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Opposing effects of cyclooxygenase-2 selective inhibitors on oxygen-glucose deprivation-induced neurotoxicity

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Abstract

Cyclooxygenase-2 inhibitors protect against excitotoxicity in vitro yet provide conflicting results in in vivo models of ischemia. To bridge the gap in understanding the discrepancies among these studies, the effects of different cyclooxygenase-2 inhibitors were studied in an in vitro model of ischemia. Oxygen-glucose deprivation (OGD) induced cyclooxygenase-2 protein expression in neuronal cortical cultures. Cyclooxygenase-2 inhibitors exhibited opposing effects on neuronal death induced by OGD. The acidic sulfonamides, *N*-(2-cyclohexyloxy-4-nitrophenyl) methanesulfonamide (NS-398) and *N*-(4-nitro-2-phenoxyphenyl)-methanesulfonamide (nimesulide), aggravated neuronal death by enhancing OGD-induced increases in extracellular glutamate and intracellular Ca^{2+} levels. In contrast, 1-[(4-methylsulfonyl)-phenyl]-3-tri-fluoromethyl-5-(4-fluorophenyl)pyrazole (SC-58125) dose-dependently protected cultures against OGD by suppressing increases in extracellular glutamate and intracellular Ca^{2+} levels. The NS-398-induced aggravation of neuronal death was lost if the inhibitor was added only following the OGD. The timing of inhibitor application also determined its effects on *N*-methyl-D-aspartate (NMDA)-induced excitoxicity. NS-398 was protective when added both during and post-NMDA exposure, but not if NS-398 was also applied for 60 min prior to the insult. In contrast, SC-58125 afforded protection against NMDA in the presence or absence of a pre-incubation period. This study demonstrates that certain cyclooxygenase-2 inhibitors have opposing effects on neuronal survival depending on the timing of application and the nature of the insult. These results may account for the discrepancies among previous studies which used different inhibitors and different models of neurotoxicity.

Keywords: Cyclooxygenase-2; Oxygen-glucose deprivation; NMDA; Ca^{2+}; Primary cortical culture; Excitotoxicity

1. Introduction

Cyclooxygenases are rate-limiting enzymes that catalyze the biosynthesis of prostaglandins from arachidonic acid. The constitutive isoform, cyclooxygenase-1, is present in nearly all cells types. The inducible isoform, cyclooxygenase-2, is normally absent from most cell types but is constitutively present in the brain, particularly in the cortex and hippocampus, and is regulated by physiological synaptic activity (Yamagata et al., 1993). Additionally, cyclooxygenase-2 is strongly induced by pathological events associated with the excessive activation of *N*-methyl-D-aspartate (NMDA) receptors, such as cerebral ischemia (Planas et al., 1995; Ohtsuki et al., 1996; Collaço-Moraes et al., 1996; Nogawa et al., 1997; Miettinen et al., 1997), seizures (Yamagata et al., 1993; Chen et al., 1995) and spreading depression (Caggiano et al., 1996; Miettinen et al., 1997). A causative role for cyclooxygenase-2 in excitotoxicity is supported by the findings that cyclooxygenase-2-deficient mice are less susceptible to both ischemic brain injury and NMDA-mediated neurotoxicity (Iadecola et al., 2001), and that neuronal overexpression of human cyclooxygenase-2 in transgenic mice potentiates kainate acid-induced seizures in vivo as well as glutamate neurotoxicity in vitro (Kelley et al., 1999). Furthermore, NMDA exposure induces cyclooxygenase-2 expression in

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cultured mouse cortical neurons and pharmacological inhibition of this enzyme protects against NMDA-mediated neurotoxicity (Carlson, 2003; Hewett et al., 2000).

The large body of evidence linking cyclooxygenase-2 overexpression with excitotoxicity has generated interest in the development of cyclooxygenase-2 inhibitors for the treatment of neurodegenerative diseases. However, studies investigating the effects of cyclooxygenase inhibition in in vivo models of ischemia and kainate acid-induced seizures have yielded conflicting results. In rodent models of focal cerebral ischemia, the cyclooxygenase-2 selective inhibitor, *N*-(2-cyclohexyloxy-4-nitrophenyl)methanesulfonamide (NS-398), either reduces ischemic damage (Nogawa et al., 1997; Sugimoto and Iadecola, 2003) or has no significant effect on infarct volume (Hara et al., 1998). The non-selective cyclooxygenase inhibitor, α-methyl-4-(2-methylpropyl)benzeneacetic acid (ibuprofen), reduces infarct volume following transient focal ischemia but increases infarct volume in a model of permanent focal ischemia (Cole et al., 1993). Furthermore, the cyclooxygenase-2 selective inhibitor, *N*-(4-nitro-2-phenoxyphenyl)-methanesulfonamide (nimesulide), affords partial protection against kainate-induced oxidative damage in rats (Candelario-Jalil et al., 2000), while cyclooxygenase inhibitors, including NS-398, exaggerate kainic acid-induced seizure activity and mortality in mice (Baik et al., 1999). Clearly, further mechanistic information on the role of cyclooxygenase-2 in neurotoxicity is required before deciding whether inhibition of the activity of this enzyme represents a valid neuroprotective strategy.

We sought to bridge the gap in understanding the discrepancies among the studies that used pharmacological inhibition of cyclooxygenase-2 to assess the role of this enzyme in neurotoxicity. This study examined the effects of various cyclooxygenase-2 selective inhibitors on oxygen-glucose deprivation (OGD)-induced glutamate release, Ca^{2+} influx and neuronal death in mixed neuronal/glial cortical cultures. Of the three cyclooxygenase-2 selective inhibitors used, two were from the class of acidic sulfonamides, NS-398 and nimesulide, and the other was from the class of diarylheterocyclics, 1-[(4-methylsulfonyl)phenyl]-3-tri-fluoromethyl-5-(4-fluorophenyl)pyrazole (SC-58125).

2. Materials and methods

2.1. Materials

Tissue culture dishes and plates were purchased from either Du Pont-Life Technologies (Burlington, ON, Canada) or VWR Canlab (Mississauga, ON, Canada). Minimal essential medium (with Earle's salt and L-glutamine) and fetal bovine serum were purchased from Wisent Canadian Laboratories (St-Bruno, QC, Canada) and horse serum was obtained from Hyclone Laboratories (Logan, UT, USA). Uridine, 5-fluoro-2′ -deoxy-uridine, poly-L-lysine, propidium iodide, NMDA, glutamate, tetrodotoxin, (5*S*,10*R*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]cyclohepten-5,10-imine maleate (MK-801), the rabbit anti-actin antibody (A-2066) and the anti-rabbit Immunoglobulin G (IgG) peroxidase antibody (A-9169) were purchased from Sigma (St. Louis, MO, USA). The cyclooxygenase-2 goat polyclonal antibody (sc-1747) and the bovine anti-goat IgG peroxidase (sc-2350) were purchased from Santa Cruz Biotechnology, (Santa Cruz, CA, USA). Protein reagent and sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) standards were purchased from Bio-Rad Laboratories (Richmond, CA, USA). Polyvinylidene difluoride membranes and the ECL Plus™ kit were purchased from Amersham Pharmacia Biotech (Baie D'Urfé, QC, Canada). Fluo-4, AM, fluorescein-5-isothiocyanate (FITC) and the Amplex Red Glutamic Acid/Glutamate Oxidase Assay Kit (A-12221) were bought from Molecular Probes (Eugene, OR, USA). The cyclooxygenase-2 selective inhibitors, NS-398, nimesulide and SC-58125 were purchased from Cayman Chemical (Ann Arbor, MI, USA).

2.2. Preparation of primary cortical cultures

All procedures using animals were approved by the Institute for Biological Sciences Animal Care Committee. Primary cortical cultures were prepared from E15 to E17 mouse fetuses (C57B/6 × SV129) or from E18 rat (Sprague–Dawley) fetuses as previously described (Black et al., 1995). Following dissection of the cortical region of the fetal brain, cells were dissociated and resuspendend in plating medium consisting of minimal essential medium supplemented to 25 mM glucose, 10% fetal bovine serum, 10% horse serum and 2 mM glutamine. Cells were plated at a density of 1.6×10^6 cells/ml in poly-L-lysine-coated 12-well plates or 35-mm dishes. Cultures were treated with 15 μg/ml of 5-fluoro-2′ -deoxyuridine and 35 μg/ml uridine at 4 days in vitro to minimize glial growth. At 7 days in vitro, one-half of the medium was replaced with medium identical to plating medium, except for the absence of fetal bovine serum. Experiments were performed on mixed neuronal/glial cell cultures at 7–15 or 15–19 days in vitro for mouse and rat cortical cultures, respectively.

2.3. Oxygen-glucose deprivation

OGD was performed as previously described (Tauskela et al., 1999). Cultures were washed twice in a glucose-free balanced salt solution (BSS) at room temperature. The BSS used for mouse cultures had the following composition: 140 mM NaCl, 3.5 mM KCl, 0.4 mM KH_2PO_4, 5 mM $NaHCO_3$, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$ and 20 mM HEPES, pH 7.4. The BSS used on rat cultures was composed of: 140 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 20 mM HEPES and 0.03 mM glycine, pH 7.4. Cells were then exposed to an anaerobic environment (95% N_2/5% CO_2, O_2 partial pressure of 10–15 Torr) by placing cultures in a 37 °C incubator housed in an anaerobic glovebox (Forma Scientific, Marjetta, OH, USA).

OGD was terminated by removing cultures from the anaerobic incubator, returning the cells to their stored medium and placing them in a standard incubator maintained at 37 °C in 95% O_2/5% CO_2. Control cultures were exposed to BSS with glucose (15 mM for mouse and 3 mM for rat cultures) and kept in the standard incubator. Mouse and rat cultures were exposed to 120–150 and 60–75 min OGD, respectively.

To test the effects of cyclooxygenase-2 inhibition on OGD-induced neuronal death, cyclooxygenase-2 selective inhibitors were applied in the following ways: (1) to the media following OGD, (2) to both the glucose-free BSS during OGD and to the media following OGD, or (3) to only the glucose-free BSS during OGD. Cells in three to four wells of the same 12-well plate were exposed to OGD alone and to OGD + inhibitor to eliminate any effects of inter-plate variability.

2.4. NMDA and glutamate exposure

Mouse and rat cortical cultures were exposed to 500 μM (20–30 min) or 35 μM NMDA (10–20 min), respectively, in glucose-containing BSS at room temperature. Following NMDA exposure, cells were washed in BSS, returned to their medium and placed in a standard incubator. Glutamate exposure was conducted in a similar fashion. Cortical cultures were exposed to 50 μM glutamate in glucose-containing BSS for 5–10 min at room temperature, then washed, returned to their media and placed in the standard incubator.

To test the effects of cyclooxygenase-2 inhibition on glutamate- and NMDA-induced neuronal death, the cyclooxygenase-2 inhibitors were either added: (1) to both the BSS during the insult and to the media afterwards, or (2) to BSS for a 60-min pre-incubation, then to both the BSS during the insult and to the media afterwards.

2.5. Assessment of neuronal death

Neuronal injury was assessed by exposing cells to propidium iodide and measuring fluorescence intensities with a Cytofluor 2350 platereader (Millipore, Bedford, MA, USA) (Tauskela et al., 2001). Unless indicated otherwise, neuronal injury was examined 24 h following insults. Briefly, media was removed from cultures and replaced with BSS containing 4.5 μM propidium iodide. Following a 30-min incubation at room temperature, the fluorescent intensity (Ex = 530 ± 20 nm; Em = 645 ± 20 nm) from four locations within each well was measured on a plate reader. Fluorescent intensities were background-corrected by subtracting the fluorescence measured in cell-free wells containing propidium iodide. The percentage of propidium iodide uptake was determined by subtracting the fluorescence measured in untreated sister cultures containing propidium iodide, then normalizing values to the fluorescence representing 100% neuronal death, which was obtained by exposing sister cultures to 10 μM NMDA for 24 h. Thus, the percentage of propidium iodide uptake is equivalent to the percentage of dead cells above control levels.

2.6. Western blot analysis of cyclooxygenase-2

Cell lysates collected from mouse cortical cultures at different time-points following OGD were subjected to SDS-PAGE (10% acrylamide) and proteins were transferred to a polyvinylidene difluoride membrane. After blocking (5% milk powder in Tris-buffered saline with 0.5% Tween-20 for 1 h at room temperature), the membranes were incubated with an anti-cyclooxygenase-2 antibody (1:1000) for 1 h, followed by incubation with a horseradish peroxidase-conjugated anti-goat IgG. The bands were visualized by enhanced chemiluminescence. β-actin immunoreactivity (1:3000) was used as an internal control to quantify the relative amount of cyclooxygenase-2 protein. Cyclooxygenase-2 and β-actin bands were quantified using Molecular Dynamics Personal Densitometer SI.

2.7. Intracellular Ca^{2+} assay

Intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) were determined using platereader measurements of fluorescence intensity from cells loaded with the Ca^{2+}-sensitive, high-affinity fluorescent dye, fluo-4, AM ($K_d \cong 345$ nM; Ex = 485 ± 20 nm; Em = 530 ± 20 nm) (Tauskela et al., 2003). Cultures were washed in BSS and loaded with 4.5 μM fluo-4, AM in BSS for 60 min at 37 °C. Control cultures and cultures to be subjected to OGD were then washed with glucose-containing and glucose-free BSS, respectively, and allowed to equilibrate for 30 min at room temperature. The buffer was then replaced with glucose-containing or glucose-free BSS ± cyclooxygenase-2 inhibitor. The initial fluorescence intensity was measured from four locations within each well and plates were placed in the anaerobic or standard incubator at 37 °C. Immediately following OGD, plates were washed once and fluorescence intensities were measured again. The fold-change in fluorescence was calculated by dividing the final reading of each well by its initial reading.

A cell-free assay was used to ensure that the inhibitors had no effect on the fluorescence signal of fluo-4, AM. The fluorescence produced by 5 μM FITC, the basic fluorophore component of fluo-4, AM, was compared to that of FITC + inhibitor using a fluorescent platereader.

2.8. Measurement of the extracellular glutamate concentration

Buffer solutions were collected from cultures immediately following exposure to OGD and stored at −80 °C. Glutamate concentrations were determined using a commercially available Amplex Red kit. Glutamate in the collected samples was enzymatically converted to H_2O_2, which in turn reacted with Amplex Red reagent to produce the

fluorescent product, resorufin, which was detected using a fluorescent platereader.

2.9. Whole-cell recordings

Electrophysiological methods used in this study have been previously described (Mealing et al., 2001). Briefly, rat cortical neurons cultured on 35-mm culture dishes, were perfused continuously at 1 ml/min at 22 °C using a solution containing (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 10 HEPES, 3 glucose, at pH 7.4. The perfusion solution also contained 1 μM tetrodotoxin, 30 μM glycine, and 1 μM strychnine. Patch pipettes (2–4 MΩ resistance) were constructed from 1.5 mm outer diameter/1.0 mm inner diameter Pyrex 7740 glass (Corning, Big Flats, MN, USA). A modified drug application device (DAD-12; ALA Scientific Inst., Westbury, NY, USA) was used to rapidly apply glutamate or glutamate plus cyclooxygenase-2 inhibitors. The pipette solution contained (in mM): 140 CsCl, 1.1 EGTA, 10 HEPES, 2 Mg-ATP at pH 7.2.

Whole-cell currents were acquired using an Axopatch 1-D amplifier equipped with a CV-4 headstage with a 1 GΩ feedback resistor (Axon Inst., Foster City, CA, USA). Voltage command and current acquisition were accomplished using a Digidata 1200 interface and pClamp 6.0 software (Axon Inst.). For data analysis, the fractional block of glutamate-evoked currents was calculated according to the formula:

$$B = I - I_B/I$$

where I is the steady-state current evoked by glutamate and I_B is the current evoked by glutamate in the presence of a potential blocker.

Current-clamp ($I=0$) experiments were conducted to monitor membrane potential for a period of 5 min after inhibitor application to determine if they caused any immediate changes in ion homeostasis.

2.10. Statistical analysis

Three to six wells were investigated per condition from a minimum of three different platings. Data is presented as the mean ± S.E.M. Statistical comparisons were made by Student's *t*-test or analysis of variance (ANOVA). When significant differences were observed, Dunnett's test was employed for multiple comparisons. Statistical significance was inferred at $P<0.05$.

3. Results

3.1. Effect of oxygen-glucose deprivation on cyclooxygenase-2 protein expression in mouse cortical cultures

Western Blot analysis was used to study cyclooxygenase-2 protein expression immediately following 130 min of OGD and at different time-points following re-oxygenation. The antibody directed against the cyclooxygenase-2 protein detected a double band of approximately 70 kDa, consistent with previous studies (Habib et al., 1993; Kaufmann et al., 1996). Cyclooxygenase-2 was constitutively expressed at low levels in untreated mouse cultures and the levels were not altered immediately following OGD. However, a significant increase in cyclooxygenase-2 protein was observed 15–120 min following OGD (Fig. 1).

3.2. Effect of cyclooxygenase inhibitors on oxygen-glucose deprivation-induced cell death in primary cortical cultures

Hewett et al. (2000) have shown that application of the cyclooxygenase-2 selective inhibitor, NS-398, during and following NMDA exposure attenuates NMDA-induced neuronal death in mouse cortical cultures. The effect of NS-398 on OGD-induced neuronal death was investigated, given the key role for NMDA receptor activation in this process (Goldberg and Choi, 1993). In an effort to inhibit the newly synthesized cyclooxygenase-2 following OGD, 60 μM NS398 was added to the media of cultures immediately following the insult. This dose of NS-398 was not toxic under normoxic and normoglycemic conditions (data not

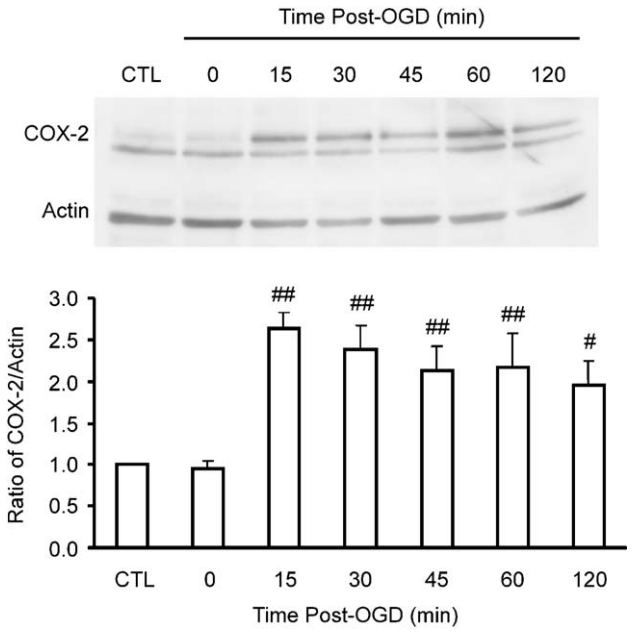

Fig. 1. Cyclooxygenase-2 protein expression increases upon re-oxygenation following OGD in mouse cortical cultures. Cultures were exposed to OGD for 130 min. Cyclooxygenase-2 protein expression was assessed by Western blot analysis using protein extracted immediately following OGD (0 min) as well as at 15, 30, 45, 60 and 120 min of re-oxygenation. The antibody directed against the cyclooxygenase-2 protein detected a double band of approximately 70 kDa. β-Actin immunoreactivity was used as an internal control to quantify the relative amount of cyclooxygenase-2 protein. Data shown is the mean ± S.E.M. from experiments performed using four separate platings of cells. CTL = normoxic/normoglycemic conditions. $^{\#,\#\#}$Significantly ($^{\#}P<0.05$, $^{\#\#}P<0.001$) different than basal levels of cyclooxygenase-2 in CTL, as determined by one-way ANOVA.

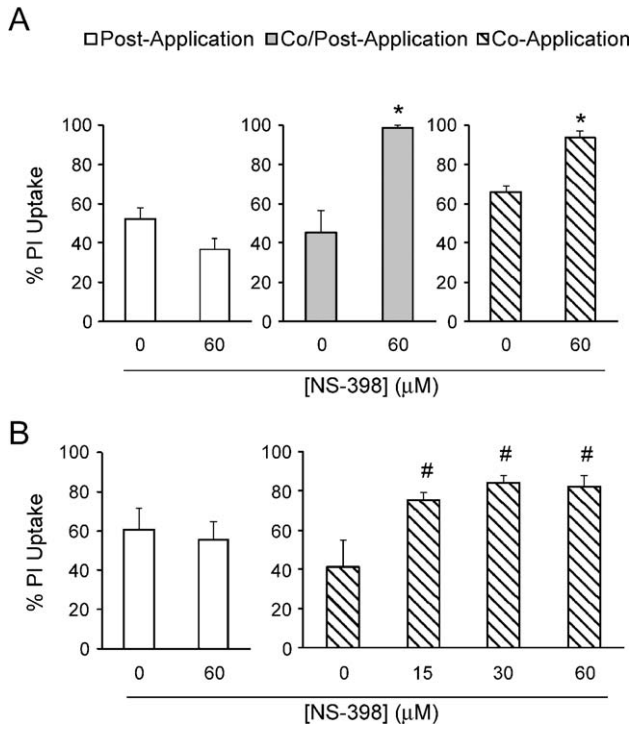

Fig. 2. Application of NS-398 during, but not following, OGD aggravates neurotoxicity. Mouse (A) and rat (B) cortical cultures were subjected to OGD and NS-398 was either added: (1) to the media following the insult (post-application), (2) to both the glucose-free BSS during OGD and to the media following the insult (co/post-application), or (3) to only the glucose-free BSS during OGD (co-application). Neuronal death was assessed 24 h later by propidium iodide (PI) uptake. Data represents the mean ± S.E.M. obtained from experiments performed using three to seven different platings of cells. *,#Significant ($P<0.05$) difference in % PI uptake compared to OGD performed in the absence of NS-398, as determined by Student's *t*-test and one-way ANOVA, respectively.

shown, $n=4$) nor does it affect cyclooxygenase-1 activity in vitro (Rosenstock et al., 1999). NS-398 had no significant effect on OGD-induced toxicity in either mouse (Fig. 2A) or rat (Fig. 2B) cortical cultures.

The lack of protection could be due to the inability of the inhibitor to permeate the cell membrane quickly enough to inhibit the rapidly synthesized cyclooxygenase-2 following OGD. In a cell-based system, NS-398 required a 30-min pre-incubation period to inhibit the enzymatic activity of cyclooxygenase-2 by half (Greig et al., 1997). To determine whether the addition of NS-398 both during and following OGD would have a greater effect on protection, mouse cultures were exposed to OGD in the presence of 60 μM NS-398 then returned to NS-398 (60 μM)-containing media. Surprisingly, these cultures exhibited significantly more neuronal death than their drug-free counterparts (Fig. 2A). The presence of NS-398 during OGD exposure alone (no post-incubation) was sufficient to aggravate neuronal death (Fig. 2A). To establish whether NS-398-induced aggravation of death is concentration- or species-dependent, rat cortical cultures were subjected to OGD in the presence of 15–60 μM NS-398. NS-398 significantly aggravated death at all doses tested (Fig. 2B).

Given these unexpected results, the effects of nimesulide and SC-58125 were tested in rat cultures. Nimesulide, like NS-398, is a cyclooxygenase-2 selective inhibitor from the class of acidic sulfonamides, whereas the cyclooxygenase-2 selective SC-58125 is a diarylheterocyclic. Similarly to NS-398, nimesulide significantly aggravated neuronal death when applied during OGD (Fig. 3A). Co-application of SC-58125 during OGD, however, provided significant neuroprotection as assessed both 24 and 48 h following the insult (Fig. 3B and C). Neither nimesulide nor SC-58125 were toxic under normoxic and normoglycemic conditions (data not shown, $n=4$ and $n=3$, respectively). Similar results were observed in mouse cortical cultures (data not shown, SC-58125 ($n=3$) and nimesulide ($n=3$)).

While all three inhibitors are known to inhibit cyclooxygenase-2, the opposite effects of these drugs suggest that either SC-58125 or NS-398/nimesulide act on additional pathways. Indeed, cyclooxygenase-2-independent effects have been reported for NS-398 and nimesulide (Saeed et al., 1998; Bozza et al., 2002; Vartiainen et al., 2001). Furthermore, NS-398 is reported to aggravate kainic acid-induced neuronal damage in vivo but fails to suppress prostaglandin E(2) production (Baik et al., 1999). It is

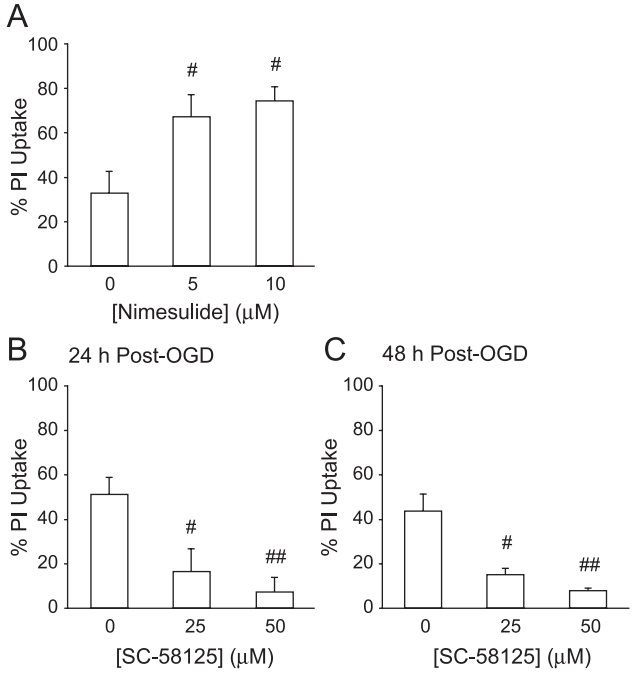

Fig. 3. OGD-induced neurotoxicity is aggravated by nimesulide and attenuated by SC-58125. Rat cortical cultures were subjected to OGD in the presence of nimesulide (A) or SC-58125 (B, C). Following OGD, cultures were washed and returned to their media. Neuronal death was assessed 24 h (A, B) or 48 h (C) later by propidium iodide (PI) uptake. Data represents the mean ± S.E.M. obtained from experiments performed using three to four different platings of cells. #,##Significant ($^{\#}P<0.05$, $^{\#\#}P<0.001$) difference in % PI uptake compared to OGD performed in the absence of inhibitor, as determined by one-way ANOVA.

unclear whether this aggravation is due to inhibition of cyclooxygenase-2 or is cyclooxygenase-2-independent. To address this concern in our model of excitotoxicity, the degree of death in cultures exposed to NS-398 (60 μM) during OGD was compared to that of cultures that were first pre-incubated with SC-58125 (50 μM, 90 min) prior to the insult then exposed to OGD in the presence of both SC-58125 (50 μM) and NS-398 (60 μM). Pre-incubation with SC-58125 should result in the formation of tightly bound complexes between SC-58125 and cyclooxygenase-2, such that NS-398 would not be able to bind cyclooxygenase-2 when applied during OGD (DeWitt, 1999). Inactivation of cyclooxygenase-2 by pre-incubating rat cortical cultures with SC-58125 prior to OGD did not attenuate NS-398-induced aggravation of death (Fig. 4). These results suggest that NS-398 exacerbates OGD-induced neuronal death by a cyclooxygenase-2-independent pathway.

3.3. The effects of cyclooxygenase-2 inhibitors on extracellular glutamate levels

Monitoring neuronal morphology during OGD revealed that cells treated with NS-398 or nimesulide become swollen and granular in appearance 5–10 min before cells that are not treated with these inhibitors. Since subjecting cortical neurons to OGD is associated with a large increase in the extracellular glutamate concentration ($[glutamate]_{ex}$) (Goldberg and Choi, 1993), and given that neurons begin to swell within 1–2 min of glutamate exposure (Choi et al., 1987), the effect of these inhibitors on $[glutamate]_{ex}$ was examined. Cultures were treated with NS-398 (60 μM) or

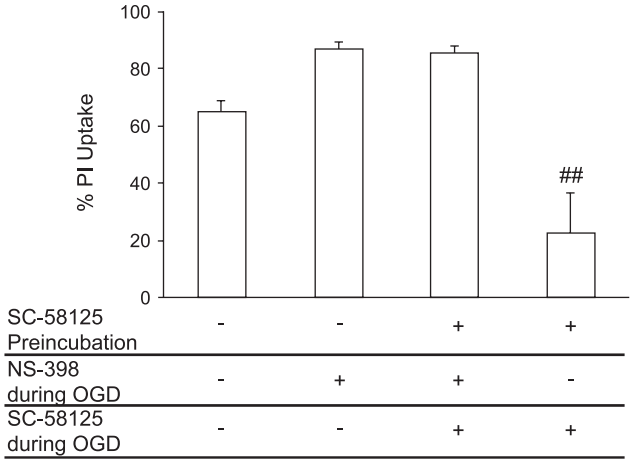

Fig. 4. Pre-incubation of cells with SC-58125 does not eliminate NS-398-induced aggravation of OGD-mediated neurotoxicity. Rat cortical cultures were pre-incubated in BSS containing or lacking SC-58125 (50 μM, 90 min) then exposed to OGD in the presence or absence of NS-398 (60 μM) and/or SC-58125 (50 μM). Following OGD, cultures were washed and returned to their media. Neuronal death was assessed 24 h later by propidium iodide (PI) uptake. Data represents the mean ± S.E.M. obtained from experiments performed using three different platings of cells. ##Significant ($P<0.001$) difference in % PI uptake compared to OGD performed in the absence of inhibitors, as determined by one-way ANOVA.

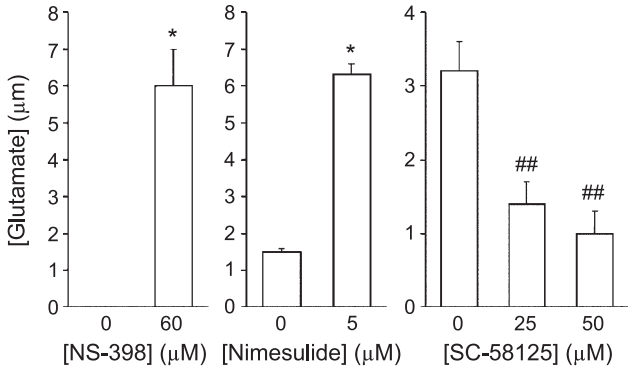

Fig. 5. The extracellular glutamate concentration is increased by NS-398 and nimesulide and decreased by SC-58125 during OGD. Rat cortical cultures were exposed to OGD in the presence or absence of cyclooxygenase-2 inhibitors. Immediately following OGD, buffer was removed and saved for $[glutamate]_{ex}$ measurements using a commercially available Amplex Red kit. Data represent the mean ± S.E.M. obtained from experiments performed using three different platings of cells. ##Significant ($P<0.001$) difference compared to OGD performed in the absence of SC-58125, as determined by one-way ANOVA. *Significantly ($P<0.05$) different compared to OGD performed in the absence of inhibitor, as determined by Student's *t*-test.

nimesulide (5 μM) during OGD (approximately 60–65 min), which was terminated when swelling of cells treated with the inhibitors became apparent. These cultures exhibited a significant increase in $[glutamate]_{ex}$ compared to their drug-free counterparts (Fig. 5). Conversely, treating cultures with SC-58125 during OGD (approximately 70–75 min), lead to a significant and dose-dependent reduction in $[glutamate]_{ex}$ (Fig. 5). In these experiments, OGD was terminated once swelling became apparent in cells not exposed to the cyclooxygenase-2 inhibitor in order to detect the reduction in $[glutamate]_{ex}$.

3.4. The effects of cyclooxygenase-2 inhibitors on intracellular Ca^{2+} levels

Increases in $[glutamate]_{ex}$ result in neuronal death through the overactivation of NMDA receptors and excessive influx of Ca^{2+} (Goldberg and Choi, 1993). To verify whether an increase in $[Ca^{2+}]_i$ is responsible for NS-398- and nimesulide-induced aggravation of death, $[Ca^{2+}]_i$ was examined in rat cortical cultures loaded with the fluorescent Ca^{2+}-sensitive dye, fluo-4, AM, and subjected to OGD in the presence or absence of the cyclooxygenase-2 inhibitors. The fluorescence intensity of cells was measured immediately following OGD and normalized to initial values measured prior to the insult. The final fluorescence intensity of cells kept under normoxic/normoglycemic conditions was half their initial value, indicating minor cellular dye leakage during the course of the experiment (Fig. 6). Neither NS-398 (60 μM) nor nimesulide (5 μM) affected fluorescence intensities under these conditions. While a significant rise in fluorescence intensity, and thus $[Ca^{2+}]_i$, was observed in all cultures exposed to OGD, the increase was

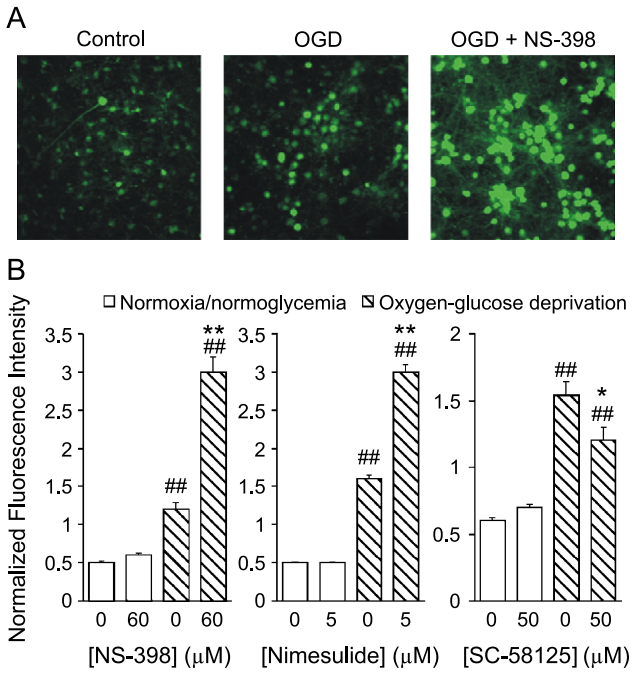

Fig. 6. Intracellular Ca^{2+} concentration is increased by NS-398 and nimesulide and decreased by SC-58125 during OGD. $[Ca^{2+}]_i$ was examined in rat cortical cultures loaded with the fluorescent Ca^{2+}-sensitive dye, fura-4, AM. The fluorescence intensity was measured immediately following OGD and normalized to initial values measured prior to the insult. Cultures were exposed to OGD in the absence or presence of NS-398, nimesulide or SC-58125. Data represents the mean ± S.E.M. obtained from experiments performed using three to four different platings of cells. ##Significant ($P<0.001$) difference compared to controls kept under normoxic/normoglycemic conditions in the absence of inhibitor, as determined by one-way ANOVA. *,**Significantly (*$P<0.05$, **$P<0.001$) different compared to OGD performed in the absence of inhibitor, as determined by Student's *t*-test.

significantly greater in cells exposed to 60 μM NS-398 or 5 μM nimesulide (Fig. 6).

In contrast, SC-58125 lowered Ca^{2+} influx during OGD as exhibited by the significant reduction in fluo-4 fluorescence in SC-58125-treated cultures compared to cultures exposed to OGD in the absence of SC-58125 (Fig. 6). The reduction in fluorescence caused by SC-58125 was not due to quenching of fluo-4 fluorescence. In a cell-free assay, none of the inhibitors altered the fluorescence of FITC, the basic fluorophore component of fluo-4, AM, suggesting that the decrease in fluo-4 fluorescence observed in SC-58125-treated cultures was due to a reduction in cellular $[Ca^{2+}]_i$ (data not shown, $n=2$).

3.5. Effects of NMDA receptor antagonism on NS-398-induced aggravation of death

To determine whether the increase in $[glutamate]_{ex}$ and subsequent influx of Ca^{2+} via NMDA receptor activation is directly responsible for NS-398-induced aggravation of death, the effect of co-applying NS-398 and the competitive NMDA receptor antagonist, MK-801, during OGD was examined. Co-application of 1 μM MK-801 with 60 μM NS-398 during OGD completely abolished the NS-398-induced aggravation of death, suggesting that an NS-398-induced increase in $[glutamate]_{ex}$ and $[Ca^{2+}]_i$ during OGD are sufficient to cause neuronal death (data not shown, $n=5$).

3.6. The effects of cyclooxygenase-2 inhibitors on glutamate currents

To evaluate whether NS-398 and SC-58125 directly modulate glutamate receptors and alter Ca^{2+} influx, patch-clamp studies were conducted. Whole-cell glutamate-induced currents were measured in cultured rat cortical neurons held at −60 mV in response to a 2-s application of 50 μM glutamate followed by a 3-s co-application of glutamate (50 μM) and NS-398 (60 μM) or SC-58125 (50 μM). No significant change in glutamate-induced currents was observed with NS-398 or SC-58125 (0% block ($n=5$) and 3.9 ± 2.5% block ($n=7$), respectively; data not shown). Furthermore, neither compound significantly affected membrane resting potential when cells were examined in current-clamp mode over a short term. With NS-398, cell membrane potential was initially 61 ± 2 mV ($n=6$), and was 55 ± 6 mV ($n=5$) after a 5-min monitoring period. With SC-58125, the initial membrane potential was 60 ± 3 mV ($n=5$) and 59 ± 4 mV ($n=5$) 5 min later (data not shown).

3.7. The effect of cyclooxygenase-2 inhibitors on glutamate- and NMDA-induced neuronal death

To determine whether NS-398-induced aggravation of neuronal death is specific to OGD, its effects on glutamate

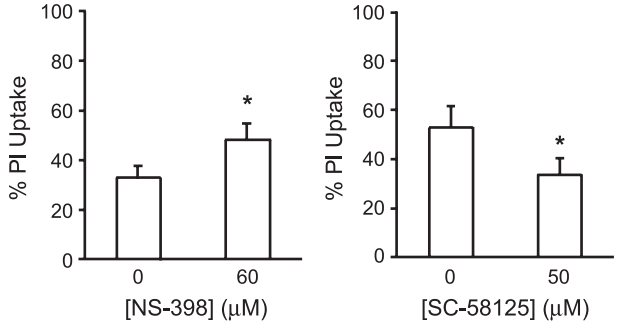

Fig. 7. Glutamate-mediated neurotoxicity is aggravated by NS-398 and attenuated by SC-58125. Rat cortical cultures were pre-incubated in BSS containing or lacking 60 μM NS-398 or 50 μM SC-58125 for 60 min. Cultures were then exposed to 50 μM glutamate in the presence or absence of NS-398 or SC-58125. Following a 5- to 10-min glutamate exposure, cultures were washed and returned to media containing or lacking 60 μM NS-398 or 50 μM SC-58125. Neuronal death was assessed 24 h later by propidium iodide (PI) uptake. Data represents the mean ± S.E.M. obtained from experiments performed using four to nine different platings of cells. *Significant (*$P<0.05$) difference in % PI uptake compared to glutamate exposure performed in the absence of inhibitor, as determined by Student's *t*-test.

and NMDA toxicity were studied. Since the duration of glutamate and NMDA exposure (10–20 min) is much shorter than that of OGD (60–75 min), rat cultures were pre-incubated with NS-398 (60 μM) for 60 min prior to the insult to enhance cell loading. NS-398 (60 μM) was also added during and following both glutamate and NMDA exposure (pre/co/post-incubation). NS-398 significantly exacerbated death induced by glutamate (Fig. 7), although not to as large a degree as observed when NS-398 was applied during OGD. Pre/co/post-incubation of NS-398 had no effect on NMDA toxicity (Fig. 8A), however, a co/post-incubation protocol significantly protected both rat and mouse cultures, consistent with mouse culture data published by Hewett et al. (2000). In rat cultures, the percentage of propidium iodide uptake in cells treated with NMDA alone was 46 ± 4% compared to 30 ± 4% in cells treated to NMDA and NS-398 (Fig. 8B). In mouse cultures, the percentage of propidium iodide uptake in cells treated with NMDA alone was 47 ± 8% compared to 28 ± 7% in cells treated to NMDA and NS-398 (data not shown, $n = 11$).

Next, the effect of SC-58125 on glutamate- and NMDA-induced toxicity was tested. SC-58125 (50 μM) provided significant protection against both glutamate and NMDA-induced neurotoxicity when added to cultures 60 min prior to the insult as well as during and following glutamate or NMDA exposure (Figs. 7 and 8C, respectively). SC-58125 also provided significant neuroprotection against NMDA-induced death in the absence of the 60-min pre-incubation period (Fig. 8D).

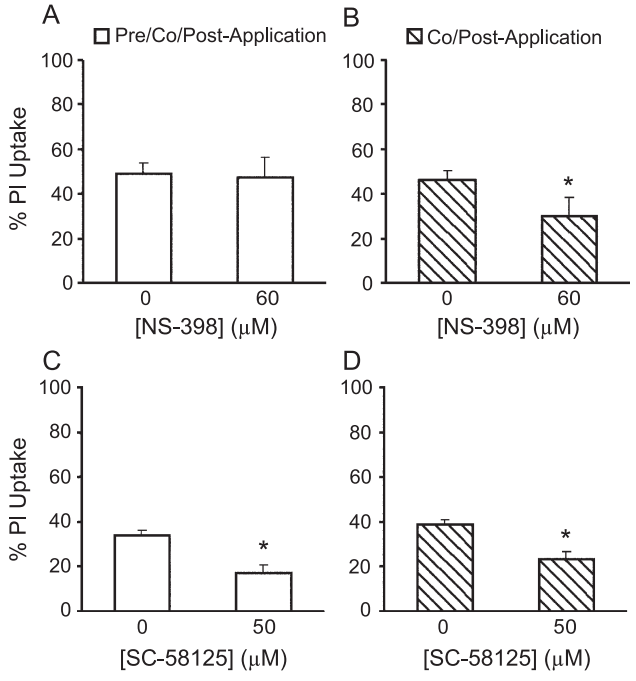

Fig. 8. The effects of NS-398 and SC-58125 on NMDA-mediated neurotoxicity. Rat cortical cultures were exposed to 35 μM NMDA for 10–20 min and 60 μM NS-398 (A, B) or 50 μM SC-58125 (C, D) was either added: (1) to BSS for a 60-min pre-incubation, then to both the BSS during NMDA exposure and to the media afterwards (pre/co/post-application) or (2) to both the BSS during NMDA exposure and to the media afterwards (co/post-application). Neuronal death was assessed 24 h later by propidium iodide (PI) uptake. Data represents the mean ± S.E.M. obtained from experiments performed using 3–10 different platings of cells. *Significant (*$P < 0.05$) difference in % PI uptake compared to NMDA exposure performed in the absence of inhibitor, as determined by Student's *t*-test.

4. Discussion

The key findings of this study are as follows: (1) OGD leads to a rapid increase in cyclooxygenase-2 protein expression upon reoxygenation in mixed neuronal/glial cortical cultures; (2) the structurally distinct cyclooxygenase-2 inhibitors, NS-398/nimesulide and SC-58125, have opposing effects on OGD-induced neuronal death, Ca^{2+} influx and glutamate release when applied during OGD and (3) the effect of cyclooxygenase-2 inhibitors on neuronal death is determined by the timing of inhibitor application and the model of excitotoxicity in which the inhibitor is employed.

The presence of NS-398 during OGD significantly aggravated neuronal death. The exacerbation of death induced by NS-398 was dose-dependent and was observed in both mouse and rat cortical cultures. This observation was mimicked by the structurally similar cyclooxygenase-2 inhibitor, nimesulide, suggesting that basal levels of cyclooxygenase-2 activity are required for neurons to cope with the immediate stress imposed by OGD. However, the neuroprotective effect of SC-58125 on OGD suggests that cyclooxygenase-2 activation during OGD is deleterious. The diametrically opposed results imply that either of these inhibitors possess cyclooxygenase-2-independent effects in addition to their ability to inhibit cyclooxygenase-2. The fact that NS-398 continued to exacerbate death in spite of cyclooxygenase-2 inhibition by SC-58125 pre-incubation supports the belief that NS-398-induced aggravation of death is independent of cyclooxygenase-2 inhibition. Cyclooxygenase-2-independent effects have been reported for both nimesulide and NS-398 (Bozza et al., 2002; Vartiainen et al., 2001; Saeed et al., 1998). At low concentrations (0.01–0.1 μM), nimesulide potentiated adrenalin-induced aggregation of human platelets, which predominantly express cyclooxygenase-1, through activation of Ca^{2+} signalling (Saeed et al., 1998). Other cyclooxygenase inhibitors tested, including NS-398, did not have this effect. In human neutrophils, NS-398, but not SC-58125, inhibited lipid body formation induced by arachidonic acid and oleic acid (Bozza et al., 2002). This effect was independent of cyclooxygenase-2 since NS-398 inhibited arachidonic acid-induced lipid body formation in leukocytes of both wild-type and cyclooxygenase-2-deficient mice. In a different study, NS-398 provided cyclooxygenase-2-independent protection against hypoxic/reoxygenation injury in mixed spinal cord cultures, possi-

bly through the increased activation of the extracellular signal-regulated kinase (ERK) pathway (Vartiainen et al., 2001). Furthermore, Baik et al. (1999) reported that NS-398 aggravated kainic acid-induced neuronal damage in vivo and increased prostaglandin E(2) levels approximately 2 times more than kainic acid alone, though inhibition of cyclooxygenase-2 is expected to lead to decreased prostaglandin synthesis.

In our model, NS-398 and nimesulide exacerbated neuronal death by enhancing the OGD-induced increase in $[glutamate]_{ex}$ resulting in excessive Ca^{2+} influx via NMDA receptor activation. Interestingly, NS-398 aggravated neuronal death only when present during, but not following, OGD. The timing of NS-398 application also determined its effect on NMDA-induced neurotoxicity. NS-398 was only protective when applied both during and post (co/post) NMDA exposure. It was not protective if the cells were also pre-incubated with the inhibitor (pre/co/post) to ensure its presence within the cells during the insult. It appears then that the cellular context in which NS-398 is present determines its effects. NS-398 likely aggravates neuronal death in the presence of factors upregulated during excitotoxic insults. This is further supported by the finding that NS-398 exacerbated glutamate-induced excitoxicity.

In contrast to NS-398, SC-58125 was significantly neuroprotective against OGD-, glutamate- and NMDA-mediated neurotoxicity. It is interesting to note that application of SC-58125 during OGD alone was sufficient to protect cultures, despite the fact that cyclooxygenase-2 expression increased only after reoxygenation. This could be due, in part, to the incomplete removal of SC-58125 following OGD and to its long half-life (over 24 h) (Nakayama et al., 1998) allowing it to inhibit newly synthesized cyclooxygenase-2 upon reoxygenation.

While the exact mechanisms by which cyclooxygenase-2 overexpression leads to neuronal death are unknown, it is believed cyclooxygenase-2 contributes to neurotoxicity via the generation of superoxide, a by-product of prostaglandin synthesis. Pepicelli et al. (2002) reported that NMDA-induced cyclooxygenase-2 activity is an important source of free radicals that contribute to neuronal impairment via lipid peroxidation. It has also been suggested that cyclooxygenase-2-mediated excitotoxicity involves cell cycle deregulation. Overexpression of human cyclooxygenase-2 in a mouse model of Alzheimer's disease leads to the increased phosphorylation of the retinoblastoma tumour suppressor protein (pRB) (Xiang et al., 2002), which regulates the cell cycle through its interactions with E2F family members. Additionally, cyclooxygenase-2 overexpression exacerbates glutamate-induced excitotoxicity in cortical–hippocampal cultures also by increasing pRB phosphorylation (Mirjany et al., 2002). Our current finding that OGD significantly increased cyclooxygenase-2 expression, together with our previous observation that cortical cultures lacking E2F1 are less susceptible to OGD (Gendron et al., 2001), support this hypothesis.

The marked decrease in $[Ca^{2+}]_i$ and $[glutamate]_{ex}$ afforded by the application of SC-58125 during OGD indicate that basal levels of cyclooxygenase-2 also play an important role in OGD-induced neuronal death. This is consistent with the findings of Pepicelli et al. (2002) showing that acute activation of hippocampal NMDA receptors in freely moving rats triggered an almost immediate increase of prostaglandin E(2) efflux, suggesting that synthesis of new cyclooxygenase-2 protein is not required for the initial burst of prostaglandin production. The reduction in $[Ca^{2+}]_i$ produced by SC-58125 was not due to this inhibitor acting directly on glutamate receptors, as determined by patch-clamp studies. The decrease in $[glutamate]_{ex}$ and $[Ca^{2+}]_i$ may have occurred through decreased synthesis of prostaglandin E(2), which has been shown to stimulate the release of glutamate from astrocytes and induce increases in $[Ca^{2+}]_i$ in astrocytes and in neurons (Bezzi et al., 1998).

The findings of the current study indicate that certain cyclooxygenase-2 inhibitors may act on cyclooxygenase-2-independent pathways and the outcome depends, not only on the inhibitor used, but also on the timing of application and on the type of insult. These findings may explain why studies investigating the effect of pharmacological inhibition of cyclooxygenase-2 in in vivo models of cerebral ischemia have yielded conflicting results (Nogawa et al., 1997; Sugimoto and Iadecola, 2003; Hara et al., 1998; Cole et al., 1993). This study underlines the need for caution when using cyclooxygenase-2 inhibitors to study various models of disease to ensure that cyclooxygenase-2-independent effects are not falsely associated with the role of this enzyme. Furthermore, since we and others (Cole et al., 1993; Baik et al., 1999) have shown that certain cyclooxygenase inhibitors exacerbate neuronal death during excitotoxic insults, great care should be taken if such inhibitors are to be used for the treatment of neurodegenerative diseases. Indeed, a meta-analysis of long-term safety studies for the presently available cyclooxygenase-2 selective inhibitors, celecoxib (CLASS) and rofecoxib (VIGOR) suggests a potential increase in cardiovascular events, including myocardial infarction, ischemic stroke and transient ischemic attacks (Mukherjee et al., 2001). A better understanding of the role of cyclooxygenase-2 and of cyclooxygenase-2 selective inhibitors is thus needed before deciding whether inhibition of this enzyme represents a valid neuroprotective strategy.

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