Inhibitors of Cyclooxygenase-2, but Not Cyclooxygenase-1 Provide Structural and Functional Protection against Quinolinic Acid-Induced Neurodegeneration

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Received February 3, 2003; accepted April 2, 2003

ABSTRACT

Cyclooxygenases (COXs) are implicated in neurodegenerative processes associated with acute and chronic neurological diseases. Given the potential utility of COX inhibitors in treating these disorders, we examined the nonselective COX inhibitor flurbiprofen, the specific COX-1 inhibitor valeryl salicylate (VS), and the COX-2 inhibitor N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide (NS-398) for their abilities to protect striatal neurons against a quinolinic acid (QA)-induced excitotoxic lesion. Rats were administered COX inhibitors 10 min before a unilateral QA lesion of the striatum, and then tested 2 to 3 weeks later in a battery of motor tasks (bracing, placing, akinesia, and apomorphine-induced rotations). Lesion volume was assessed using immunohistochemical methods 1 month after lesioning. Orally administered flurbiprofen (50 mg) was highly neuroprotective, preserving 84 to 99% of motor performance (ED$_{50}$ = 8.6–9.7 mg) while reducing lesion volume 75% (ED$_{50}$ = 3.2 mg). The identities of the COX isoforms associated with QA-induced neurodegeneration were determined using VS and NS-398. Oral VS was ineffective in virtually all indices of functional neuroprotection. In contrast, oral NS-398 was highly effective, preserving approximately 83% of motor performance at 2 mg (ED$_{50}$ = 0.1–0.4 mg), and reducing lesion volume 100% (ED$_{50}$ = 0.4 mg). Similar results were obtained using inhaled flurbiprofen (2 mg), which preserved 88 to 100% of motor performance while reducing striatal lesion size 92%. These results demonstrate that COX-2 inhibition protects neurons from acute, excitotoxic neurodegeneration. Moreover, formulating a nonselective COX inhibitor into an inhalable preparation dramatically improves its potency in treating acute neuronal damage, a situation where the rapidity of drug delivery and onset of action is critical to clinical efficacy.

One of the first steps in excitotoxic neuronal damage involves the hyperstimulation of N-methyl-D-aspartate receptors (Choi, 1992) leading to a massive Ca$^{2+}$ influx that activates, among other processes, the Ca$^{2+}$-dependent phospholipases A$_2$. These phospholipases A$_2$ cleave membrane phospholipids to yield arachidonic acid, which is converted by cyclooxygenases (COXs; Hurley et al., 2002) into prostaglandin (PG)G$_2$. PGG$_2$ is subsequently reduced to prostacyclin (PGI$_2$) and PGE$_2$, which is metabolized to thromboxane (TXA$_2$) by thromboxane synthase. TXA$_2$ is highly neurotoxic, and may contribute to neurodegeneration. Among the products of COX-1 activity is the neuroprotective prostacyclin (PGI$_2$) which is converted by cyclooxygenases (COXs; Hurley et al., 2002) into prostaglandin (PG)G$_2$. PGG$_2$ is subsequently reduced to prostacyclin (PGI$_2$) and PGE$_2$, which is metabolized to thromboxane (TXA$_2$) by thromboxane synthase. TXA$_2$ is highly neurotoxic, and may contribute to neurodegeneration. Among the products of COX-1 activity is the neuroprotective prostacyclin (PGI$_2$). The identities of the COX isoforms associated with QA-induced neurodegeneration were determined using VS and NS-398. Oral VS was ineffective in virtually all indices of functional neuroprotection. In contrast, oral NS-398 was highly effective, preserving approximately 83% of motor performance at 2 mg (ED$_{50}$ = 0.1–0.4 mg), and reducing lesion volume 100% (ED$_{50}$ = 0.4 mg). Similar results were obtained using inhaled flurbiprofen (2 mg), which preserved 88 to 100% of motor performance while reducing striatal lesion size 92%. These results demonstrate that COX-2 inhibition protects neurons from acute, excitotoxic neurodegeneration. Moreover, formulating a nonselective COX inhibitor into an inhalable preparation dramatically improves its potency in treating acute neuronal damage, a situation where the rapidity of drug delivery and onset of action is critical to clinical efficacy.

ABBREVIATIONS: COX, cyclooxygenase; PG, prostaglandin; CNS, central nervous system; NSAID, nonsteroidal anti-inflammatory drug; FLURBI, flurbiprofen; QA, quinolinic acid; NeuN, neuron-specific nuclear protein; DARPP-32, dopamine and adenosine 3′,5′-monophosphate-regulated phosphoprotein, 32 kDa; HPLC, high-pressure liquid chromatography; ANOVA, analysis of variance; VS, valeryl salicylate.
rodegenerative syndromes has promoted the development of neuroprotective treatment strategies involving COX inhibitors, such as the nonsteroidal anti-inflammatory drugs (NSAIDs). Although epidemiological studies suggest that NSAIDS may be protective in chronic neurodegenerative conditions (McGeer et al., 1996), little is known of their clinical efficacy in treating acute neurodegeneration. If COX inhibition blocks neurodegenerative aspects of excitotoxicity, then neurodegenerative disorders with an excitotoxic component may benefit from this treatment. Moreover, the rapidity of onset of COX inhibition may play a crucial role in protecting neurons impacted by acute neurological insults, such as those associated with ischemia and/or trauma (Dash et al., 2000; Strauss, 2000; Iadeola et al., 2001). This need may be served by formulations of COX inhibitors that can be delivered by the pulmonary route, allowing rapid entry of a drug into the circulation.

In an attempt to determine whether inhibition of COX activity can suppress acute neurodegeneration, we have used an animal model of excitotoxicity (Beal et al., 1991) that allows us to investigate the relative contributions of the COX-1 and COX-2 isoforms to excitotoxic neurodegenerative processes. This was done by comparing the neuroprotective efficacy of a currently prescribed, nonselective NSAID (flurbiprofen) with specific, experimental COX-1 and COX-2 inhibitors (valeryl salicylate and NS-398, respectively). In addition, we examined the neuroprotective efficacy of an NSAID administered using a novel pulmonary delivery system that optimizes the timeliness of delivery while decreasing the neuroprotective dose of the drug.

**Materials and Methods**

Male Fischer 344 rats (80 g; Taconic Farms, Germantown, NY) were used in all studies. Rats were housed in pairs in polypropylene cages with free access to food and water. The vivarium was maintained on a 12-h light/dark cycle (lights on at 7:00 AM) with a room temperature of 22 ± 1°C and relative humidity level of 50 ± 5%. All studies were approved by Alkermes Institutional Animal Care and Use Committee and were conducted in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals; Registration Certificate No. A5896.

**Preparation of Inhalable Flurbiprofen.** Flurbiprofen, excipients (1 g/l), and ammonium bicarbonate (8 g/l) were mixed into a spray drying solution with ethanol/water (70:30 v/v/v) as the solvent. The solution was then introduced into a NIRO spray dryer at 40 ml/min and atomized into droplets with a rotary atomizer at 20,000 rpm. The droplets contact the drying gas and the dry particles collected with a 6-inch cyclone. The final loading density of flurbiprofen in the particles is 20%.

**Drug Administration.** All animals were fasted for 12 h before drug administration. Rats (n = 8) were administered either flurbiprofen (FLURBI; 2, 10, or 50 mg), N-[2-(cylohexyloyloxy)-4-nitrophene-nyl]-methanesulfonamide (NS-398; 2 or 10 mg), valeryl salicylate (2, 10, or 50 mg) by the oral route, or an inhalable formulation of 20% flurbiprofen (2 mg) 10 to 15 min before injection of quinolinic acid (QA). Vehicles consisted of either blank powder for inhalable flurbiprofen, or 1% Tween in distilled water for the orally administered flurbiprofen. Rats (n = 12) were administered 5 mg of powder containing 1 mg of flurbiprofen, or were orally administered 1 mg of flurbiprofen in 1% carboxymethylcellulose solution. Blood samples (500 μl in heparinized tubes) were obtained at 0, 2, 5, 15, 30, 60, 120, 240, and 360 min after administration. Only four samples were taken from each rat. The blood samples were centrifuged, plasma removed, and then rapidly frozen and stored at −80°C until assayed.

Plasma flurbiprofen levels were assayed using HPLC with UV detection. Briefly, rat plasma samples (200 μl) were spiked with ketoprofen as an internal standard and then extracted using Oasis HLB extraction cartridges (Waters, Milford, MA). Samples were loaded onto a conditioned column, washed with 1 ml of deionized water, and then eluted with 1 ml of methanol. The eluate was dried and reconstituted with 1 ml of 0.1% trifluoroacetic acid/acetonitrile (60:40 v/v%). Flurbiprofen and ketoprofen were separated using a Luna C18 (5 μm; 150 × 3.0-mm i.d.; Phenomenex, Torrance, CA) column with a guard column. The column temperature was 35°C and samples were maintained at 25°C with a refrigerated autosampler. The injection volume was 5 μl and the flow rate was 0.4 ml/min. Materials were eluted from the column with a gradient consisting of 0.1% trifluoroacetic acid (A) and 100% acetonitrile (B), using the following parameters. Initial conditions: 50:50; 4 min, 50:50, isocratic; 6 min, 40:60, linear gradient; 10 min, 40:60, isocratic; 11 min, 20:80, linear gradient; and 13 min, 20:80, isocratic. Eluates were detected by monitoring at λ = 254 nm.

**Surgery.** Immediately after drug administration, rats were anesthetized with ketamine (25 mg/kg), xylazine (1.3 mg/kg), and acepromazine (0.25 mg/kg intramuscularly) and positioned in a stereotaxic instrument (Kopf, Tujunga, CA). A midline incision was made in the scalp and a hole drilled through the skull for injection of QA (225 nmol in phosphate-buffered saline) at the following coordinates: 1.2 mm anterior, 2.6 mm lateral to bregma, and 5.5 mm ventral to the surface of the brain (Emeric et al., 1996). QA was infused into the striatum using a 28-gauge blunt-tip syringe (Hewlett Packard, Palo Alto CA) in a volume of 1 μl over 5 min. The injection cannula was left in place for an additional 2 min to allow the QA to diffuse from the needle tip, after which the cannula was removed, the bone window waxed over, and the overlying skin sutured closed. A similar procedure was followed on the contralateral side, with the exception that only vehicle was injected. The rats were then injected with lactated Ringer’s solution (10 ml, subcutaneous) to prevent dehydration and allowed to recover on a heating pad. Surgery was timed so that QA was injected exactly 10 min after administration of COX inhibitors.

**Behavioral Testing.** Four tests were used to measure unilateral motor impairment. Placement and akinesia tests (Schallert and Tillerson, 2000) were performed 27 days after QA lesions. The placement test requires holding a rat parallel to the edge of a tabletop in such a way as to allow it to place its forelimb atop the table in response to stimulation of its whiskers by contact with the table edge. For each trial, the subjects were held with their limbs hanging unsupported and then placed with their bodies parallel to and within the distance of their whiskers (approximately 4 cm) from the edge of the table. Each rat was tested in 10 consecutive trials per forelimb, and the total number of times the rat placed its forelimb on top of the table was recorded. In the akinesia test, the rat was supported on one forelimb and allowed to move independently. The number of “steps” taken with each weighted forelimb was recorded over 30 s. Rats were tested in the braking task 34 days after surgery. Subjects were individually placed on a smooth stainless steel surface and gently pushed laterally a distance of 90 cm at a rate of approximately 20 cm/s. The number of braces made with the forelimb opposing the
direction of movement was recorded. Each trial involved moving the rat twice on each side.

Apomorphine-induced rotations were recorded 4-weeks after surgery to further assess the extent of damage to the striatum (Carman et al., 1991). Rats were administered apomorphine (1 mg/kg s.c.) and then placed into a cylindrical acrylic container. Each 360 degree rotation made by the rat was counted over a 30-min trial period. Partial rotations and reversals were not recorded. Rats were tested in this paradigm once a week for 2 weeks, with the data for the third trial presented under Results. Apomorphine-treated rats with QA-induced lesions of the striatum typically rotate 150 times/30 min (Nakao et al., 1998).

**Histology.** At the conclusion of behavioral testing, all animals were sacrificed for histological analysis. Rats were anesthetized with ketamine, xylazine, and acepromazine solution and then transcardially perfused with heparinized phosphate-buffered saline (5000 U/l, 20 ml, pH 7.4, 0–4°C), followed by 4% Zamboni’s fixative (500 ml, 0–4°C). The brains were then removed, placed in 30% phosphate-buffered sucrose (pH 7.4), and stored (48 h, 0–4°C). Before sectioning, the brains were rapidly frozen in methylbutane (−60°C), mounted on a freezing microtome, and 40-μm-thick sections cut and stored in a solution of 30% sucrose/30% ethylene glycol in phosphate-buffered saline at −20°C until processed for assessment of lesion size and DARPP-32 immunohistochemistry.

**Immunohistochemistry.** Sections were processed for the histochemical visualization of DARPP-32-like or NeuN-like immunoreactivity using biotin-labeled antibodies (Hau et al., 1981). Endogenous peroxidases were eliminated with a 20-min incubation in 0.1 M sodium periodate in Tris-buffered saline. Background staining was suppressed with a 1-h incubation in Tris-buffered saline (pH 7.4) containing 3% normal goat serum, 2% bovine serum albumin, and 0.05% Triton X-100. The sections were then incubated in the primary antibodies, either DARPP-32 (1:25; Cell Signaling Technology Inc., Figure 1.

**Pretreatment with oral FLURBI suppresses the deterioration of motor performance scores in rats receiving QA lesions.** Rats were administered vehicle (Veh) or FLURBI (2, 10, or 50 mg p.o.) 10 min before receiving unilateral QA lesions of the striatum. Data represent the mean ± S.E.M. of motor performance scores of rats (n = 8) in the placement test (A), bracing test (B), akinesia test (C), and apomorphine-induced rotations (D). Performance of the impaired limb (contralateral to brain side receiving lesion) is compared with that of the unimpaired limb (ipsilateral to lesion) in A, B, and C. Rotation scores are compared with vehicle group in D. *p < 0.05 and **p < 0.01, respectively; a and aa, vehicle control group (p < 0.05 and 0.01, respectively) two-way ANOVA + Tukey’s test (A–C), one-way ANOVA + Dunnett’s test (D).

**TABLE 1. Relative potency of COX inhibitors as neuroprotectants**

The ED₅₀ estimates of drug potency were derived using nonlinear regression fitting of a sigmoidal dose-response curve to the data, with the minimum constrained to the value obtained for the mean performance of the lesioned side from the vehicle-treated group, or 0 for lesion size. The maximum value of the curve was constrained to the mean performance of the unlesioned limb, or 0 for lesion size. The maximum value of the curve was constrained to the mean performance of the unlesioned limb, or 0 for lesion size.

<table>
<thead>
<tr>
<th>Assessment</th>
<th>Drug</th>
<th>ED₅₀, 95% CI</th>
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<tr>
<td>Placement test</td>
<td>Flurbiprofen</td>
<td>9.7, 6.5–14.0</td>
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<tr>
<td>Bracing test</td>
<td>Flurbiprofen</td>
<td>8.6, 7.4–9.9</td>
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<tr>
<td>Akinesia test</td>
<td>Flurbiprofen</td>
<td>9.5, 6.5–13.9</td>
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<tr>
<td>Apomorphine-induced rotations</td>
<td>Flurbiprofen</td>
<td>11, 4.6–27.0</td>
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<tr>
<td>Lesion volume</td>
<td>Valeryl salicylate</td>
<td>41, 10–165</td>
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<tr>
<td>DARPP-32 immunoreactivity</td>
<td>Valeryl salicylate</td>
<td>1.6, 0.01–30.0</td>
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N.D., not determined.
Beverly, MA), or NeuN (1:100; Chemicon International, Temecula, CA) for 48 h at room temperature. After several washes, sections were sequentially incubated in the biotinylated IgG secondary antibody (1:200; Vector Laboratories, Burlingame, CA) for 60 min and the avidin-biotin (ABC Elite) substrate (1:500) for 75 min. DARPP-32-immunostained sections were then reacted in a chromogen solution containing Tris-buffered saline, 0.05% 3,3' dianinobenzidine, and 0.005% H2O2. All sections were mounted on chrome alum-treated slides, air-dried, and coverslipped with Permount. Control sections were treated in an identical manner except for the substitution of the primary antibody solvent or an irrelevant IgG matched to the protein concentration of the primary antibody.

**Volumetric Measurements.** Lesion volume was quantified using a point counting procedure, (Cavalieri procedure; Gundersen and Jensen, 1987) and StereoInvestigator software (MicroBrightField, Colchester, VT). Stereological measures were made using a microscope (BX-60; Olympus, Tokyo, Japan), integrated with a computer-controlled three-dimensional motorized stage (Ludl Electronic Products, Hawthorne, NY), and a high-sensitivity charge-coupled device videocamera system (Hitachi, Tokyo, Japan). Every sixth section throughout the rostrocaudal extent of the NeuN-stained lesion was analyzed. The StereoInvestigator software internally calculates the total volume for each case. Because of the shrinkage of the lesioned striatum over time, we

**Fig. 2.** Photomicrographs of NeuN-immunoreactive neurons in the rat striatum. Note the almost complete loss of NeuN-positive neurons in the section from a rat receiving a unilateral QA injection and vehicle treatment (A and E). In contrast, substantial neuroprotection is seen in QA-lesioned rats receiving 2 (B and F), 10 (C and G), or 50 (D and H) mg of oral flurbiprofen. The high magnification photomicrographs in E–H were taken from the corresponding areas enclosed by the squares illustrated in A–D. Bar, 2500 μm (A–D); 125 μm (E–H).
normalized the volume of the lesioned striatum \((V_N)\) to the intact side in all animals by applying the formula \(V_N = V_{\text{Lesion}} \cdot (V_{\text{Intact side}} / V_{\text{Lesion side}})^{-1}\).

Neuronal Counts. Counts of DARPP-32-immunopositive neurons were performed using the MicroBrightfield stereological software and stereological principles (Gundersen et al., 1988). The total number of neurons was estimated by the optical fractionator method using a 100× plan-apo oil immersion objective with a 1.4 numerical aperture. For each tissue section analyzed, section thickness was assessed empirically (approximately 12.5 μm), and upper and lower "guard zones" with a 4- to 5-μm thickness were established before measurements. The striatum was outlined under a low magnification (4×) and approximately 5% of the outlined region was analyzed using a systematic random sampling design. The total number of neurons \((N)\) for each case was calculated using the following formula: \(\Sigma N_{\text{Total}} = (N_1 + N_2 + N_3) \cdot 12\). The coefficients of error were calculated according to the procedure of Gundersen and colleagues as estimates of precision. Values of \(<0.10\) were accepted (West et al., 1996).

Data Analysis. The \(ED_{50}\) estimates of drug potency in the placement, akinesia, and bracing tests were derived using nonlinear regression fitting of a sigmoidal dose-response curve to the data (GraphPad Prism; GraphPad Software Inc., San Diego CA). The \(ED_{50}\) estimates of drug potency were derived using nonlinear regression fitting of a sigmoidal dose-response curve to the data, with the minimum constrained to the value obtained for the mean motor performance of the lesioned side from the vehicle-treated group, or zero for lesion size. The maximum value of the curve was constrained to the mean motor performance of the lesioned side from the vehicle-treated group, or zero for lesion size. The maximum value of the curve was constrained to the mean level of performance by the unlesioned limb, or the size of the lesion from the vehicle-treated group. The significance of the differences between populations was assessed using multiway ANOVA followed by Bonferroni’s post hoc comparison matrix, except for the placement test, on which Kruskal-Wallis tests were performed as the nature of scoring the placement task skews the distribution.

Results

Rats receiving vehicle treatment before the QA lesion showed significant deficits in the performance of the placement, bracing, and akinesia tests by the limb contralateral to the lesioned side. This was evidenced by an average decrease of 94 ± 1.1, 31 ± 6.9, and 56 ± 3.1% in the number of placements, braces, and steps taken, respectively, relative to the limb ipsilateral to the lesioned hemisphere. In the apomorphine-induced rotation test, all vehicle-treated animals rotated 140 ± 8.1 times in 30 min. Lesion volumes in vehicle-treated animals were 6.1 ± 0.6 mm³ and survival of DARPP-32-immunoreactive neurons was 29.7 ± 5.5%.

Orally administered flurbiprofen dose dependently spared rats from QA-induced decrements in the performance of a number of motor assessments (Fig. 1; Table 1). Specifically, the number of placements by the impaired limb of rats treated with 50 mg of flurbiprofen was not significantly different from the unlesioned side (Fig. 1A), constituting a 74% decrease in lesion volume compared with vehicle-treated rats \((p < 0.01)\), with no significant differences between the three doses (Table 1; Figs. 2 and 3A). Furthermore, the number of DARPP-32-immunoreactive neurons was 290, 340, and 355% higher in the striata of rats receiving 2 (\(p < 0.05\)), 10 (\(p < 0.01\)), and 50 mg \((p < 0.01)\) of flurbiprofen compared with vehicle-treated animals (Fig. 3B).

After establishing the neuroprotective efficacy of flurbiprofen in this model, we attempted to enhance its pharmacokinetics by formulating flurbiprofen into inhalable microparticles. Comparison of the plasma pharmacokinetics of flurbiprofen (1 mg) administered through oral and pulmonary routes of delivery indicated that inhaled flurbiprofen yielded maximal plasma levels of 79 ± 9.0 to 84 ± 7.3 μg/ml (statistically indistinguishable) by 2 to 5 min after inhalation (Fig. 4). Detectable levels of inhaled flurbiprofen (9.5 ± 1.5 μg/ml) were found in the plasma as long as 6 h after administration, with the area under the curve equal to 9310 μg ml⁻¹ min. In contrast, 1 mg of flurbiprofen administered orally reached a maximum plasma level of 3.6 ± 0.6 μg/ml by...
5 min after dosing. Plasma levels six h after oral administration were maintained at approximately 3 \( \mu \text{g/ml} \), with the area under the curve equal to 1260 \( \mu \text{g} \cdot \text{ml}^{-1} \). Administration of 2 mg of flurbiprofen via the pulmonary route fully protected limb performance as assessed in the placement task (Fig. 5A). Similar results were observed in the akinesia test (Fig. 5C) and apomorphine-induced rotations (Fig. 5D), where performance levels were not significantly different from unlesioned animals. Interestingly, the bracing task did not reveal any lesion effect in the animals administered inhalable flurbiprofen or blank powder (Fig. 5B). Consistent with the behavioral data, the lesion volumes of rats insufflated with flurbiprofen (Figs. 6C and 7A) were 92% smaller than those in vehicle-treated animals (Figs. 6, A and B, and 7A), and there was a 74% increase in survival of DARPP-32-immunoreactive neurons (Figs. 6, D–F, and 7B).

To determine which isofrom of COX was responsible for the neuroprotective efficacy of flurbiprofen, the effects of selective COX inhibitors were investigated. The COX-1-selective agent valeryl salicylate exerted no notable neuroprotective effects. Even at the highest dose tested (50 mg), limb performance was not significantly improved in either the placement or akinesia tasks (Fig. 8, A and C), or in the number of apomorphine-induced rotations (Fig. 8D). Only in the bracing test did the 50-mg dose of VS improve motor function to levels observed on the unlesioned side (\( p < 0.05 \)).

In contrast, rats receiving either 2 or 10 mg of the COX-2-selective inhibitor NS-398 orally before the QA lesion showed significant sparing of limb performance in the placement task compared with vehicle-treated animals (Fig. 9A; Table 1). The 10-mg dose of NS-398 fully protected limb function on the side contralateral to the QA injection. Similar results
were observed in the performance of the bracing (Fig. 9B) and akinesia tests (Fig. 9C), where rats pretreated with 10 mg of NS-398 demonstrated contralateral limb performance that was not significantly different from that of the ipsilateral limb. NS-398 also dose dependently reduced the number of apomorphine-induced rotations relative to vehicle-treated rats (Fig. 9D). Stereological analysis indicated that 2 mg of NS-398 resulted in a lesion volume 90% smaller than vehicle (p < 0.01), whereas no lesions were apparent after pretreatment with 10 mg of NS-398 (Figs. 10, A–C, and 11A). Similarly, the 2- and 10-mg doses of NS-398 significantly increased DARPP-32 immunoreactivity compared with vehicle treatment (p < 0.01; Figs. 10, D–F, and 11B).

Discussion

High levels of COX-2 expression are routinely observed in association with acute and chronic neurodegenerative diseases. Excitotoxic processes seem to enhance COX-2 activity, both through increased production of substrates and by elevating COX-2 expression, thereby exacerbating neuronal damage through free-radical and prostanoid-mediated mechanisms (Kukreja et al., 1986; Kelley et al., 1999; Manev et al., 2000). Given the close association of COX-2 with neurodegenerative states, we set about comparing the relative neuroprotective efficacy of the currently prescribed, nonselective COX-1/COX-2 inhibitor flurbiprofen, as well as selective inhibitors of COX-1 and COX-2 in an animal model of acute, excitotoxic neurodegeneration.

The mixed COX-1/COX-2 inhibitor flurbiprofen was found to be a highly efficacious neuroprotectant from both histological and behavioral standpoints. COX-1 activity apparently has little involvement in excitotoxic neurodegeneration because the COX-1 selective inhibitor VS showed little or no neuroprotective efficacy over the dose range tested, which should be sufficient to completely inhibit COX-1 activity (Bhattacharyya et al., 1995). Moreover, COX-1 does not seem to produce any neuroprotective agents in the short term (Teismann and Ferger, 2001; Zhang and Rivest, 2001; Lin et al., 2002) because inhibition of COX-1 by VS neither increased lesion size nor impaired motor performance to a greater extent than vehicle treatment. In contrast, the selective COX-2 inhibitor NS-398 was a very potent and efficacious suppressor of excitotoxic neurodegeneration in vivo. This was manifested not only by a significant reduction in lesion area and preservation of DARPP-32-immunoreactivity neurons, but also functionally, as indicated by the preserva-

Fig. 6. Photomicrographs of NeuN- (left) and DARPP-32 (right)-immunoreactive neurons in the striata of rats receiving unilateral QA injections. The high-power photomicrographs (insert) are from the area enclosed in the square in the corresponding panels. Sections from animals who received either no additional treatment (A and D) or vehicle (B and E) before QA administration showed a significant depletion of immunoreactive neurons (a–e). In contrast, pretreatment with 2 mg of flurbiprofen (FLURBI) administered via pulmonary insufflation provided significant neuroprotection against QA-induced excitotoxic damage (c and f). Scale bar, 1.75 mm (A–F), 85 μm (a–f).
before receiving unilateral lesions of the striatum with QA. Veh or an inhalant formulation containing 2 mg of FLURBII 10 min received only QA or were insufflated with either the inhalant vehicle

Significant role for COX-2 in acute neurodegeneration involv- (Carman et al., 1991). Nonetheless, these data support a

between fully healthy versus functionally impaired neurons

function present despite histological evidence of relatively small lesions. This may reflect the influence of environmental factors upon behavioral performance at any given time, as well as the inability of the histological markers to distinguish between fully healthy versus functionally impaired neurons (Carman et al., 1991). Nonetheless, these data support a significant role for COX-2 in acute neurodegeneration involving excitotoxic processes (Hewett et al., 2000).

The potent and significant neuroprotection offered by both NS-398 and flurbiprofen that could be administered by inhalation. Pharmacokinetic studies indicated that the C{\text{max}} for inhaled flurbiprofen (1 mg) was 24 times higher than an equivalent oral dose. Moreover, the increase in plasma levels after pulmonary administration of flurbiprofen was too rapid to accurately resolve. Although the T_{\text{max}} for oral flurbiprofen was also on the order of 5 min, the oral formulation used in this study (flurbiprofen in aqueous Tween) is a departure from the typical tablet or capsule, which would require more time to dissolve and be systemically absorbed (T_{\text{max}}: 0.7–2 h; Davies, 1995). Therefore, it seems that not only does the pulmonary delivery route allow rapid entry of flurbiprofen into the circulation, it achieves higher plasma concentrations than an equivalent, orally administered dose (Davies, 1995).

The pharmacodynamic characteristics of the inhalable flurbiprofen formulation are as dramatic as its pharmacokinetics. Inhalation of 2 mg of flurbiprofen afforded almost complete neuroprotection, as indicated by >90% retention of motor function relative to the unlesioned side, and >90% reduction of lesion volume compared with vehicle-treated control. Moreover, 2 mg of inhaled flurbiprofen was 2 to 6 times more effective than the same dose administered orally, which was only slightly more efficacious from a neurobehavioral standpoint than vehicle. Indeed, oral administration of 50 mg of flurbiprofen was necessary to provide almost complete neuroprotection (66–86% preservation of behavioral and histological indices). Together, the pharmacokinetic and pharmacodynamic observations indicate that the pulmonary route of drug administration is capable of rapidly delivering an agent into the circulation and hence, the brain, whereas achieving higher plasma levels than oral administration. Moreover, acute, as opposed to chronic, administration of a COX-2 inhibitor in close temporal association with the onset of a neurological insult may prove to be the most effective way to minimize neuronal damage (Gilroy et al., 1999; Dash et al., 2000).

though COX-2 inhibitors were acutely neuroprotective in the above-mentioned studies, selective inhibition of COX-2 may present liabilities. Selective COX-2 inhibition increases chemotactic eicosanoid (leukotriene B_{4}) formation via 5-lipoxygenase, possibly as a compensatory response to the anti-inflammatory effects of COX-2 inhibition. COX-2 selective inhibitors also carry the burden of unwanted cardiovascular effects, resulting from the unbalanced inhibition of cyclooxygenases (Cheng et al., 2002). Furthermore, the efficacy of selective COX-2 inhibitors in treating chronic neurodegeneration has yet to be established in clinical trials, despite the apparent effectiveness of the nonselective COX inhibitors (Rogers et al., 1993; McGeer et al., 1996; Stewart et al., 1997; Veld et al., 2000; Zandi et al., 2002). Together, these observations support the consideration of nonselective COX inhibitors for the treatment of neurodegenerative syndromes.

The promising therapeutic potential of NSAIDs for treating various neurodegenerative diseases raises the question of how to optimize the delivery method of these drugs to effectively protect neurons, particularly against acute CNS insults. For example, enhancing the rapidity of onset of an NSAID may make a critical difference in preserving neurons after acute trauma or ischemic attacks. Given that drug administration via pulmonary pathways often yields pharmacokinetics comparable with those of intravenous delivery (Vanbever et al., 1999), we created a formulation of flurbiprofen that could be administered by inhalation. Pharmacokinetic studies indicated that the C_{\text{max}} for inhaled flurbiprofen (1 mg) was 24 times higher than an equivalent oral dose. Moreover, the increase in plasma levels after pulmonary administration of flurbiprofen was too rapid to accurately resolve. Although the T_{\text{max}} for oral flurbiprofen was also on the order of 5 min, the oral formulation used in this study (flurbiprofen in aqueous Tween) is a departure from the typical tablet or capsule, which would require more time to dissolve and be systemically absorbed (T_{\text{max}}: 0.7–2 h; Davies, 1995). Therefore, it seems that not only does the pulmonary delivery route allow rapid entry of flurbiprofen into the circulation, it achieves higher plasma concentrations than an equivalent, orally administered dose (Davies, 1995).

The pharmacodynamic characteristics of the inhalable flurbiprofen formulation are as dramatic as its pharmacokinetics. Inhalation of 2 mg of flurbiprofen afforded almost complete neuroprotection, as indicated by >90% retention of motor function relative to the unlesioned side, and >90% reduction of lesion volume compared with vehicle-treated control. Moreover, 2 mg of inhaled flurbiprofen was 2 to 6 times more effective than the same dose administered orally, which was only slightly more efficacious from a neurobehavioral standpoint than vehicle. Indeed, oral administration of 50 mg of flurbiprofen was necessary to provide almost complete neuroprotection (66–86% preservation of behavioral and histological indices). Together, the pharmacokinetic and pharmacodynamic observations indicate that the pulmonary route of drug administration is capable of rapidly delivering an agent into the circulation and hence, the brain, whereas achieving higher plasma levels than oral administration. Moreover, acute, as opposed to chronic, administration of a COX-2 inhibitor in close temporal association with the onset of a neurological insult may prove to be the most effective way to minimize neuronal damage (Gilroy et al., 1999; Dash et al., 2000).

The powerful and significant neuroprotection offered by both NS-398 and flurbiprofen is consistent with the involvement of COX-2 in inflammation and neurodegeneration. Both mixed and COX-2-selective inhibitors suppressed neuronal death in an 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mouse model of Parkinson’s disease (Teismann and Ferger 2001), with the COX-2 inhibitor being more potent. Similarly, NS-398 blocks LPS- and NMDA-induced neuron death in vitro (Hewett et al., 2000; Araki et al., 2001). Al-
Fig. 8. Orally administered valeryl salicylate is less effective than flurbiprofen or NS-398 in mitigating the motor performance deficits of rats receiving QA lesions. Rats received either vehicle (Veh) or valeryl salicylate (2, 10, or 50 mg p.o.) 10 min before receiving unilateral lesions of the striatum with QA. Data represent the mean ± S.E.M. of motor performance scores of rats (n = 8) in the placement test (A), bracing test (B), akinesia test (C), and apomorphine-induced rotations (D). Performance of the impaired limb ( Contralateral to brain side receiving lesion) is compared with that of the unimpaired limb ( Ipsilateral to lesion) in A, B, and C. Rotation scores are compared with vehicle group in D. * and **, performance is significantly different from corresponding unimpaired limb (p < 0.05 and 0.01, respectively) two-way ANOVA + Tukey’s test.

Fig. 9. Orally administered NS-398 improves the motor performance scores of rats receiving QA lesions. Rats received either vehicle (Veh) or NS-398 (2 or 10 mg p.o.) 10 min before receiving unilateral lesions of the striatum with QA. Data represent the mean ± S.E.M. of motor performance scores of rats (n = 8) in the placement test (A), bracing test (B), akinesia test (C), and apomorphine-induced rotations (D). Performance of the impaired limb (Contralateral to brain side receiving lesion) is compared with that of the unimpaired limb (Ipsilateral to lesion) in A, B, and C. Rotation scores are compared with vehicle group in D. * and **, performance is significantly different from corresponding unimpaired limb (p < 0.05 and 0.01, respectively); a, vehicle control group (p < 0.01) two-way ANOVA + Tukey’s test (A–C), one-way ANOVA + Dunnett’s test (D).
The underlying mechanisms responsible for the profound neuropsychervation observed after the pulmonary administration of such small amounts of flurbiprofen remain unclear. After an acute insult to the brain, COX-2 expression increases in two phases. Initially, glutamate receptor activation rapidly increases neuronal COX-2 expression (Hewett et al., 2000; Manev et al., 2000) and the production of reactive oxygen species. In addition to suppressing necrosis, reducing free-radical damage to the mitochondria by COX-2 inhibition would reduce the probability of neuronal apoptosis manifested many days after the initial insult (Luetjens et al., 2000). Subsequent to this initial insult, cellular inflammatory processes would increase the amount of active COX-2 available at the lesion site (Luo et al., 1998), expanding the neurodegeneration beyond the initial area impacted (Barone and Feuerstein, 1999). Based on our observations, rapid suppression of the initial, glutamate receptor-stimulated activation of COX-2 expression by inhaled flurbiprofen seems sufficient to reduce the immediate neuronal damage that would trigger subsequent neurodegeneration by inflammatory mechanisms. Therefore, an inhalable, rapidly acting preparation of flurbiprofen holds promise as a neuroprotectant in cases where the time to achieve effective concentrations in the target organ is a critical factor, as in acute CNS insults.

In summary, the current investigation compares the efficacy of three different classes of COX inhibitors dispensed using two different administration modalities to an animal model of excitotoxic neurodegeneration. We demonstrate significant histological preservation and functional protection with both nonselective and COX-2-selective inhibitors, with even more robust effects achieved by using pulmonary over oral routes of administration. Thus, an inhalable formulation of a NSAID may have a significant impact on the severity of acute neurological insults, such as stroke and trauma (Hurely et al., 2002), where time is critical in establishing a therapeutically effective dose and where COX-2 activity in the CNS is the target.

References

Fig. 10. Photomicrographs of NeuN-immunoreactive neurons in the rat striatum. The high-magnification photomicrographs in E–H were taken from the areas enclosed by the squares illustrated in the corresponding A–D. Note the almost complete loss of NeuN-positive neurons in the section from a rat treated with vehicle before receiving a unilateral QA injection (A and D). In contrast, almost complete neuroprotection is seen in QA-lesioned rats receiving 2 (B and E) or 10 (C and F) mg of oral NS-398. Scale bar, 2200 μm (A–C); 110 μm (B, D, and F).

Fig. 11. Histological indices of QA-induced neurodegeneration are reduced in rats treated with orally administered NS-398. Data represent the mean ± S.E.M. of lesion volume (A) and DARPP-32 immunoreactivity (B) of rats (n = 10) administered either vehicle or NS-398 (2 and 10 mg) 10 min before receiving unilateral lesions of the striatum with QA. *, **, and *** indices are significantly different from the vehicle control group (p < 0.05 and 0.01) two-way ANOVA + Dunnett’s test. Dashed line equals control level.


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