

# Cyclooxygenase-2 Contributes to *N*-Methyl-D-aspartate-Mediated Neuronal Cell Death in Primary Cortical Cell Culture<sup>1</sup>

SANDRA J. HEWETT, TRACY F. ULIASZ, ANIRUDDHA S. VIDWANS, and JAMES A. HEWETT

Department of Pharmacology, Program in Neuroscience, University of Connecticut Health Center, Farmington, Connecticut

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## ABSTRACT

Cyclooxygenase isozymes (COX-1 and COX-2) are found to be constitutively expressed in brain, with neuronal expression of COX-2 being rapidly induced after numerous insults, including cerebral ischemia. Because overactivation of *N*-methyl-D-aspartate (NMDA) receptors has been implicated in the cell loss associated with ischemia, we characterized the expression of the COX isozymes in murine mixed cortical cell cultures and used isozyme-selective inhibitors to determine their relative contribution to NMDA receptor-stimulated prostaglandin (PG) production and excitotoxic neuronal cell death. Immunocytochemical analysis of mixed cortical cell cultures revealed that COX-2 expression was restricted to neurons, whereas COX-1 was expressed in both neurons and astrocytes. Brief exposure to NMDA (5 min; 100  $\mu$ M) elicited a time-dependent accumulation of PGs in the culture medium that preceded neuronal cell death and correlated with the induction of COX-2 mRNA.

COX-1 expression remained unchanged. Flurbiprofen, a non-selective COX-1/COX-2 inhibitor, blocked NMDA-stimulated PG production and attenuated neuronal death in a concentration-dependent manner. Similar results were obtained with the specific COX-2 inhibitor NS-398 (10–30  $\mu$ M) but not with the selective COX-1 inhibitor valeryl salicylate (10–300  $\mu$ M). Inhibition of total constitutive COX activity with aspirin (100  $\mu$ M, 1.5 h) before NMDA exposure did not prevent subsequent NMDA-mediated neuronal cell death. However, neuronal injury in aspirin-pretreated cultures was attenuated by flurbiprofen administration after NMDA exposure. Finally, the protection afforded by COX-2 inhibition was specific for NMDA because neither flurbiprofen nor NS-398 protected neurons against kainate-mediated neurotoxicity. Together, these results support the conclusion that newly synthesized COX-2 protein contributes to NMDA-induced neuronal injury.

Free arachidonic acid is metabolized to prostaglandins (PGs) and thromboxanes via the enzyme cyclooxygenase (COX; for a review, see Smith et al., 1991). There are two known COX isoforms, COX-1 and COX-2, which are 90% similar in amino acid sequence and 60% homologous (Smith and DeWitt 1995). Although the isoforms catalyze the same reaction, the genes encoding the different isoforms differ in their regulation at the transcriptional level. COX-1 is constitutively synthesized in many tissues, whereas COX-2, which is normally undetectable in most tissues, can be rapidly induced by proinflammatory cytokines *in vitro* or after inflammatory insults *in vivo* (for a review, see Hla et al., 1999). Curiously, both isoforms appear to be constitutively expressed in normal rat forebrain neurons but not astrocytes (Yamagata et al., 1993; Breder et al., 1995). Furthermore, neuronal COX-2 expression *in vivo* is rapidly induced by *N*-methyl-D-aspartate (NMDA) receptor-dependent synaptic

activity (Yamagata et al., 1993; Miettinen et al., 1997), after seizures (Yamagata et al., 1993; Adams et al., 1996), by direct excitotoxin injection (Adams et al., 1996; Miettinen et al., 1997), by spreading depression (Miettinen et al., 1997), and by cerebral ischemia (Collaco-Moraes et al., 1996; Miettinen et al., 1997; Nogawa et al., 1997; Nakayama et al., 1998). In addition to the animal studies described, up-regulation of COX-2 has been reported to occur in human brain after a lethal cerebral ischemic insult (Iadecola et al., 1999). These data suggest a potential role for COX-2 in activity-dependent neuronal plasticity and in hypoxia- or excitatory amino acid-induced neuronal cell death.

With respect to the latter, brain cells rapidly release arachidonic acid from cellular membrane phospholipids after ischemia *in vivo* and NMDA-mediated excitotoxicity *in vitro* (Yoshida et al., 1986; Dumuis et al., 1988; Sanfeliu et al., 1990). Furthermore, preischemic administration of COX but not lipoxygenase inhibitors ameliorated delayed hippocampal CA1 neuronal death in gerbils after transient forebrain ischemia (Sasaki et al., 1988; Nakayama et al., 1998). In rats,

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**ABBREVIATIONS:** PG, prostaglandin; COX, cyclooxygenase; ASA, aspirin; CS, calf serum; ir, immunoreactivity; LDH, lactate dehydrogenase; GFAP, glial fibrillary acidic protein; MS, media stock; MEM, modified Eagle's medium; NGS, normal goat serum; NSE, neuron-specific enolase; NMDA, *N*-methyl-D-aspartate; PCR, polymerase chain reaction; PPAR, peroxisome proliferator-activated receptor; PLA<sub>2</sub>, phospholipase A<sub>2</sub>; PI, propidium iodide.

nonselective inhibition of COX reduced brain infarct volume after transient but not permanent forebrain ischemia (Cole et al., 1993). More recently, selective inhibition of COX-2 protected against both global and focal ischemia in rats (Nogawa et al., 1997; Nakayama et al., 1998). Together, these results imply that COX-2 contributes to the demise of central nervous system neurons during an ischemic insult. However, a direct link between neuronal COX-2 activity and cell death remains to be demonstrated.

Because the overactivation of glutamate receptors, particularly of the NMDA subtype, has been implicated in the processes that underlie cell loss associated with ischemia, the goal of this study was to determine the relative contribution of COX-1 and/or COX-2 to NMDA-stimulated prostaglandin production in murine mixed cortical cell cultures and to test the hypothesis that COX-2 activity in neurons specifically contributes to excitotoxic neuronal injury.

## Materials and Methods

**Cell Culture.** Mixed cortical cultures containing both neurons and astrocytes were prepared from postnatal and fetal mice. Briefly, astrocytes were first obtained from aseptically dissected cerebral cortices of 1- to 3-day-old postnatal pups (CD-1; Charles River Laboratories, Wilmington, MA) and plated onto Falcon Primaria (Becton Dickinson, Lincoln Park, NJ) 15-mm multiwell dishes in media stock (MS) supplemented with 10% FBS (Hyclone, Logan, UT), 10% calf serum (CS; Hyclone), 10 ng/ml epidermal growth factor (Life Technologies, Grand Island, NY), and 50 I.U./ml penicillin, 50  $\mu$ g/ml streptomycin (Life Technologies). MS is composed of modified Eagle's medium (MEM, Earle's salts; Mediatech, Herndon, VA) supplemented with 2 mM glutamine and 20 mM glucose. Confluent astrocyte monolayers (9–11 days in vitro) were exposed to 8  $\mu$ M cytosine arabinoside (Sigma Chemical Co., St. Louis, MO) for 2 days to inhibit and eliminate the growth of microglia, macrophages, and oligodendrocytes. Cells were maintained thereafter in maintenance media (MS plus 10% CS and antibiotics). Cortical neurons were obtained from the cerebral cortices of embryonic day 15 animals and plated at a density of 3.0 to 3.5 hemispheres/plate/10 ml on an established astrocyte monolayer (12–24 days in vitro) in MS supplemented with 5% CS and 5% FBS. After 5 to 7 days in vitro, mixed cultures were exposed to 8  $\mu$ M cytosine arabinoside for 2 days. Cells were then shifted into maintenance medium, and the medium was changed twice weekly. Experiments were performed on mixed cultures between 14 and 16 days in vitro. All cultures were kept at 37°C in a humidified 6% CO<sub>2</sub>-containing atmosphere.

**Western Blot Analysis.** To test COX antibody isozyme specificity, COX-2 and COX-1 enzymes obtained from stimulated murine macrophage lysate (0.5  $\mu$ g; Transduction Laboratories, Lexington, KY) and ram seminal vesicles (0.25  $\mu$ g; Cayman Chemical, Ann Arbor, MI), respectively, were subjected to SDS-7.5% polyacrylamide gel electrophoresis and transferred to nitrocellulose. Membranes were incubated overnight at 4°C in TTBS (pH 7.4) consisting of 25 mM Tris-buffered saline, 0.1% Tween 20, and 10% nonfat dry milk. After blocking endogenous biotin sites with Avidin-Biotin Block (20 min, 25°C; Vector Laboratories, Burlingame, CA), membranes were incubated with primary antibody (Cayman Chemical) to either COX-2 (rabbit polyclonal, 1:5000) or COX-1 (mouse monoclonal 1:5000 or rabbit polyclonal 1:5000). Blots were sequentially incubated with appropriate species-specific biotinylated secondary antibodies (1:4000; Vector Laboratories) and streptavidin-linked horseradish peroxidase (1:20,000; Zymed, South San Francisco, CA), and results were visualized on X-ray film by chemiluminescence (ECL Western blotting kit and Hyperfilm; Amersham, Arlington Heights, IL).

**Immunocytochemistry.** COX-1 and COX-2 proteins were detected in cultures by indirect immunofluorescence. Thirteen-day-old mixed cultures and 3- to 4-week-old astrocyte cultures were fixed with a freshly prepared solution of 50% methanol, 50% acetone (15 min) and permeabilized with 0.25% Triton X-100 in 10 mM PBS (7 min). After blocking with 10% normal goat serum (NGS) in PBS (4°C overnight or 2 h at 25°C), cultures were double labeled (4°C overnight or 2 h at 25°C) with mouse anti-COX-1 (1:100; Cayman Chemical) and either rat anti-gial fibrillary acidic protein (GFAP; 1:1000; Zymed) or rabbit anti-neuron-specific enolase (NSE; DiaSorin, Stillwater, MN) to detect COX-1 in astrocytes and/or neurons, respectively. COX-1, GFAP, and NSE were visualized after a 1-h (25°C) incubation with goat anti-mouse CY3 (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA), goat anti-rat Alexa (1:400; Molecular Probes, Eugene, OR), and goat anti-rabbit Bodipy FL (1:75; Molecular Probes), respectively.

To assess COX-2 expression in neurons, cultures were stained for NSE and COX-2 in series because both antibodies were of rabbit origin. Cultures were first labeled with rabbit anti-NSE and goat anti-rabbit Bodipy FL as above, refixed with Triton X-100, and reblocked in 10% NGS/PBS. Next, cultures were labeled with rabbit anti-COX-2 (1:200 Cayman Chemical; 2 h, 25°C) followed by goat anti-rabbit CY3 (1:200; Jackson ImmunoResearch Laboratories; 1 h, 25°C). No CY3 immunofluorescence was detected in cultures when anti-COX-2 was omitted. To determine the presence of COX-2 in astrocytes, cultures were double labeled with rabbit anti-COX-2 and rat anti-GFAP, followed by goat anti-rabbit CY3 and goat anti-rat Alexa as described earlier.

All antibodies were diluted in PBS containing 2% NGS. Images were acquired with an Olympus IX-70 microscope outfitted with epifluorescence and a Spot CCD camera (Diagnostic Instruments, Inc., Sterling Heights, MI) and processed using Adobe Photoshop software.

**Drug Exposure.** Exposure to NMDA (Sigma Chemical Co.) either alone or in the presence of other compounds was carried out for 5 min at room temperature in a HEPES-buffered salt solution containing 120 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl<sub>2</sub>, 1.8 mM CaCl<sub>2</sub>, 20 mM HEPES, 15 mM glucose, and 0.01 mM glycine (pH 7.4). After 5 min, the exposure solution was washed away (3  $\times$  750  $\mu$ l) and replaced by MS supplemented with glycine (0.01 mM). Exposure to kainate (Sigma Chemical Co.) alone or in the presence of other compounds as indicated was carried out at 37°C in MS. MK-801 (10  $\mu$ M; Research Biochemicals Inc., Natick, MA) was included with kainate to prevent NMDA receptor activation after the release of endogenous glutamate.

**Measurement of COX Metabolites.** Mixed cortical cell cultures were pretreated with 30  $\mu$ M arachidonic acid (BIOMOL, Plymouth Meeting, PA) 1 to 2 h before NMDA exposure (5 min, 100  $\mu$ M). Supernatants were collected at the times indicated after NMDA exposure and frozen at -80°C. The amount of COX metabolites (total PGs) accumulated in the cell culture supernatants was measured at room temperature via enzyme immunoassay according to the manufacturer's instructions (Cayman Chemical). For the time course, PG levels from wash controls at each time point were subtracted from the levels in experimental conditions to yield PG production specific to NMDA stimulation. Arachidonic acid was prepared as a 12 mM stock solution in DMSO under anaerobic conditions.

**Assessment of Neuronal Cell Injury.** In most cases, neuronal cell death was quantitatively assessed by the measurement of lactate dehydrogenase (LDH) released into the cellular bathing medium 20 to 24 h after experimentation. LDH activity was quantified by the rate of oxidation of NADH, which was followed spectrophotometrically at 340 nm (Koh and Choi 1987). The small amount of LDH present in the medium of parallel cultures subjected to sham wash (generally <15% of total) was subtracted from the levels in experimental conditions to yield the LDH activity specific to experimental injury. This specific efflux of LDH is linearly proportional to the number of neurons damaged or destroyed (Koh and Choi, 1987).

Activity was either scaled to the mean value obtained after a 5-min exposure to NMDA alone (set at 100%) or expressed as the percentage of total neuronal LDH activity (100%), which was determined in each experiment by assaying the supernatant of parallel cultures exposed to 300  $\mu$ M NMDA for 20 to 24 h. In addition, the time course of NMDA-induced neuronal cell death was assessed via propidium iodide (PI) staining (Molecular Probes). PI (10  $\mu$ g/ml, 10 min) was added to culture wells at intervals ranging from 10 min to 4.5 h after NMDA exposure. The PI was removed by gentle washing, and cultures were fixed with 4% paraformaldehyde in PBS (20 min). Images of PI fluorescence were acquired with an Olympus IX-70 microscope outfitted with epifluorescence and a Spot CCD camera (Diagnostic Instruments, Inc.) and processed using Adobe Photoshop software.

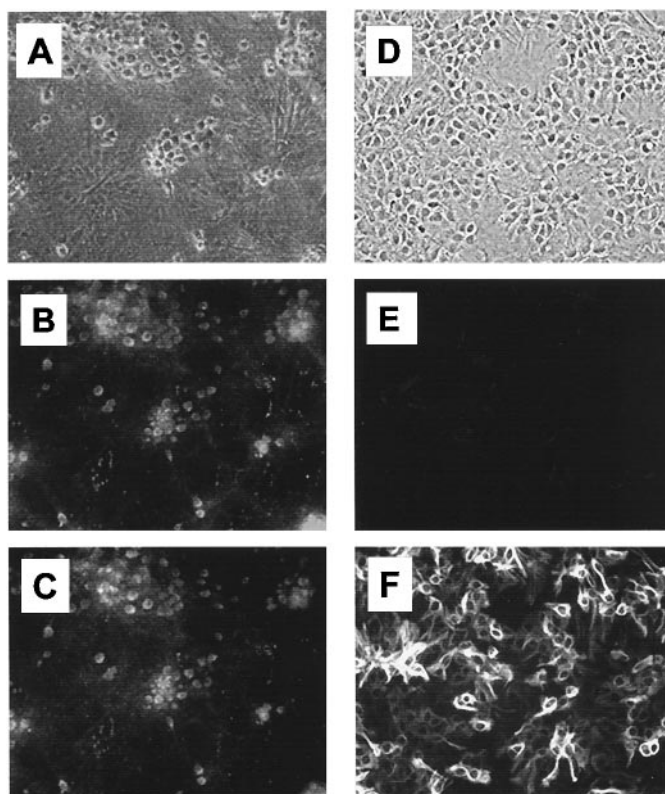
#### Reverse Transcription-Polymerase Chain Reaction (PCR)

**Analysis.** Total RNA was extracted from cells grown in 24-well tissue culture dishes using TRIZOL reagent (Life Technologies). Duplicate wells were combined, and RNA was resuspended in 20  $\mu$ l of water. One half of each RNA sample was subjected to first-strand cDNA synthesis using Moloney murine leukemia virus reverse transcriptase (400 U; Life Technologies) as previously described (Hewett, 1999). Reactions were performed in 20- $\mu$ l volumes at 40–42°C in a water bath for 1 h. The other half of each RNA sample was incubated similarly in the absence of reverse transcriptase to test for genomic DNA contamination (none detected). PCR amplicon pairs for analysis of COX-2 cDNA were 5'-TTCAAAAGAAGTGCTGGAAAA GGT-3' (sense) and 5'-GATCATCTCTACCTGAGTGTCTTT-3' (antisense). COX-1 cDNA amplicon pairs were 5'-TGTTACAGTCTTG GCCAACAGCT-3' (sense) and 5'-AGCGCATCAACACGGACGC CTGTT-3' (antisense).  $\beta$ -Actin cDNA amplicons were 5'-GTGGG CCGCTCTAGGCACCAA-3' (sense) and 5'-CTCTTTGATGTCACG CACGAT TTC-3' (antisense).  $\beta$ -Actin mRNA was assessed to control for the amount and the integrity of RNA in each sample. Each PCR was performed on 1  $\mu$ l of cDNA sample using *Taq* DNA polymerase (1 U; Fisher Scientific, Pittsburgh, PA) in a total volume of 25  $\mu$ l in a Perkin-Elmer Cetus (Norwalk, CT) 2400 DNA thermal cycler. Each cycle consisted of a denaturation step (94°C for 30 s), an annealing step (45 s), and a primer extension step (72°C, 1 min). Annealing temperatures and cycle number were as follows: COX-2 (63°C, 30 cycles); COX-1 (66°C, 25 cycles), and  $\beta$ -actin (63°C, 23 cycles). PCR products were separated by electrophoresis in 2% agarose and detected by ethidium bromide staining using a UV transilluminator. Results were recorded on Polaroid film.

## Results

Mixed murine cortical cell cultures were analyzed for the presence of constitutively expressed COX-1 and COX-2 in neurons and/or astrocytes with commercially available antibodies, verified via Western blot analysis to be antigen-specific (data not shown). Double immunolabeling of mixed cultures demonstrated colocalization of COX-2 immunoreactivity (ir) with the neuron-specific marker NSE (Fig. 1, A–C). No staining was observed in the astrocyte monolayer of the mixed cultures (i.e., NSE-negative cells; Fig. 1B). To confirm the absence of constitutive COX-2 expression in untreated astrocytes, pure astrocyte cultures were double immunolabeled with COX-2 and the astrocyte-specific marker GFAP (Fig. 1, D–F). COX-2 ir was never observed in GFAP-positive cells (Fig. 1, D–F). In contrast, COX-1 ir was detected in both NSE- and GFAP-positive cells, indicating expression in both neurons and astrocytes (Fig. 2, A–C and D–F, respectively).

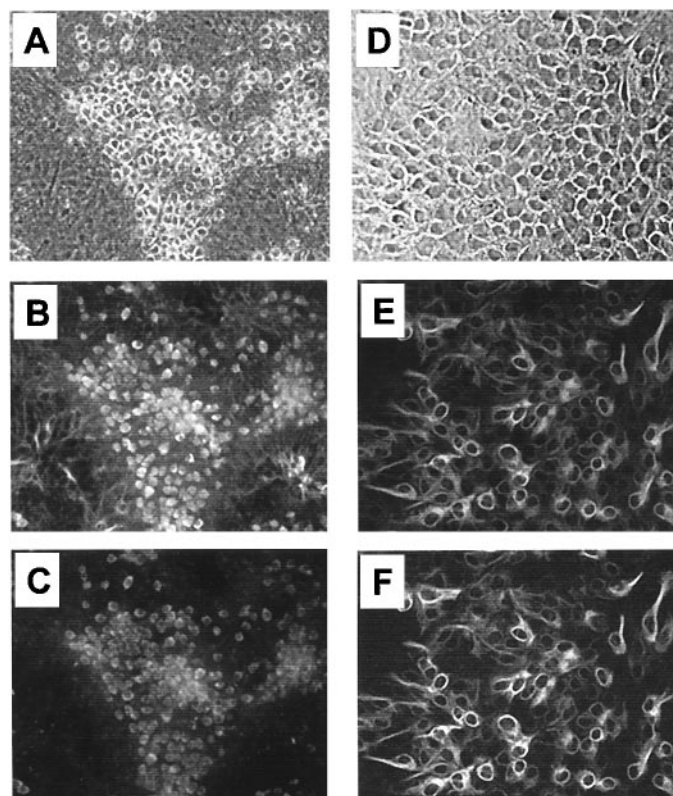
Because neuronal release of arachidonic acid can be triggered by stimulation of the NMDA receptor (Dumuis et al., 1988; Sanfeliu et al., 1990), the time course of PG production from cortical cultures after NMDA exposure was assessed.



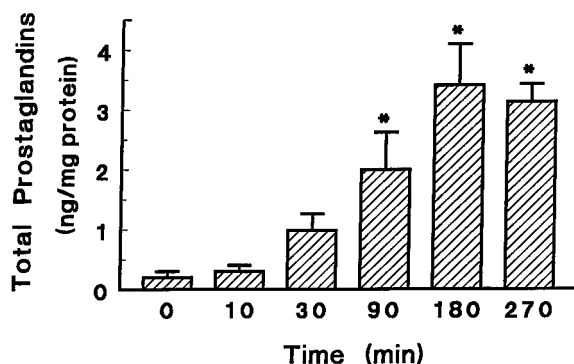
**Fig. 1.** COX-2 is constitutively expressed in neurons but not astrocytes. Protein levels of COX-2 were determined in untreated mixed cortical cell cultures (A–C) and pure astrocyte cultures (D–F) by indirect immunofluorescence. A and D, phase contrast micrographs of mixed cortical cell cultures (A) and pure astrocytes (D), respectively. B and E, COX-2 expression in the same cells as in A and D, demonstrating that cortical neurons (B) but not cortical astrocytes (B and E) stain for COX-2. C, NSE ir of the same cells as in A. F, GFAP ir of the same cells as in D.

PGs accumulated in the medium of murine mixed cortical cultures in a time-dependent manner after a brief exposure to NMDA (100  $\mu$ M, 5 min). PG accumulation was significantly elevated at 90 min and continued to increase up to 3 h after NMDA exposure (Fig. 3). Importantly, although the neurons appeared swollen in comparison with control untreated cells (compare Fig. 4, C and A), the number of PI-stained cells was not elevated (Fig. 4, B and D), indicating that the increase in PG release at 90 min was not due simply to early neuronal cell death/lysis. In contrast, PI staining was dramatically increased 4.5 h after NMDA exposure, consistent with significant neuronal injury ( $\approx$ 35%) at this later time (Fig. 4, E and F).

Interestingly, the time-dependent release of PGs was paralleled by an enhancement of COX-2 mRNA (Fig. 5), suggesting a possible link between NMDA-induced COX-2 expression and PG release. Analysis of mRNA expression via reverse transcription-PCR demonstrated that untreated cultures constitutively expressed COX-1 and low levels of COX-2, as expected given the results presented in Figs. 1 and 2. After brief exposure to NMDA (100  $\mu$ M, 5 min), COX-2 mRNA was consistently and rapidly (30 min) elevated and maintained for at least 4 h (Fig. 5, top). Later time points were not tested due to ensuing neuronal degeneration (see Fig. 4, E and F). Importantly, this rise in COX-2 mRNA was blocked by the concurrent exposure of cultures to MK-801 (Fig. 5, top), demonstrating a link between NMDA receptor



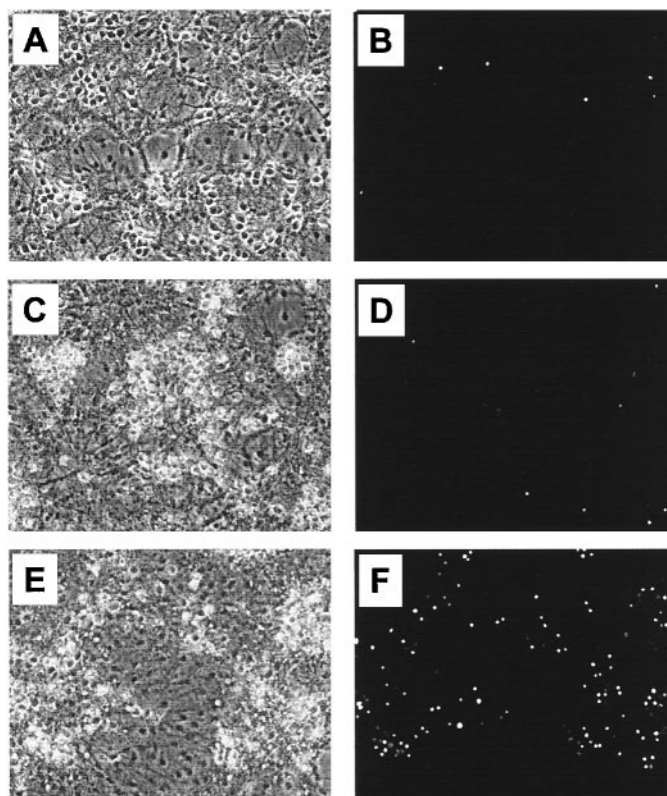
**Fig. 2.** Both neurons and astrocytes constitutively express COX-1. Protein levels of COX-1 were determined in untreated mixed cortical cell cultures (A–C) and pure astrocyte cultures (D–F) by indirect immunofluorescence. A and D, phase contrast micrographs of mixed cortical cell cultures (A) and pure astrocytes (D), respectively. B and E, COX-1 expression in the same cells as in A and D. C, NSE ir of the same cells as in A. F, GFAP ir of the same cells as in D.



**Fig. 3.** Time-dependent production of PGs after NMDA exposure. Mixed cortical cell cultures were treated with 30  $\mu$ M arachidonic acid for 1 h, washed, and then exposed to NMDA (100  $\mu$ M). After 5 min, the exposure solution was washed away and replaced by MS plus glycine, and the plates were returned to the incubator. Cell culture supernatants were collected at the times indicated, and PGs were released into the bathing medium were measured via enzyme immunoassay. Data are expressed as nanograms of PGs per milligram of cellular protein. Values represent the mean  $\pm$  S.E. ( $n = 4$  or 5 cultures per condition). \*, significantly different from time 0 as determined by ANOVA followed by Dunnett's test for multiple comparison. Significance was assessed at  $P < .05$ .

activation and COX-2 transcriptional activity. COX-1 mRNA remained unchanged over the same time frame (Fig. 5, bottom).

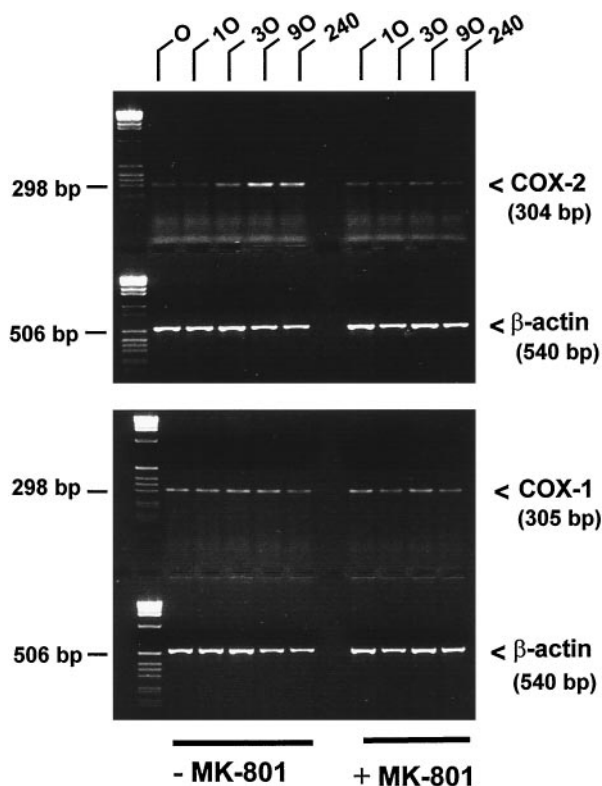
Next, pharmacological inhibitors were used to assess the relative contribution of the two COX isoforms to NMDA-induced PG production and subsequent neuronal injury.



**Fig. 4.** Release and accumulation of PGs precede neuronal cell death. Cells were exposed to 100  $\mu$ M NMDA (5 min), after which PI (10  $\mu$ g/ml, 10 min) was added to the culture wells at intervals ranging from 10 min to 4.5 h after exposure. B, PI staining of control cultures 4.5 h after sham wash. D and F, PI staining 1.5 h (D) and 4.5 h (F) after NMDA exposure. A, C, and E, phase contrast micrographs of corresponding fields.

Flurbiprofen ( $\geq 30$   $\mu$ M), a nonselective COX inhibitor (DeWitt et al., 1993), completely prevented PG production measured 1.5 h after NMDA exposure (Fig. 6A) and afforded partial protection against NMDA-induced neuronal injury as assessed 20 to 24 h later (Fig. 7A). Identical results were obtained with the selective COX-2 inhibitor NS-398 (10–30  $\mu$ M; Figs. 6C and 7C; Futaki et al., 1994; Masferrer et al., 1994) but not with valeryl salicylate (10–300  $\mu$ M), a selective COX-1 inhibitor (Figs. 6B and 7B; Bhattacharyya et al., 1995; A. S. Vidwans and J. A. Hewett, unpublished observations). The lack of effect of valeryl salicylate was not due to its inability to block constitutive COX-1 in our cultures because a 1-h pretreatment with 30 and 300  $\mu$ M decreased subsequent PG production elicited via exogenous application of arachidonic acid (15  $\mu$ M, 30 min) to  $54.3 \pm 12.3$  and  $36.7 \pm 5.2\%$  of nontreated controls, respectively. Finally, kainate neurotoxicity was unaffected, demonstrating that the protection afforded by flurbiprofen and NS-398 was specific for NMDA (Fig. 8, A and B).

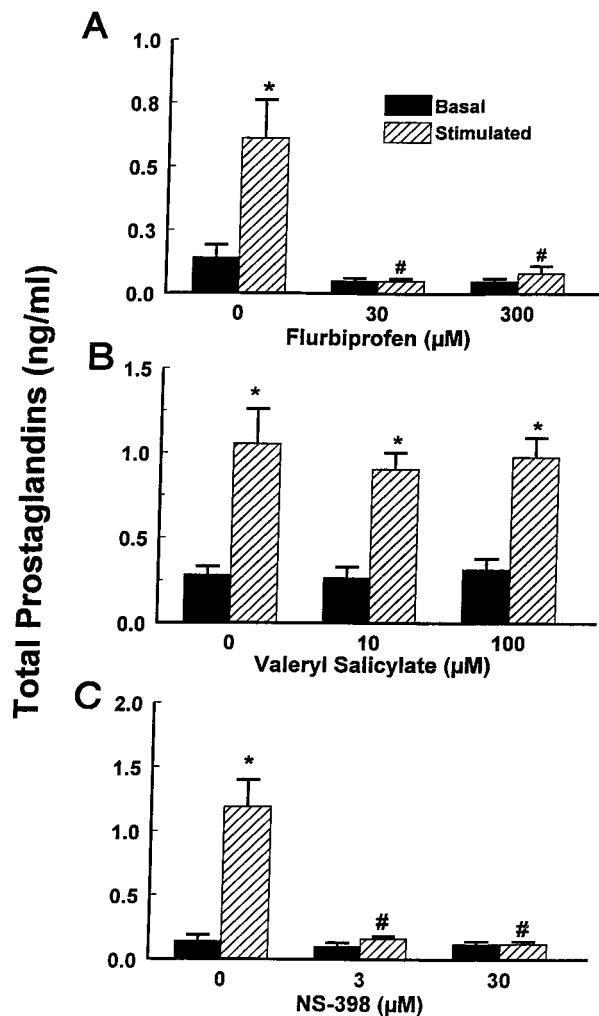
The ability of flurbiprofen and NS-398 to preserve cell viability was not due to NMDA receptor antagonism because the accumulation of  $^{45}\text{Ca}^{2+}$  after exposure to NMDA was not altered in the presence of either drug (data not shown). Furthermore, the effect of the inhibitors could not be ascribed to their potential ability to activate peroxisome proliferator-activated receptors (PPARs) (Lehmann et al., 1997). Treatment with PPAR activators Wy14643 (10–100  $\mu$ M), 15-deoxy- $\Delta^{12,14}$ -PGJ<sub>2</sub> (1–10  $\mu$ M), or docosahexaenoic acid (1–10



**Fig. 5.** NMDA receptor activation is associated with the specific up-regulation of COX-2 gene transcription. Cultures were exposed to NMDA (100  $\mu$ M, 5 min) in the presence and absence of the noncompetitive NMDA antagonist MK-801 (10  $\mu$ M). RNA was isolated 10, 30, 90, and 240 min after washout of NMDA, first-strand cDNA was synthesized, and COX-2 (top) and COX-1 (bottom) expressions were assessed by PCR as described in the text.  $\beta$ -Actin mRNA was assessed in all RNA samples as an internal control for the amount of RNA in each sample. 0 h represents the untreated control. COX-2 mRNA up-regulation was blocked by MK-801 (top). COX-1 mRNA expression is not increased by NMDA exposure. Data have been replicated with at least three different cultures.

$\mu$ M) for 3 h before and 20 to 24 h after NMDA exposure (100  $\mu$ M, 5 min) failed to reproduce the neuroprotective effects of flurbiprofen and NS-398 (data not shown). Finally, the concentrations of NS-398 used here (3–30  $\mu$ M) do not affect COX-1 activity *in vitro* (Futaki et al., 1994; Rosenstock et al., 1999). Thus, it is unlikely that these compounds are protecting against NMDA-mediated neuronal cell death by a mechanism other than COX-2 inhibition.

To distinguish between the contribution of constitutive COX-2 and new COX-2 protein synthesis to NMDA-induced neurotoxicity, constitutive COX proteins were irreversibly inhibited with aspirin (ASA; Meade et al., 1993) before NMDA exposure. A concentration of ASA (100  $\mu$ M) was used that effectively blocked  $\approx$ 85% of basal COX activity in otherwise untreated cultures (Fig. 9, inset). Pretreatment with ASA before NMDA exposure had no effect on neurotoxicity over a range of NMDA concentrations (Fig. 9A). However, NMDA-induced neurotoxicity in ASA-pretreated cultures was subsequently ameliorated by flurbiprofen (Fig. 9B). Finally, the time course of the rescue effect was determined. NS-398 (30  $\mu$ M) was added at various times after the conclusion of NMDA exposure, and neuronal injury was assessed 20 to 24 h later. Although efficacy was maximal at  $t_0$ , significant neuroprotection was still observed when NS-398 was added up to 1 h after NMDA exposure (Fig. 10). Taken together,

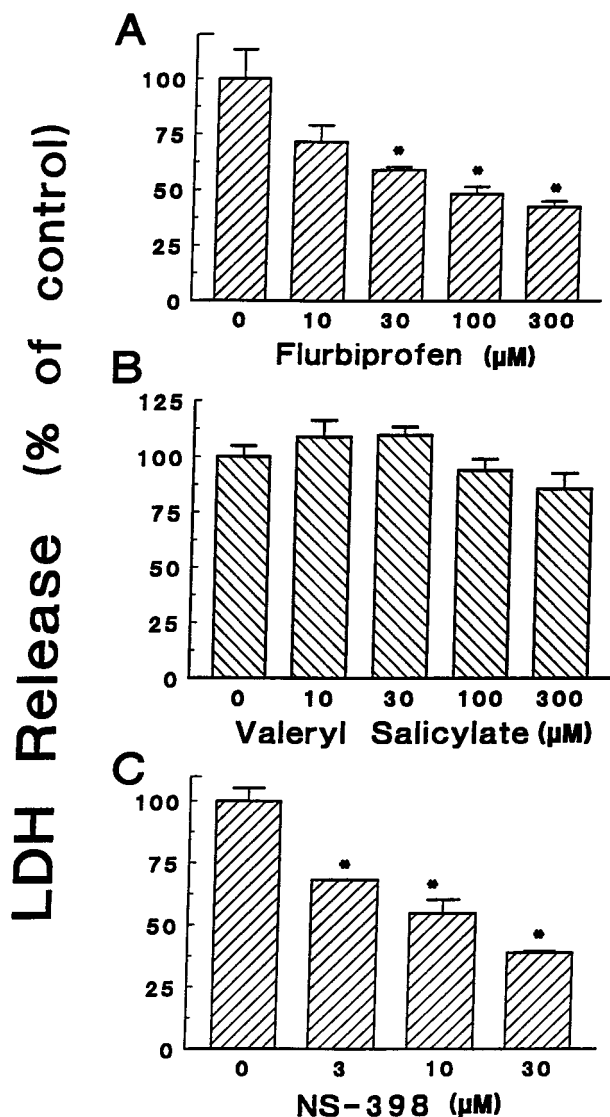


**Fig. 6.** NMDA-stimulated PG production is specifically catalyzed by COX-2. Mixed cortical cell cultures were treated with 30  $\mu$ M arachidonic acid for 1 to 2 h, washed, and then exposed to NMDA (100  $\mu$ M). After 5 min, the exposure solution was washed away and replaced with MS alone or MS containing flurbiprofen (A), valeryl salicylate (B), or NS-398 (C) at the indicated concentrations. At 1.5 h later, cell culture supernatants were collected, and PGs were measured via enzyme immunoassay. Data are expressed as nanograms of PGs per milliliter of cell culture medium. Values represent the mean  $\pm$  S.E. ( $n = 4-8$  cultures per condition). \*, significantly different from basal levels. #, significant diminution of the NMDA-stimulated levels as determined by ANOVA followed by Student-Newman-Keuls test for multiple comparison. Significance was assessed at  $P < .05$ .

these results suggest that induction of new COX-2 protein contributes to neuronal injury after NMDA exposure.

## Discussion

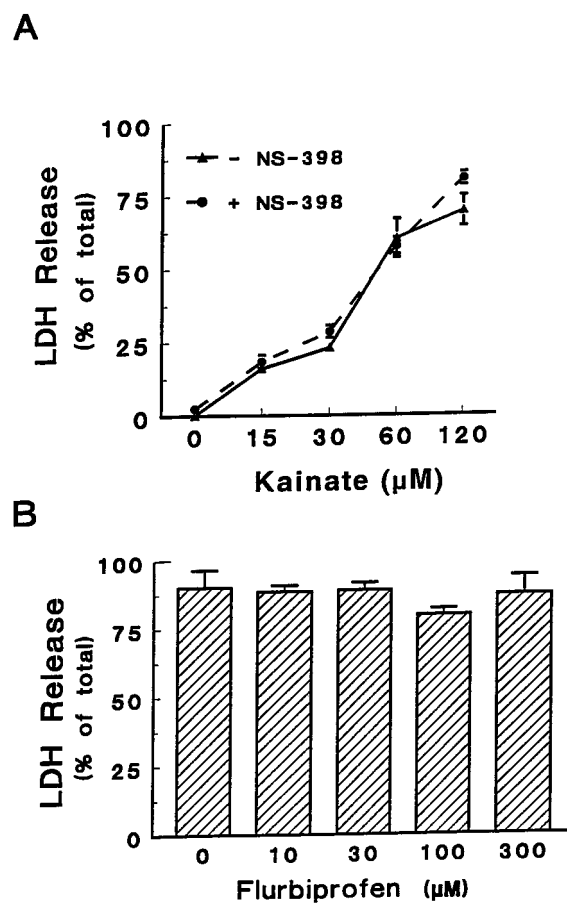
Results from the present study are the first to provide direct evidence for the contribution of newly expressed COX-2 in the pathogenesis of NMDA receptor-mediated neurotoxicity in mixed cortical cell culture. This conclusion is supported by the following evidence. First, brief exposure to a neurotoxic concentration of NMDA was followed by an increase in COX-2 mRNA expression and enzymatic activity that preceded neuronal injury. Second, nonselective pharmacological inhibition of COX enzymes blocked NMDA-induced PG production and attenuated neuronal injury. Third, these effects were reproduced by a selective inhibitor of COX-2 but



**Fig. 7.** NMDA-mediated excitotoxicity is attenuated by inhibition of COX-2. Mouse cortical cultures were exposed for 5 min to NMDA (100 or 200  $\mu\text{M}$ ) either alone or in the presence of increasing concentrations of flurbiprofen (A), valeryl salicylate (B), or NS-398 (C). The exposure solution was then washed away and replaced with MS with or without inhibitors, the cells were returned to the incubator, and neuronal cell death was assessed 20 to 24 h later. To facilitate comparison between compounds, values are expressed as the mean LDH release + S.E. ( $n = 6$ –12 from three separate experiments) normalized to the mean LDH release by cells treated with NMDA alone (100%). Actual percentage of cell death due to NMDA alone in the flurbiprofen, valeryl salicylate, or NS-398 studies was  $95.7 \pm 4.9$ ,  $63.4 \pm 4.8$ , and  $71.1 \pm 5.3$ , respectively. \*, significantly different from control as determined by ANOVA followed by Dunnett's test for multiple comparison. Significance was assessed at  $P < .05$ .

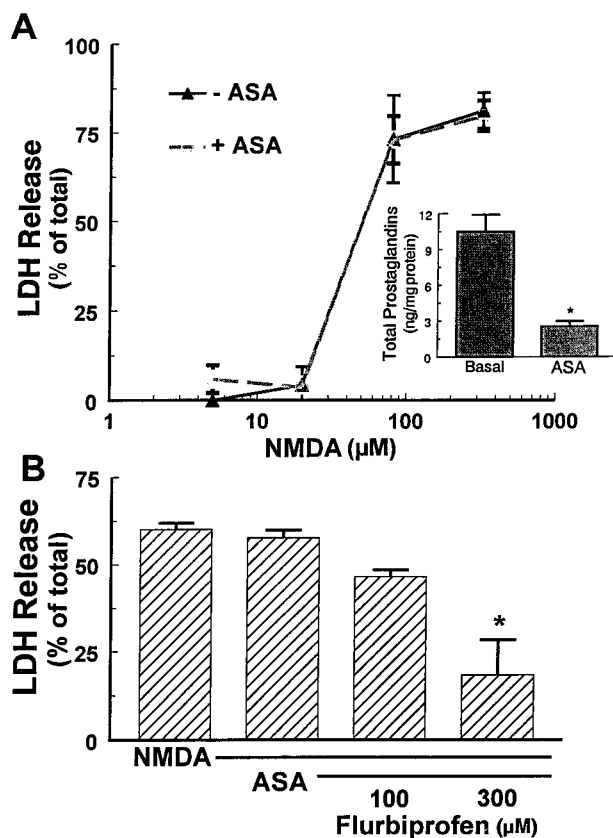
not of COX-1. Fourth, irreversible nonselective inhibition of total constitutive COX activity with ASA before NMDA exposure did not afford protection against neuronal injury, whereas treatment of ASA-pretreated cultures with a COX inhibitor remained neuroprotective. Last, significant neuroprotection was observed with NS-398 when added in a delayed fashion. Additional studies demonstrated that the neuroprotection afforded by COX-2 inhibition was specific for NMDA because kainate-mediated neuronal cell death was unaffected.

Characterization of COX in ovine (Breder et al., 1992)



**Fig. 8.** COX inhibition does not protect against kainate-mediated toxicity. A, concentration-response curve of kainate in the presence and absence of NS-398. Mixed cortical cell cultures were exposed for 24 h to increasing concentrations of kainate in the presence of MK-801 (10  $\mu\text{M}$ ) with or without NS-398 (30  $\mu\text{M}$ ) as indicated. LDH was assessed 20 to 24 h later (mean  $\pm$  S.E.;  $n = 3$ –6 cultures from three separate experiments). NS-398 did not affect injury at any tested kainate concentration as determined by repeated measures ANOVA. B, flurbiprofen does not protect against kainate-mediated toxicity. Mouse cortical cultures were exposed to kainate in the presence of MK-801 (100 and 10  $\mu\text{M}$ , respectively) with or without increasing concentrations of the nonselective COX inhibitor flurbiprofen. LDH was assessed 20 to 24 h later (mean  $\pm$  S.E.;  $n = 12$ –14 cultures from four separate experiments). Flurbiprofen did not affect kainate-mediated toxicity at any given concentration as determined by one-way ANOVA.

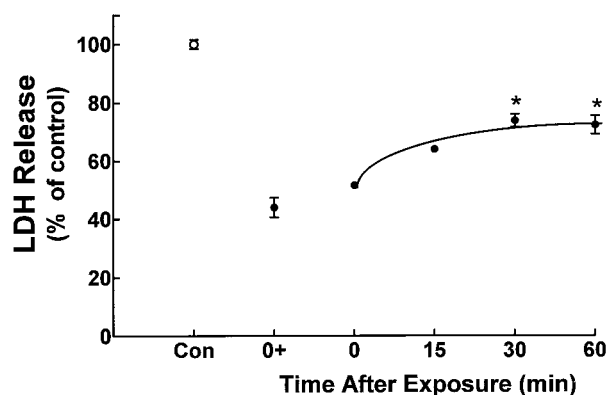
and rat (Yamagata et al., 1993; Breder et al., 1995) brain demonstrated an exclusive neuronal localization of both COX-2 and COX-1. In agreement with those studies, we found that constitutive COX-2 protein expression was restricted to murine cortical neurons. In contrast, COX-1 protein was detected in both neurons and astrocytes. Although the reason for this discrepancy is unknown, it is possible that expression of the COX-1 enzyme in normal CNS astrocytes of sheep and rat was below the level of detection of the antibody used. Alternatively, astrocyte expression may be a consequence of tissue culture preparation, because several studies have reported that cultured astrocytes have the capacity to synthesize PGs, implying the presence of some form of the COX enzyme (Keller et al., 1985; Seregi et al., 1987). Although we cannot rule out the latter, the former possibility is supported by the finding that weak but positive staining for COX-1 was detected in glia from monkey brain (Tsubokura et



**Fig. 9.** New COX-2 protein expressed after NMDA exposure contributes to neuronal injury. A, effect of ASA pretreatment on NMDA-induced neuronal injury. Mixed cortical cell cultures were treated with 100  $\mu\text{M}$  ASA for 1.5 h, washed, and exposed to NMDA (100  $\mu\text{M}$ ). After 5 min, the exposure solution was washed away and replaced by MS plus glycine, and the plates were returned to the incubator. LDH activity was assessed 20 to 24 h later. Values represent the mean LDH release  $\pm$  S.E. ( $n = 6$  from two separate experiments) expressed as a percentage of the total neuronal LDH (100%), which was determined in each experiment by assaying the supernatant of parallel cultures exposed to 300  $\mu\text{M}$  NMDA for 20 to 24 h. ASA did not affect injury at any NMDA concentration as determined by repeated measures ANOVA. Inset, effect of ASA on basal COX levels. Cultures were exposed to ASA (100  $\mu\text{M}$ ) for 1.5 h, exogenous arachidonic acid (30  $\mu\text{M}$ ) was added for 30 min (37°C), and the supernatants were removed for PG measurements as described in the text. \*, significant decrease in arachidonic acid-stimulated PG production as determined by one-way ANOVA followed by Dunnett's  $t$  test. B, effect of flurbiprofen after ASA pretreatment. Cultures were preincubated with ASA as in A and then exposed for 5 min to NMDA alone or NMDA plus flurbiprofen at the indicated concentrations. The exposure solution was washed away and replaced by MS plus glycine with or without flurbiprofen, and the plates were returned to the incubator. LDH activity was assessed 20 to 24 h later. Values represent the mean LDH release  $\pm$  S.E. ( $n = 13$  or 14 from four separate experiments) expressed as a percentage of total neuronal LDH (100%), which was determined in each experiment by assaying the supernatant of sister cultures after exposure to 300  $\mu\text{M}$  NMDA for 20 to 24 h. \*, significant diminution ( $P < .01$ ) of NMDA-induced neuronal injury as determined by Kruskal-Wallis ANOVA followed by Dunn's  $t$  test.

al., 1991). Thus, a potential species variability should not be overlooked.

Although our cultured neurons contained both COX-1 and COX-2, NMDA-stimulated PG production and neuronal cell death were prevented by NS-398 but not by valeryl salicylate, selective inhibitors of COX-2 (Futaki et al., 1994; Masferrer et al., 1994) and COX-1 (Bhattacharyya et al., 1995; A. S. Vidwans and J. A. Hewett, unpublished observation), respectively. Because valeryl salicylate was effective in in-



**Fig. 10.** Time course of rescue. Cultures were exposed to NMDA (100  $\mu\text{M}$ , 6 min) followed by the addition of NS-398 in a volume of 25  $\mu\text{l}$  (final concentration, 30  $\mu\text{M}$ ) at the indicated time in minutes after washout of NMDA. LDH activity was assessed 20 to 24 h later. Values represent the mean LDH release  $\pm$  S.E. ( $n = 6$  from two separate experiments) normalized to the mean LDH release by cells treated with NMDA alone (○, 100%). Actual percentage of cell death due to NMDA alone was  $79.5 \pm 1.6$ . ●, values significantly different from control (Con = NMDA alone). \*, values significantly different from 0+ (NS-398 added during and after NMDA exposure) as determined by ANOVA followed by Student-Newman-Keuls test for multiple comparison. For data points lacking error bars, the error falls within the confines of the symbol. Significance was assessed at  $P < .05$ .

hibiting PG production elicited by the exogenous application of arachidonic acid, these data suggest that NMDA receptor stimulation is specifically coupled to COX-2. This could be a result of the selective enhancement of COX-2 expression that occurred in our cultures after NMDA exposure. In addition, this could be related to a differential compartmentalization of the two isoforms, which may serve to separate the activities of COX-1 and COX-2 within cells (Spencer et al., 1998). However, subcellular compartmentalization of COX isoforms in neurons remains to be demonstrated. Alternatively, it could be related to kinetic properties unique to the COX-2 isoform. In this regard, Kulmacz and Wang (Kulmacz et al., 1994) reported a large intrinsic difference between COX-1 and COX-2 in initiation efficiency, with COX-2 catalytic activity being initiated at lower hydroperoxide concentrations, whereas Swinney et al. (1997) reported greater COX-2 activity under conditions of limiting substrate. Finally, the dependence of NMDA-stimulated PG production on COX-2 may be mediated through specific coupling to a distinct phospholipase.  $\text{Ca}^{2+}$  entry through the NMDA receptor activates  $\text{Ca}^{2+}$ -dependent cytosolic phospholipase A<sub>2</sub> (PLA<sub>2</sub>; Sanfeliu et al., 1990), and it has been proposed that PG synthesis occurs via two independent pathways: an intracellular cytosolic PLA<sub>2</sub>-dependent pathway selective for COX-2 and a secretory PLA<sub>2</sub> transcellular pathway that appears to have preference for COX-1 (Reddy and Herschman, 1994, 1997).

Although flurbiprofen and NS-398 completely inhibited NMDA-mediated PG production, they provided only partial protection against NMDA-induced neuronal injury when administered during and for 24 h after NMDA exposure. Although this suggests that only a subset of neurons are susceptible to COX-2-mediated cytotoxicity, it could also simply reflect the presence of multiple parallel pathways of injury. The duration of exposure was chosen because these drugs are time-dependent inhibitors of COX (Copeland et al., 1994; Greig et al., 1997). As such, their ability to block activity can be viewed in practical terms as delayed. In particular, in an

intact cellular system, NS-398 requires an incubation time of 30 min for half-maximal inhibition of COX-2 enzymatic activity, with more than 20% activity remaining even after 60 min of exposure to the drug (Greig et al., 1997). Thus, it is particularly striking that NS-398 protected against NMDA-induced neurotoxicity when given as long as 1 h after NMDA exposure. Furthermore, irreversible nonselective inhibition of total constitutive COX activity before NMDA exposure with aspirin did not affect NMDA-induced neuronal injury, whereas treatment of ASA-pretreated cultures with a COX inhibitor remained neuroprotective. Taken all together, these data provide strong evidence for the contribution of newly expressed COX-2 to NMDA-induced neuronal injury in our cortical cell culture system.

Nonsteroidal anti-inflammatory drugs along with other compounds, including prostanoids, long-chain fatty acids, and the fibrate class of hypolipidemic drugs have been shown to activate PPARs (Kliwer et al., 1997; Krey et al., 1997; Lehmann et al., 1997). PPARs ( $\alpha$ ,  $\delta$ , and  $\gamma$ ) are nuclear hormone receptors first identified in peripheral tissues that control the expression of genes involved in fatty acid and lipid metabolism (Tugwood et al., 1996). All three isoforms have since been detected in neurons (Kainu et al., 1994; Cullingford et al., 1998), although their function within the central nervous system has not been elucidated. Nevertheless, they represent a potential alternate target for flurbiprofen and NS-398. However, neither 15-deoxy- $\Delta^{12,14}$ -PGJ<sub>2</sub>, Wy14643, nor docosahexaenoic acid, activators of PPAR $\gamma$ , PPAR $\alpha$ ,  $\delta$ , or PPAR $\alpha$ ,  $\delta$ ,  $\gamma$ , respectively (Kliwer et al., 1995, 1997), mimicked the protective effect of flurbiprofen or NS-398. Furthermore, protection could not be explained by NMDA receptor antagonism because COX inhibitors did not alter NMDA-induced calcium flux. Importantly, the concentrations of NS-398 used here (3–30  $\mu$ M) do not affect COX-1 activity in vitro (Futaki et al., 1994; Rosenstock et al., 1999). Thus, inhibition of COX-2 catalytic activity appears to be the underlying mechanism by which flurbiprofen and NS-398 protect against NMDA-mediated neurotoxicity.

The exact mechanism by which inhibition of COX-2 protects against and is selective for NMDA-mediated neurotoxicity is currently under investigation. Reactive oxygen species are generated by the process of arachidonic acid metabolism through COX (Kukreja et al., 1986). In fact, production of COX-associated reactive oxygen species has been demonstrated after NMDA but not kainate receptor stimulation in vitro (Lafon-Cazal et al., 1993; Reynolds et al., 1995; but see Dugan et al., 1995). This provides a potential explanation for the selective action of COX inhibitors against NMDA-induced neurotoxicity. In addition, PGs are known to modulate neurotransmitter release (Allgaier and Meder 1995; Sekiyama et al., 1995) and have been shown to potentiate excitatory amino acid-induced synaptic depolarizations (Kimura et al., 1985; but see Akaike et al., 1994; Cazevielle et al., 1994). Thus, effective interruption of the arachidonic acid cascade through COX-2 inhibition might prevent deleterious metabolite and/or oxygen-derived free radical formation.

Although injury resulting from overactivation of NMDA receptors is unlikely to result from a single causal event, the present results indicate that effective disruption of arachidonic acid metabolism through the inhibition of COX-2 can limit NMDA-induced neurotoxicity. As such, we suggest that

inhibitors of COX-2 might prove to be therapeutically useful in neurological diseases associated with excessive NMDA receptor activation.

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**Send reprint requests to:** Dr. Sandra J. Hewett, University of Connecticut Health Center, Department of Pharmacology MC-6125, 263 Farmington Ave., Farmington, CT 06030-6125. E-mail: shewett@neuron.uhc.edu

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