PGE2 and Eicosanoid Formation in KA-Treated Brain. The content of PGE2, TXB2, 6-keto-PGF1α, PGF2α, and PGD2 in brain, spinal cord, and CSF of normal and 24-h KA-treated animals was determined. As shown in Fig. 1A, in brains from KA-treated rats there was a significant rise in the content of PGE2, PGF2α, and PGD2 of, respectively, 7-, 7-, and 3-fold over the levels in the normal brain. In contrast, the profile of eicosanoids in the spinal cord showed a KA-induced increase only in TXB2 and PGD2 (3.5- and 3-fold over the levels in the spinal cord from untreated rats) (Fig. 1B). In the CSF, there was a significant increase in PGF2α in KA-treated animals, but not the other eicosanoids (Fig. 1C). Since the tissue content of PGs may not reflect COX enzyme levels, COX activity was assessed by adding arachidonic acid to tissue homogenates and measuring PGE2 formation. As shown in Fig. 2, there was a slight increase in PGE2 biosynthesis in brains from rats treated for 6 h with KA compared with the normal brain, whereas at 24 h, PGE2 formation was significantly higher, demonstrating substantial increase in COX activity in brains from KA-treated animals. Pretreatment with dexamethasone (1 mg/kg) did not affect PGE2 brain production 6 h after KA treatment but did reduce PGE2 values to normal levels in the 24-h samples.

Induction of COX-2 and PGEs mRNA Expression in CNS after KA Treatment. The TaqMan technique was
used for quantitative analysis of COX-2 and PGEs mRNA levels in normal and KA-treated rat brain, hippocampus, cortex, and spinal cord. Twenty-four hours after KA administration both COX-2 and PGEs mRNA levels were increased in the total brain, cortex, and hippocampus, but not in the spinal cord (Fig. 3, A and B). The highest induction in PGEs mRNA was in the cortex, followed by total brain and hippocampus (10-, 6-, and 4-fold increase, respectively), whereas COX-2 was induced 5-fold in hippocampus and cortex and 4-fold in the total brain, showing a more homogeneous distribution. No changes were found either in COX-1 or PGD synthase mRNA in all the areas investigated (Fig. 3, C and D). Pretreatment of rats with dexamethasone (1 mg/kg) significantly inhibited induction of COX-2 and PGEs mRNA (–40% and –47%, respectively) (Fig. 4). Celecoxib administration had no effect on levels of COX-2 or PGEs mRNA (data not shown).

**COX-2 and PGEs Protein Expression in Different CNS Regions after KA Administration.** Western blot analysis of COX-2 and PGEs proteins in different brain regions showed protein bands at 70 and 16 kDa, 24 h after KA injection (Fig. 5, A and B). COX-2 protein expression was weakly detectable in total brain, cortex, and hippocampus of normal rats, whereas no detectable expression was found in spinal cord; none of the regions studied from normal rats expressed detectable PGEs protein. KA treatment increased COX-2 and PGEs levels in total brain and in different brain regions compared with the expression in normal rat; the only exception was spinal cord, where KA administration did not result in COX-2 or PGEs expression. The KA-induced COX-2 protein levels were greater in hippocampus and cortex than in total brain, with a more intense band for hippocampus than for cortex. On the contrary, KA-induced PGEs expression in total brain, cortex, and hippocampus was almost identical, with a strong band in all three regions.

**KA-Induced COX-2 Immunoreactivity in the Brain.** Immunocytochemical staining of normal cortex showed constitutive expression of COX-2 protein in neurons with faint immunoreactivity, whereas no immunoreactivity was evident in endothelial or glial cells (Fig. 6A). After 24 h of treatment of animals with KA, the cortex section showed increased COX-2 protein expression strictly localized in neurons (Fig. 6B), that was partially inhibited by pretreatment with dexamethasone (Fig. 6C). The increased KA-induced COX-2 expression and dexamethasone modulation could also be observed in hippocampus and almost all other brain areas (Fig. 6, D to F). The COX-2 immunoreactivity was present in the cytoplasmic area and not in the nucleus.

**Effect of Treatment with Celecoxib on PGE2 Concentration in KA-Treated Brain and on Arthritis Paw Edema.** Celecoxib dosed 20 h after KA administration dose
zymes responsible for PGE2 biosynthesis in the CNS, we observed was a direct effect of the inhibitor is not certain since the central effect could be due to a decrease in the stimulus that the CNS receives from the periphery. To better assess the central pharmacology of celecoxib and the enzymes responsible for PGE2 biosynthesis in the CNS, we studied a model of central COX-2 induction driven by kainate injection in the rat, the kainate-induced seizure model. Kainate produces a distinct profile of increased eicosanoid biosynthesis in the brain. Levels of PGE2 and PGF2α were markedly elevated above basal (7-fold), whereas PGD2 increased 3-fold; changes in TxB2 and 6-keto-PGF1α were minimal. Nevertheless, PGD2 levels were still 5- to 10-fold higher than other prostaglandins in both normal and kainate-induced brain. The increases in PGF2α levels were remarkable and confirm previous studies in this model (Simmet and Tippler, 1990). The parallel increases in PGE2 and PGF2α levels at the expense of other prostanoids suggest that specific synthases for PGE and PGF may be coordinately induced in neurons along with COX-2.

The kainate effect on eicosanoid biosynthesis in the spinal cord was not as marked as in brain tissue and showed a distinctly different pattern; substantial elevations in TxB2 and PGD2 were observed with no increases in PGE2 and PGF2α levels. The lack of homogeneity in the eicosanoid profiles after kainate treatment might be explained by either cell type or regional specificity of kainate action or by selective induction of the enzymes downstream of COX-2 in different areas of the CNS. Surprisingly, there was only a slight increase in eicosanoid content in the CSF after kainate treatment, with a profile of eicosanoids that did not resemble either the brain or spinal cord pattern. This suggests that the eicosanoids produced by neurons remain in the tissue or do not reach the point of CSF sampling. The former seems unlikely since PG levels fell in kainate-induced brain 4 h after dosing with celecoxib.

To assess the mechanism of increased prostaglandin content in the KA-treated brain, COX activity was directly assessed in homogenates of brain by direct addition of substrate arachidonic acid. The biosynthetic capacity of the tissue was clearly increased by kainate, and this enhanced enzyme activity was efficiently inhibited by pretreatment of rats with dexamethasone, an inhibitor of de novo COX-2 synthesis. Two inducible proteins are involved in the enzymatic synthesis of PGE2: COX-2 and PGEs. COX-2 and PGEs mRNA expression was assessed in total brain, cortex and hippocampus, and spinal cord. The areas most involved in KA toxicity, cortex and hippocampus, are sites of enhanced COX-2 expression; increases in PGEs mRNA expression generally paralleled that of COX-2, but were more marked in total brain and cortex than in hippocampus. As expected, expression of COX-2 and PGEs protein was generally consis-

Fig. 4. Effect of dexamethasone administration on kainate-induced increase in COX-2 and PGEs mRNA levels in brain. Dexamethasone (1 mg/kg) was orally dosed 1 h before and 8 h after 10 mg/kg kainate injection. At 24 h after kainate administration, brains were harvested and RNA was extracted. mRNA levels were measured by TaqMan PCR. Data are shown as mean ± S.E.M.; n = 5; *, p < 0.05; **, p < 0.01.

Fig. 5. Representative immunoblot analysis of COX-2 and PGEs protein in normal versus kainate-treated brain and spinal cord. Animals were injected with 10 mg/kg kainate and after 24 h, tissues were harvested and processed for the detection of protein levels by electrophoresis. A, COX-2; B, PGEs. Comparison between normal (N) and kainate-treated (KA) tissues.
tent with mRNA expression. No changes in COX-1 mRNA levels were observed, suggesting that the enzymes responsible for the increased PGEs biosynthesis in this model are the inducible proteins, COX-2 and PGEs. The hypothesis of coupling between COX-2 and PGEs is supported by the data of Murakami et al. (2000), who demonstrated that PGEs is colocalized at the microsomal level with both COX-1 and COX-2, and may be preferentially coupled with COX-2 rather than COX-1. Of interest, no changes in mRNA levels for PGD synthase were observed; thus, the increase of the PGD2 content must be due to COX-2 up-regulation. Unlike COX-2, PGEs was not detectable in the normal brain. Consistent with the mRNA data, COX-2 and PGEs proteins were not found in the spinal cord of KA animals; this could be due to insufficient assay sensitivity. Nevertheless, the absence of COX-2 in the spinal cord after kainate stimulation suggests that any increase in level is modest in this tissue.

In these studies, we found that celecoxib markedly reduced the increased PGEs content in brain after kainate treatment, at doses below those needed for anti-inflammatory activity. This suggests that celecoxib can inhibit central COX-2 independent of any peripheral action. Reduction of PG levels to
near baseline was observed 4 h after drug administration, indicating rapid clearance of centrally produced PGs and suggesting continual PG production in this model, similar to results obtained at a peripheral inflammatory site (Zhang et al., 1997).

In conclusion, this study indicates that a profound excitotoxic stimulus, kainate, elicits selective, high-level production of PGE_2 and PGF_2a, in the brain that is accompanied by coordinate high level expression of COX-2 and PGE synthase, with little change in spinal cord enzyme expression. Despite markedly elevated tissue levels of PGs in this model, negligible increases in CSF PGs were observed. The high levels of COX-2 enzymatic activity and tissue PGs found in brains from kainate-treated rats provide a useful model for evaluating the pharmacologic activity of inhibitors of enzymes in the PG biosynthetic pathway.

References


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