Journal of Neurochemistry, 2005, **93**, 1561–1567 doi:10.1111/j.1471-4159.2005.03150.x

Cyclo-oxygenase-1 and -2 differently contribute to prostaglandin E_2 synthesis and lipid peroxidation after *in vivo* activation of *N*-methyl-D-aspartate receptors in rat hippocampus

Olimpia Pepicelli,† Ernesto Fedele,†,‡ Maria Berardi,† Maurizio Raiteri,†,‡ Giulio Levi,* Anita Greco,* Maria Antonietta Ajmone-Cat* and Luisa Minghetti*

**Department of Cell Biology and Neurosciences, Istituto Superiore di Sanità, Rome, Italy*

†*Department of Experimental Medicine, Pharmacology and Toxicology Section, University of Genova, Italy*

‡*Centre of Excellence for Biomedical Research, University of Genova, Italy*

Abstract

Using intracerebral microdialysis, we reported previously that acute *in vivo* activation of NMDA glutamate receptors triggers rapid and transient releases of prostaglandin E_2 (PGE_2) and F_2-isoprostane 15-F_{2t}-IsoP in the hippocampus of freely moving rats. The formation of the two metabolites – produced through cyclo-oxygenase (COX) enzymatic activity and free radical-mediated peroxidation of arachidonic acid (AA), respectively, – was prevented by the specific NMDA antagonist MK-801, and was largely dependent on COX-2 activity. Here, we demonstrate that besides COX-2, which is the prominent COX isoform in the brain and particularly in the hippocampus, the constitutive isoform, COX-1 also contributes to prostaglandin (PG) synthesis and oxidative damage following *in vivo* acute activation of hippocampal NMDA glutamate receptors. The relative contribution of the two isoforms is dynamically regulated, as the COX-2 selective inhibitor NS398 immediately prevented PGE_2 and 15-F_{2t}-IsoP formation during the application of NMDA, whereas the COX-1 selective inhibitor SC560 was effective only 1 h after agonist infusion. Our data suggest that, although COX-2 is the prominent isoform, COX-1 activity may significantly contribute to excitotoxicity, particularly when considering the amount of lipid peroxidation associated with its catalytic cycle. We suggest that both isoforms should be considered as possible therapeutic targets to prevent brain damage caused by excitotoxicity.

Keywords: cyclo-oxygenase, excitotoxicity, isoprostane, microdialysis, neurodegeneration, prostaglandin

J. Neurochem. (2005) **93,** 1561–1567.

Excitotoxicity is an important cause of neuronal death in a variety of pathologies, including acute diseases, such as ischemia and seizures, and also infectious and chronic degenerative disorders. Among other mechanisms, cyclo-oxygenase-2 (COX-2) has been implicated in excitotoxic cell death and, currently, this inducible enzyme is regarded as a potential therapeutic target for neuroprotection.

Cyclo-oxygenase, which exists in two major isoforms (COX-1 and COX-2), catalyses the first committed step in the synthesis of prostaglandins (PGs) and other prostanoids from arachidonic acid (AA) (Smith *et al*. 1996). Cyclo-oxygenase-2 is rapidly induced in several cell types in response to mitogenic and inflammatory stimuli and has emerged as the isoform primarily responsible for prostanoid production under pathological conditions. However, in the brain, COX-2 is detectable in specific neuronal populations under physiological conditions. In these cells, COX-2 expression is regulated dynamically, being dependent on normal excitatory synaptic activity, and contributes to fundamental brain functions, such as synaptic plasticity, memory consolidation and functional hypermia (for review, see Minghetti 2004). Nonetheless, COX-2 is rapidly up-regulated after seizures or ischemia and mounting

Received December 22, 2004; revised manuscript received February 15, 2005; accepted February 15, 2005.

Address correspondence and reprint requests to Luisa Minghetti, Department of Cell Biology and Neurosciences, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy.
E-mail: luisa.minghetti@iss.it

Abbreviations used: AA, arachidonic acid; artificial CSF, artificial cerebrospinal fluid; COX, cyclo-oxygenase; 15-F2t-IsoP, F2-isoprostane; LPS, lipopolysaccharide; NOS, nitric oxide synthase; NSAIDs, non-steroidal anti-inflammatory drugs; PG, prostaglandin; ROS, reactive oxygen species.

evidence indicates that COX-2 mediates, at least in part, glutamate neurotoxicity following activation of NMDA or α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Nogawa *et al*. 1997; Nakayama *et al*. 1998; Koistinaho *et al*. 1999). The mechanisms by which COX-2 dependent neurotoxicity is exerted remain a matter of investigation. PGE_2, one of the prominent PGs, was reported to be neurotoxic (Hewett *et al*. 2000; Takadera *et al*. 2002) and to stimulate the release of glutamate from astrocytes (Bezzi *et al*. 1998) but also neuroprotective in hippocampal and cortical neuronal cultures against excitotoxic injury, lipopolysaccharide (LPS)-induced cytotoxicity or oxygen deprivation (Akaike *et al*. 1994; Thery *et al*. 1994; Kim *et al*. 2002; McCullough *et al*. 2004). Interestingly, the neuroprotective effects were generally observed at concentrations consistent with PGE_2 receptor activation (< μM), and lower than those required for toxic effects. Recently, EP2 and EP3 receptors, two of the four PGE_2 receptor subtypes, were shown to mediate neuroprotection both *in vitro* and *in vivo* (Bilak *et al*. 2004; McCullough *et al*. 2004).

Radical formation and oxidant injury play critical roles in excitotoxic damage (Facchinetti *et al*. 1998), as evidenced by the presence of lipid peroxidation products, including isoprostanes, in brain tissues from patients or animal models of different human neurological diseases (Patel *et al*. 2001; Greco and Minghetti 2004; Popoli *et al*. 2004). As COX activity and prostanoid synthesis are associated with the generation of free radicals, which are essential for sustaining the COX catalytic cycle, it has been proposed that the excitotoxic damage mediated by COX-2 involves oxidative injury rather than, or in addition to, PG toxicity. In line with this hypothesis, we have shown (Pepicelli *et al*. 2002) that *in vivo* activation of NMDA receptors in the rat hippocampus causes an immediate and transient increase of the basal levels of PGE_2, which is accompanied by the formation of the isoprostane 15-F_{2t}-IsoP (also named 8-epi-$PGF_{2\alpha}$), a compound generated *in vivo* by free radical-dependent lipid peroxidation that is widely used as a marker of oxidative stress (for review, see Greco and Minghetti 2004). In this experimental model, the formation of the two metabolites was prevented to a similar extent by the specific NMDA antagonist MK-801 and by selective/non-selective COX-2 inhibitors, indicating that the NMDA-evoked PG synthesis and free radical-mediated isoprostane formation were largely dependent on COX-2 activity (Pepicelli *et al*. 2002). COX-2 dependent oxidative damage was also demonstrated in animal models of restraint stress (Madrigal *et al*. 2003) and global ischemia (Candelario-Jalil *et al*. 2003).

Besides COX-2, the constitutive isoform, COX-1, has also been found to contribute to lipid peroxidation − measured as malondialdehyde and 4-hydroxyalkenals formation − in an animal model of global ischemia (Candelario-Jalil *et al*. 2003). In rat and human brain, COX-1 and COX-2 are present in discrete neuronal populations distributed in distinct areas, such as cerebral cortex and hippocampus, where COX-2 appears the predominant isoform, and midbrain, pons and medulla, in which COX-1 immunoreactivity prevails (Yamagata *et al*. 1993; Breder *et al*. 1995). The relative contribution of COX-1 and COX-2 activity to brain pathology and physiology has been questioned recently (Graham and Hickey 2003; Schwab and Schluesener 2003) and it is now clear that the popular paradigm by which COX-1 and COX-2 serve physiological and pathological functions, respectively is inadequate to explain an increasing number of observations (Parente and Perretti 2003).

To gain a better insight into the roles of COX isoforms in excitotoxicity, and their contribution to oxidative injury, we have extended our *in vivo* microdialysis study in freely moving rats to monitor the levels of PGE_2 and 15-F_{2t}-IsoP after co-infusion of NMDA and COX-1 or COX-2 specific inhibitors, or a combination of the two drugs. In this experimental paradigm, the agonist NMDA (with or without COX inhibitors) is administered directly into the hippocampus and the metabolites are measured in the artificial cerebrospinal fluid (CSF) infusing the microdialysis probe. This procedure allows direct and dynamic observations of the events triggered by NMDA receptor activation. Our results indicate that, although COX-2 is the predominant isoform, COX-1 is also involved in PGE_2 synthesis and 15-F_{2t}-IsoP formation. The relative contribution of each isoform is dynamically regulated, COX-2 being predominant during the acute application of the glutamatergic agonist and COX-1 in the later phase.

Materials and methods

Materials

The following drugs were purchased from the companies indicated in parentheses: NMDA (Tocris Cookson, Bristol, UK); SC560 and NS398 (Cayman Chemical, Ann Arbor, MI, USA). [^{3}H]PGE_2 (specific activity 164 Ci/mmol) from Amersham International (UK). Specific antibody for PGE_2 and standard PGE_2 (Thin-layer chromatography-purified) were from Sigma Chemical (St Louis, MO, USA). Specific enzyme immunoassay for 15-F_{2t}-IsoP (detection limit: 2 pg/mL) was from Cayman Chemical.

Microdialysis surgery

The *in vivo* experimental procedures were approved by the Ethics Committee of the Section of Pharmacology and Toxicology, University of Genoa, according to European legislation on the use and care of laboratory animals (CEE 86/609). As reported previously (Fedele *et al*. 1996), male Sprague–Dawley rats (250–300 g; CD-COBS, Charles River, Calco, Italy) were anaesthetised with Equitesin (4 ml/kg body i.p.), placed in a stereotaxic frame (incisor bar 3.5 mm below horizontal zero) and implanted with a homemade microdialysis probe that was positioned transversely in the dorsal hippocampi according to the following co-ordinates based on the atlas by Paxinos and Watson (1986): anteroposterior distance (AP) = +3.8, dorsoventral distance (H) = +6.5 from the interaural

line. The dialysis fibre (AN69HF Hospal S.p.A., Bologna, Italy; 0.3 mm outer diameter with more than 40 000 molecular mass cut-off) was covered with epoxy glue to confine dialysis to the area of interest (10 mm glue-free zone); the dialysis probe, held straight by a tungsten wire inside, was inserted transversely so that the glue-free zone was exactly located in the target area. The tungsten wire was withdrawn, stainless steel cannulae (22-gauge diameter, ≈15 mm long) were glued to the ends of the fibre, bent up and fixed vertically to the skull with dental cement and modified Eppendorf tips. After a 24 h recovery period, rats were placed into observation cages and probes infused at a flow rate of 5 μL/min (CMA/100 microinjection pump, Carnergie Medicine, Stockholm, Sweden) with artificial CSF containing (in mM): NaCl (145), KCl (3), $CaCl_2$ (1.26), $MgCl_2$ (1), buffered at pH 7.4 with 2 mM phosphate buffer. Following a stabilisation period of 1 h, consecutive samples were collected every 20 min and kept at −80°C until analysed. At the end of the experiment, rats were killed with an anaesthetic overdose and the correct position of the probe was verified by optical examination of the fibre tract.

NMDA and COX inhibitor treatments

NMDA (1 mM) was infused into the probe for 20 min after three control samples had been collected to estimate analyte basal extracellular levels. When used, inhibitors were added 20 min before NMDA and infused till the end of the experiment. The selective COX-1 inhibitor SC560 (IC_{50} for COX-1: 9 nM; IC_{50} for COX-2 6.3 μM, as calculated using human purified enzymes), and the selective COX-2 inhibitor NS398 (IC_{50} for COX-1: 75 μM; IC_{50} for COX-2: 1.77 μM), were dissolved in pure dimethyl sulfoxide (DMSO) at a concentration of 10 mM and diluted to final concentration into artificial CSF. DMSO (0.3% in the final solutions infused in the probe) used in the experiments did not affect analyte basal levels (data not shown). The concentrations of the glutamatergic agonist and the inhibitors were chosen on the basis of previous *in vivo* and *in vitro* studies (Niwa *et al*. 2001; Pepicelli *et al*. 2002). It has to be noted that, due to several technical reasons, the amount of a drug delivered to the tissue through the dialysis probe, has to been assumed to reach 10–20% of its original concentration. Therefore, tissue concentrations of the two inhibitors would be 3–6 μM in our experiments. In addition, it has to be taken into account that the inhibitors have to cross cell membranes to produce significant enzyme inhibition.

PGE_2 and 15-F_{2t}-IsoP determinations

Microdialysis eluates were collected and immediately stored at −80°C until tested to avoid *ex vivo* auto-oxidation. PGE2 was measured by a specific radioimmunoassay as described previously (Minghetti and Levi 1995; detection limit: 25 pg/mL). 15-F_{2t}-IsoP, also known as 8-epi-$PGF_{2\alpha}$, was measured by a colourimetric enzyme immunoassay (detection limit: 2 pg/mL; Cayman Chemical). As described previously (Pepicelli *et al*. 2002), anti-15-F_{2t}-IsoP Ig cross-reactivity with other prostaglandins was less than 1% (0.02% for PGE_2), whereas cross-reactivity of the antibody for PGE_2 with prostaglandins of the $F_{2\alpha}$ series was less than 1.5%. Interference of artificial CSF with the enzymatic assay was excluded by performing two series of determinations carried out in standard buffer and in artificial CSF spiked with known amounts of PGE_2 or 15-F_{2t}-IsoP. No difference in the values obtained could be detected.

Statistics and expression of results

The data presented are expressed as pg/mL and given as mean ± SEM. As the NMDA-evoked effects, in the absence of inhibitors, were not different amongst the various experimental sets, data were pooled. Differences were analysed by two-way ANOVA followed Mann–Whitney *U*-test and considered significant at the level of $p < 0.05$.

Results

Local administration of 1 mM NMDA by reverse dialysis for 20 min caused a massive increase in extracellular PGE_2 (Fig. 1) and 15-F_{2t}-IsoP (Fig. 2). As described previously (Pepicelli *et al*. 2002), PGE_2 reached maximal levels during the 20 min infusion of NMDA, remained elevated for 40 min following agonist application and slowly decreased in the subsequent fractions. As to 15-F_{2t}-IsoP release (Fig. 2), the peak and the subsequent decline were delayed compared with

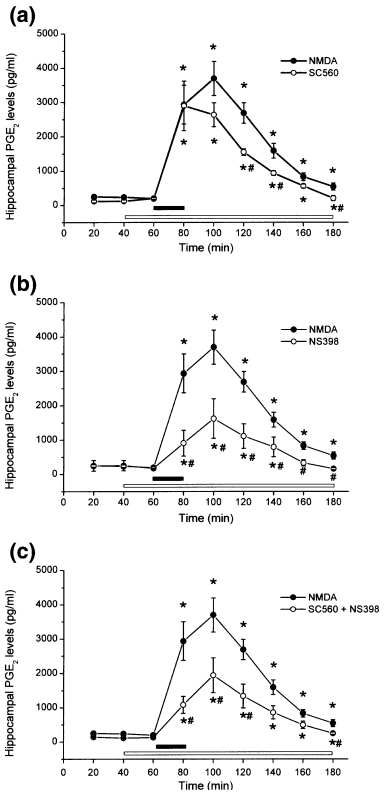

Fig. 1 Effects of COX-1 and COX-2 selective inhibitors on the NMDA-induced increase of PGE_2 extracellular levels in the rat hippocampus. The graph shows the effects of a 20-min (horizontal filled bar) infusion of NMDA (1 mM, $n = 20$) on the extracellular levels of PGE_2. Co-infusion (horizontal unfilled bar) of 30 μM SC560 (a) ($n = 5$), or 30 μM NS398 (b) ($n = 6$) or the combination of the two drugs (c) ($n = 6$) prevented the effects of NMDA (1 mM) to different extents. Experiments were carried out 24 h after surgery on un-anaesthetised rats. Each point represents mean ± SEM of 5–20 independent experiments. *$p < 0.05$ versus basal; #$p < 0.05$ versus NMDA (1 mM).

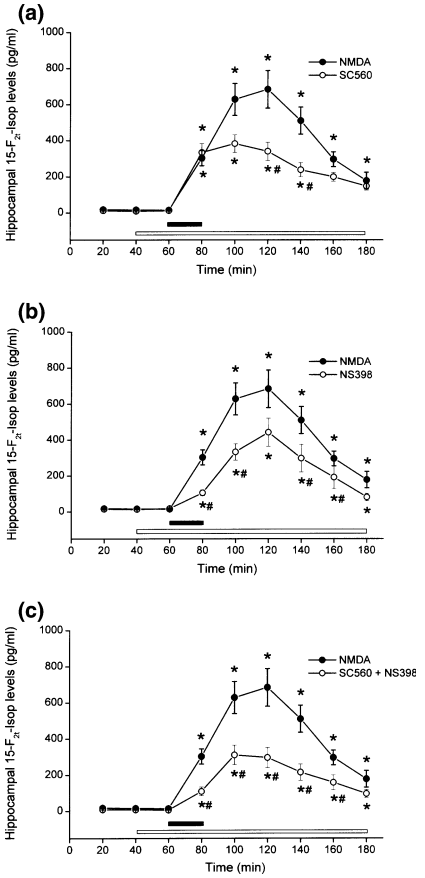

Fig. 2 Effects of COX-1 and COX-2 selective inhibitors on the NMDA-induced increase of 15-F_{2t}-IsoP extracellular levels in the rat hippocampus. The graph shows the effects of a 20-min (horizontal filled bar) infusion of NMDA (1 mM, $n = 20$) on the extracellular levels of 15-F_{2t}-IsoP. Co-infusion (horizontal unfilled bar) of 30 μM SC560 (a) ($n = 5$), or 30 μM NS398 (b) ($n = 6$) or the combination of the two drugs (C) ($n = 6$) prevented the effects of NMDA (1 mM) to different extents. Experiments were carried out 24 h after surgery on un-anaesthetised rats. Each point represents mean ± SEM of 5–20 independent experiments. *$p < 0.05$ versus basal; #$p < 0.05$ versus NMDA (1 mM).

the pattern seen with PGE_2. Furthermore, the presence of the NMDA receptor antagonist MK-801 (30 μM) in the infusion stream greatly diminished the levels of both metabolites (not shown).

As shown in Fig. 1a, the presence of the COX-1 inhibitor SC560 (30 μM) in the infusion stream did not diminish the levels of PGE_2 during NMDA infusion or the following 20 min. However, the levels of PGE_2 in the subsequent two fractions were significantly lower in the presence of the COX-1 inhibitor compared to the agonist alone. On the contrary, the COX-2 specific inhibitor NS398 (30 μM) greatly diminished the levels of hippocampal PGE2 throughout the entire experiment (Fig. 1b), indicating a prominent role of COX-2 and confirming our previous observations (Pepicelli *et al.* 2002). The co-administration of the two inhibitors, used at the same concentrations as in Fig. 1(a and b), significantly decreased the release of PGE_2 induced by NMDA infusion. The extent of inhibition was similar to that obtained in the presence of NS398 alone. NS398 or SC560 had no effect on the basal levels of PGE_2, measured before NMDA administration.

Then, we analysed the effects of the two COX inhibitors on NMDA-induced release of 15-F_{2t}-IsoP (Fig. 2). Concerning PGE_2, the COX-1 selective inhibitor SC560 did not affect the formation of 15-F_{2t}-IsoP during NMDA infusion (80 min fraction and the following 20 min), but it significantly reduced the levels of the metabolite in the following two fractions (Fig. 2a). On the contrary, the presence of NS398 in the infusion stream caused an immediate decrease of 15-F_{2t}-IsoP during NMDA infusion, which lasted for the entire experiment, with the exception of the 120 min fraction containing the highest levels of 15-F_{2t}-IsoP (Fig. 2b). These results suggest that both isoforms of COX participate in radical formation and lipid peroxidation induced by NMDA receptor activation. However, the roles of COX-1 and COX-2 appear to be time-dependent, in that COX-2 contributes actively to lipid peroxidation during the acute phase of NMDA receptor stimulation ($t = 80$ and 100 min) and COX-1 plays a role in a later phase ($t = 120$–140 min). The hypothesis that both isoforms contribute to lipid peroxidation upon NMDA stimulation was further supported by the additive inhibition of the two inhibitors SC560 and NS398, when infused together into the probe. In this case, the release of 15-F_{2t}-IsoP was significantly and permanently reduced during the entire infusion period (Fig. 2c).

To further analyse the relative contribution of the two COX isoforms to PG synthesis and isoprostane formation, we compared the percentage of inhibition of SC560, NS398 or the combination of the two drugs on the maximal release of each metabolite (80-, 100- and 120-min fractions). The percentage of inhibition was calculated taking the NMDA-evoked metabolite concentration in each fraction as 100%. As shown in Fig. 3, during the administration of the glutamatergic receptor agonist (80-min fraction) and in the following fraction (100-min fraction), both PGE_2 and 15-F_{2t}-IsoP were dependent largely on COX-2 activity. However, the activity of COX-1 became evident subsequently, as the inhibition of both PGE_2 and 15-F_{2t}-IsoP by SC560 increased with the time after NMDA infusion to become statistically relevant in the 100 and 120-min fraction (Fig. 3). Interestingly, while PGE_2 inhibition by NS398 remained substantially constant in the three fractions, the effect of the COX-2 inhibitor on 15-F_{2t}-IsoP release decreased with time. This diminished inhibition by NS398 was concomitant to an increased inhibition by SC560, suggesting a compensatory activity of COX-1. Indeed, when both inhibitors were present PGE_2 as well as 15-F_{2t}-IsoP were permanently inhibited.

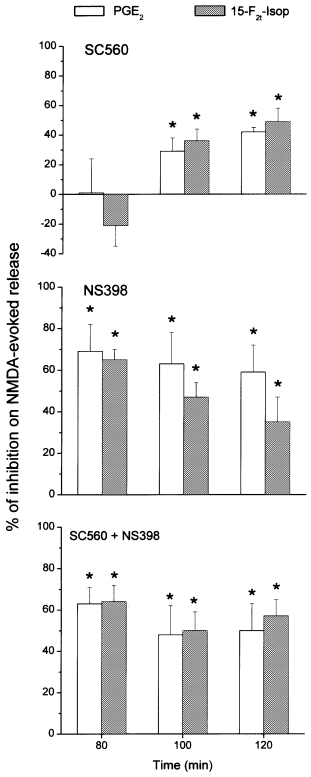

Fig. 3 Comparison of the inhibitory effects of SC560, NS398 or their combination on NMDA induced PGE_2 and 15-F_{2t}-IsoP levels. The percentage of inhibition (fractions $t = 80$, 100 and 120 min) was calculated taking the NMDA-evoked metabolite concentration in each corresponding fraction as 100%. *$p < 0.05$ versus NMDA (1 mM).

Discussion

We have reported previously that acute activation of NMDA glutamate receptors in the hippocampus of freely moving rats triggers a rapid and transient release of PGE_2 and 15-F_{2t}-IsoP. The formation of these two metabolites, which are produced through the enzymatic activity of COX and free radical-mediated peroxidation of AA, respectively, was prevented by the specific NMDA antagonist MK-801, and by arachidonyl trifluoromethyl ketone, a specific inhibitor of cytosolic phospholipase A_2. This PLA_2 isoform is known to be involved in the release of AA from membrane phospholipids after ischemia and excitotoxic lesions (Phillis and O'Regan 2003). In addition, the release of both PGE_2 and 15-F_{2t}-IsoP was significantly inhibited by non-selective COX inhibitors as well as by the COX-2 selective inhibitor NS-398, suggesting that the NMDA-evoked prostaglandin synthesis and free radical-mediated lipid peroxidation are largely dependent on COX-2 activity.

Here, we demonstrate that besides COX-2, which is the prominent COX isoform in the brain and particularly in the hippocampus, the constitutive isoform COX-1 also contributes to PG synthesis and oxidative damage following *in vivo* acute activation of hippocampal NMDA glutamate receptors. The relative contribution of the two isoforms appears dynamically regulated, as PGE_2 and 15-F_{2t}-IsoP are almost exclusively dependent on COX-2 activity during and 20 min after the acute application of the receptor agonist. The contribution of COX-1 activity becomes significant only subsequently, as the inhibition of both PGE_2 and 15-F_{2t}-IsoP by the COX-1 selective inhibitor SC560 increases with the time after NMDA infusion. Interestingly, in this late phase, the inhibition of PGE_2 by the COX-2 selective inhibitor NS398 remained substantially constant, whereas the effect on 15-F_{2t}-IsoP release decreased with time. This diminished inhibition of 15-F_{2t}-IsoP formation by NS398 was concomitant to an increasing inhibitory effect by SC560, suggesting that radical formation and lipid peroxidation are sustained by the interplay of the two COX isoforms.

COX-1-dependent PGE_2 release and lipid peroxidation are consistent with the neuronal expression of COX-1 in several brain regions, including hippocampus, and in primary mixed cortical cultures (Breder *et al*. 1995; Kaufmann *et al*. 1997; Hewett *et al*. 2000). Additionally, astrocytes express COX-1 *in vitro* (Hewett *et al*. 2000) and COX-1 positive microglial cells have been observed in Alzheimer's disease brains and in age-matched control subjects (Yermakova *et al*. 1999). Several studies have shown that COX-1 is not up-regulated after ischemic brain injury (Nogawa *et al*. 1997; Nakayama *et al*. 1998; Koistinaho *et al*. 1999) or glutamate intrahippocampal injection (Kolko *et al*. 2002), thus supporting a predominant role of the COX-2 isoform in these acute conditions. More recently, Candelario-Jalil *et al*. (2003) reported that both isoforms were involved in PGE_2 synthesis and oxidative damage following transient global cerebral ischemia in gerbils. In this study, a selective COX-2 inhibitor, rofecoxib, and a COX-1 selective inhibitor, valeryl salicylate, were both effective in decreasing the levels of PGE_2 in hippocampal homogenates. The inhibitory activity of valeryl salicylate was more potent after 2 h of reperfusion than after 24 h, whereas the opposite was true for rofecoxib. Interestingly, both inhibitors prevented the accumulation of the markers of lipid peroxidation MDA and 4-HAD, to a similar extent 48 h after ischemia.

Besides COX-1 and -2, a third variant of COX, named COX-3, has been identified recently from canine and human cerebral cortex cDNAs (Chandrasekharan *et al*. 2002). COX-3 is a product of the *COX-1* gene, but retains intron 1 in its mRNA. Within the brain, the highest levels of COX-3 mRNA are detected in cerebral cortex, where the expression of this COX-1 variant gene accounts for $\approx 5\%$ of COX-1. Like its counterpart COX-1, COX-3 is not induced by acute inflammatory stimulation (Shaftel *et al*. 2003). To date, the functional role of COX-3 is largely unknown and the expression of a full-length, catalytically active COX-3 protein is still questioned (Warner and Mitchell 2004).

The notion that COX-2 participates in excitotoxicity via excessive production of free radicals has become widely

accepted, although the specific mechanisms of COX-2-induced oxidative stress are not well known. While it is generally reported that COX activity is accompanied by generation of oxygen radicals (Chan and Fishman 1980; Kontos *et al*. 1980; Iadecola and Alexander 2001), this concept has been challenged recently. Using an animal model of excitotoxicity produced by microinjection of NMDA into the somatosensory cortex of mice, Manabe *et al*. (2004) reported that the COX-2 inhibitor NS398 attenuates brain damage and PGE_2 synthesis but not the associated formation of oxygen radicals, measured by means of the cell permeable fluorescent dye hydroethidine, which is oxidized to ethidium by superoxide but not other oxygen radicals. Additionally, the genetic deletion of COX-2 did not reduce the NMDA-dependent formation of oxygen radicals and the injection of a PGE_2 stable analogue abolished the protection exerted by NS398. Thus, the authors suggest that although oxidative stress plays a major role in glutamate-mediated neurotoxicity, COX-2 is not a major source of superoxide and that prostanoids, rather than reactive oxygen radicals, are responsible for COX-2 dependent neuronal damage.

Still, the present study, as well as other previous reports (Pepicelli *et al*. 2002; Candelario-Jalil *et al*. 2003; Madrigal *et al*. 2003), clearly show that COX activity is associated with lipid peroxidation. This apparent conflict can be reconciled by a recent study in which Jiang *et al*. (2004) elegantly demonstrate that oxygen radicals are not necessary for the induction of COX-2-dependent oxidative stress. Using cell-free systems, as well as PC12 cells over-expressing COX-2, the authors reported that during the COX catalytic cycle, no oxygen or thiyl radicals are produced. The only radical species generated were carbon-centred radicals that were sufficient to cause phospholipid peroxidation. Interestingly, in intact PC12 cells, the oxidation was not random among the different classes of phospholipids, but it mainly involved phosphatidylserine (PtdSer), one of the major glycerophospholipids in neural tissue. In a cell-free system, PtdSer was not directly utilized by COX-2 but was rather co-oxidized in the presence of AA, suggesting that PtdSer was the substrate for the carbon-centred radicals generated during COX catalytic cycle (Jiang *et al*. 2004). On the basis of these observations, it is tempting to speculate that the formation of $15F_{2t}$-Isop is achieved through the peroxidation of glycerolphospholipids by carbon-centred radicals generated through the catalytic activity of COX-2, which is triggered by a cascade of events, involving NMDA receptor stimulation, Ca^{2+} influx, $cPLA_2$ activation and culminating with a massive AA release (Pepicelli *et al*. 2002; Phillis and O'Regan 2003). As COX-1 and COX-2 have identical catalytic activities, the same mechanisms of carbon-centred radical formation can be envisaged for COX-1.

Although COX-1 and -2 have nearly identical kinetic properties and suicide inactivation, there are some subtle differences in peroxide requirement and substrate specificity (Smith *et al*. 1996). COX-1, but not COX-2, shows a negative allosterism at low AA concentration, suggesting that COX-2 may be a better competitor for AA released within the cells. In fibroblasts and endotoxin-activated macrophages, COX-1 was not able to convert endogenous AA into PGs, suggesting that this isoform requires exogenous substrate and that neighbouring cells may regulate COX-1 activity by providing the substrate (Herschman 1996; Dubois *et al*. 1998). Thus, the delayed COX-1-dependent PGE_2 synthesis and lipid peroxidation observed in our system may be linked to the less favourable availability of the substrate, when compared to COX-2, which is the prominent isoform expressed by hippocampal neurones. Alternatively, COX-2 and COX-1 may not be co-localised in the same cells and the delayed involvement of COX-1 could be related to substrate supply from the surrounding cells.

In conclusion, our data show that both COX-1 and COX-2 contribute to the synthesis of PG and oxidative damage following *in vivo* acute activation of hippocampal NMDA glutamate receptors. Although COX-2 is the prominent isoform, COX-1 activity may be significant particularly when considering the levels of lipid peroxidation associated with COX catalytic cycle. Thus, our observations suggest that both isoform should be considered as therapeutic targets to prevent neuronal damage by excitotoxicity.

Acknowledgements

This work was supported by grants from FIRB MIUR 2001 (Meccanismi di vulnerabilita′ neuronale: identificazione di nuovi target biologici per lo sviluppo di terapie innovative) to MR and from Istituto Superiore di Sanità (Ricerca Corrente) Grant no. 2ADI to LM. We thank Mr Claudio Di Giuseppe for technical assistance.

References

Akaike A., Kaneko S., Tamura Y., Nakata N., Shiomi H., Ushikubi F. and Narumiya S. (1994) Prostaglandin E2 protects cultured cortical neurons against *N*-methyl-D-aspartate receptor-mediated glutamate cytotoxicity. *Brain Res.* **663**, 237–243.

Bezzi P., Carmignoto G., Pasti L., Vesce S., Rossi D., Rizzini B. L., Pozzan T. and Volterra A. (1998) Prostaglandins stimulate calcium-dependent glutamate release in astrocytes. *Nature* **391**, 281–285.

Bilak M., Wu L., Wang Q., Haughey N., Conant K., St Hillaire C. and Andreasson K. (2004) PGE_2 receptors rescue motor neurons in a model of amyotrophic lateral sclerosis. *Ann. Neurol.* **56**, 24024–24028.

Breder C. D., Dewitt D. and Kraig R. P. (1995) Characterization of inducible cyclooxygenase in rat brain. *J. Comp. Neurol.* **355**, 296–315.

Candelario-Jalil E., Gonzalez-Falcon A., Garcia-Cabrera M., Alvarez D., Al-Dalain S., Martinez G., Leon O. S. and Springer J. E. (2003) Assessment of the relative contribution of COX-1 and COX-2 isoforms to ischemia-induced oxidative damage and neurodegeneration following transient global cerebral ischemia. *J. Neurochem.* **86**, 545–555.

Chan P. H. and Fishman R. A. (1980) Transient formation of superoxide radicals in polyunsaturated fatty acid-induced brain swelling. *J. Neurochem.* **35**, 1004–1007.

Chandrasekharan N. V., Dai H., Roos K. L., Evanson N. K., Tomsik J., Elton T. S. and Simmons D. L. (2002) COX-3, a cyclooxygenase-1 variant inhibited by acetaminophen and other analgesic/antipyretic drugs: cloning, structure, and expression. *Proc. Natl Acad. Sci. USA* **99**, 13 926–13 931.

Dubois R. N., Abramson S. B., Crofford L., Gupta R. A., Simon L. S., Van De Putte L. B. and Lipsky P. E. (1998) Cyclooxygenase in biology and disease. *FASEB J.* **12**, 1063–1073.

Facchinetti F., Dawson V. L. and Dawson T. M. (1998) Free radicals as mediators of neuronal injury. *Cell. Mol. Neurobiol.* **18**, 667–682.

Fedele E., Jin Y., Varnier G. and Raiteri M. (1996) *In vivo* microdialysis study of a specific inhibitor of soluble guanylyl cyclase on the glutamate receptor/nitric oxide/cyclic GMP pathway. *Br. J. Pharmacol.* **119**, 590–594.

Graham S. H. and Hickey R. W. (2003) Cyclooxygenases in central nervous system diseases: a special role for cyclooxygenase 2 in neuronal cell death. *Arch. Neurol.* **60**, 628–630.

Greco A. and Minghetti L. (2004) Isoprostane as biomarkers and mediators of oxidative injury in infant and adult central nervous system diseases. *Curr. Neurovascular Res.* **1**, 341–354.

Herschman H. R. (1996) Prostaglandin synthase 2. *Biochim. Biophys. Acta* **1299**, 125–140.

Hewett S. J., Uliasz T. F., Vidwans A. S. and Hewett J. A. (2000) Cyclooxygenase-2 contributes to *N*-methyl-D-aspartate-mediated neuronal cell death in primary cortical cell culture. *J. Pharmacol. Exp. Ther.* **293**, 417–425.

Iadecola C. and Alexander M. (2001) Cerebral ischemia and inflammation. *Curr. Opin. Neurol.* **14**, 89–94.

Jiang J., Borisenko G. G., Osipov A. *et al.* (2004) Arachidonic acid-induced carbon-centered radicals and phospholipid peroxidation in cyclo-oxygenase-2-transfected PC12 cells. *J. Neurochem.* **90**, 1036–1049.

Kaufmann W. E., Andreasson K. I., Isakson P. and Worley P. F. (1997) Cyclooxygenases and the central nervous system. *Prostaglandins* **54**, 601–624.

Kim E. J., Kwon K. J., Park J. Y., Lee S. H., Moon C. H. and Baik E. J. (2002) Neuroprotective effects of prostaglandin E2 or cAMP against microglial and neuronal free radical mediated toxicity associated with inflammation. *J. Neurosci. Res.* **70**, 97–107.

Koistinaho J., Koponen S. and Chan P. H. (1999) Expression of cyclooxygenase-2 mRNA after global ischemia is regulated by AMPA receptors and glucocorticoids. *Stroke* **30**, 1900–1905.

Kolko M., Nielsen M., Bazan N. G. and Diemer N. H. (2002) Secretory phospholipase A (2) induces delayed neuronal COX-2 expression compared with glutamate. *J. Neurosci. Res.* **69**, 169–177.

Kontos H. A., Wei E. P., Povlishock J. T., Dietrich W. D., Magiera C. J. and Ellis E. F. (1980) Cerebral arteriolar damage by arachidonic acid and prostaglandin G2. *Science* **209**, 1242–1245.

Madrigal J. L., Garcia-Bueno B., Moro M. A., Lizasoain I., Lorenzo P. and Leza J. C. (2003) Relationship between cyclooxygenase-2 and nitric oxide synthase-2 in rat cortex after stress. *Eur. J. Neurosci.* **18**, 1701–1705.

Manabe Y., Anrather J., Kawano T., Niwa K., Zhou P., Ross M. E. and Iadecola C. (2004) Prostanoids, not reactive oxygen species, mediate COX-2-dependent neurotoxicity. *Ann. Neurol.* **55**, 668–675.

McCullough L., Wu L., Haughey N., Liang X., Hand T., Wang Q., Breyer R. M. and Andreasson K. (2004) Neuroprotective function of the PGE2 EP2 receptor in cerebral ischemia. *J. Neurosci.* **24**, 257–268.

Minghetti L. and Levi G. (1995) Induction of prostanoid biosynthesis by bacterial lipopolysaccharide and isoproterenol in rat microglial cultures. *J. Neurochem.* **65**, 2690–2698.

Minghetti L. (2004) Cyclooxygenase-2 (COX-2) in inflammatory and degenerative brain diseases. *J. Neuropathol. Exp. Neurol.* **63**, 901–910.

Nakayama M., Uchimura K., Zhu R. L., Nagayama T., Rose M. E., Stetler R. A., Isakson P. C., Chen J. and Graham S. H. (1998) Cyclooxygenase-2 inhibition prevents delayed death of CA1 hippocampal neurons following global ischemia. *Proc. Natl Acad. Sci. USA* **95**, 10 954–10 959.

Niwa K., Haensel C., Ross M. E. and Iadecola C. (2001) Cyclooxygenase-1 participates in selected vasodilator responses of the cerebral circulation. *Circ. Res.* **88**, 600–608.

Nogawa S., Zhang F., Ross M. E. and Iadecola C. (1997) Cyclo-oxygenase-2 gene expression in neurons contributes to ischemic brain damage. *J. Neurosci.* **17**, 2746–2755.

Parente L and Perretti M. (2003) Advances in the pathophysiology of constitutive and inducible cyclooxygenases: two enzymes in the spotlight. *Biochem. Pharmacol.* **65**, 153–159.

Patel M., Liang L. P., Roberts L. J. and 2nd. (2001) Enhanced hippocampal F2-isoprostane formation following kainate-induced seizures. *J. Neurochem.* **79**, 1065–1069.

Paxinos G. and Watson C. (1986) *The Rat Brain in Stereotaxic Coordinates*. Second Edition. Academic Press, New York.

Pepicelli O., Fedele E., Bonanno G., Raiteri M., Ajmone-Cat M. A., Greco A., Levi G. and Minghetti L. (2002) *In vivo* activation of *N*-methyl-D-aspartate receptors in the rat hippocampus increases prostaglandin E (2) extracellular levels and triggers lipid peroxidation through cyclooxygenase-mediated mechanisms. *J. Neurochem.* **81**, 1028–1034.

Phillis J. W. and O'Regan M. H. (2003) The role of phospholipases, cyclooxygenases, and lipoxygenases in cerebral ischemic/traumatic injuries. *Crit. Rev. Neurobiol.* **15**, 61–90.

Popoli P., Pintor A., Tebano M. T. *et al.* (2004) Neuroprotective effects of the mGlu5R antagonist MPEP towards quinolinic acid-induced striatal toxicity: involvement of pre- and post-synaptic mechanisms and lack of direct NMDA blocking activity. *J. Neurochem.* **89**, 1479–1489.

Schwab J. M. and Schluesener H. J. (2003) Cyclooxygenases and central nervous system inflammation: conceptual neglect of cyclooxygenase 1. *Arch. Neurol.* **60**, 630–632.

Shaftel S. S., Olschowka J. A., Hurley S. D., Moore A. H. and O'Banion M. K. (2003) COX-3: a splice variant of cyclooxygenase-1 in mouse neural tissue and cells. *Brain. Res. Mol. Brain. Res.* **119**, 213–215.

Smith W. L., Garavito R. M. and DeWitt D. L. (1996) Prostaglandin endoperoxide H synthases (cyclooxygenases)-1 and -2. *J. Biol. Chem.* **271**, 33157–33160.

Takadera T., Yumoto H., Tozuka Y. and Ohyashiki T. (2002) Prostaglandin E (2) induces caspase-dependent apoptosis in rat cortical cells. *Neurosci. Lett.* **317**, 61–64.

Thery C., Dobbertin A. and Mallat M. (1994) Downregulation of in vitro neurotoxicity of brain macrophages by prostaglandin E2 and a beta-adrenergic agonist. *Glia* **4**, 383–386.

Warner T. D. and Mitchell J. A. (2004) Cyclooxygenases: new forms, new inhibitors, and lessons from the clinic. *FASEB J.* **18**, 790–804.

Yamagata K., Andreasson K. I., Kaufmann W. E., Barnes C. A. and Worley P. F. (1993) Expression of a mitogen-inducible cyclooxygenase in brain neurons: regulation by synaptic activity and glucocorticoids. *Neuron* **11**, 371–386.

Yermakova A. V., Rollins J., Callahan L. M., Rogers J., O'Banion M. (1999) Cyclooxygenase-1 in human Alzheimer's, and control brain: quantitative analysis of expression by microglia and CA3 hippocampal neurons. *J. Neuropathol. Exp. Neurol.* **58**, 1135–1146.