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Antiinflammatory and neuroprotective actions of COX2 inhibitors in the injured brain

Kenneth I. Strauss, Ph.D.*

*Mayfield Neurotrauma Research Lab, Department of Neurosurgery, University of Cincinnati College of Medicine, 231 Albert Sabin Way, ML515, Cincinnati, OH 45267 (kstrauss@uc.edu)

Abstract

Overexpression of COX2 appears to be both a marker and an effector of neural damage after a variety of acquired brain injuries, and in natural or pathological aging of the brain. COX2 inhibitors may be neuroprotective in the brain by reducing prostanoid and free radical synthesis, or by directing arachidonic acid down alternate metabolic pathways. The arachidonic acid shunting hypothesis proposes that COX2 inhibitors' neuroprotective effects may be mediated by increased formation of potentially beneficial eicosanoids. Under conditions where COX2 activity is inhibited, arachidonic acid accumulates or is converted to eicosanoids via lipoxygenases and cytochrome P450 (CYP) epoxygenases. Several P450 eicosanoids have been demonstrated to have beneficial effects in the brain and/or periphery. We suspect that arachidonic acid shunting may be as important to functional recovery after brain injuries as altered prostanoid formation per se. Thus, COX2 inhibition and arachidonic acid shunting have therapeutic implications beyond the suppression of prostaglandin synthesis and free radical formation.

Introduction

The role of cyclooxygenase-2 (COX2) and its inhibitors in the brain must be examined in the larger context of its role in arachidonic acid metabolism (Figure 1). Perturbations or insults to the brain activate phospholipases, releasing arachidonic acid from membrane stores (Dumuis et al., 1988; Gardiner et al., 1981). Cyclooxygenase-2 catalyzes the conversion of arachidonic acid and molecular oxygen into vasoactive prostaglandins, producing reactive oxygen free radicals in the process. COX2 is the dominant player in a complex and interlocking metabolic pathway that converts a structural membrane lipid into a plethora of biologically active eicosanoids, many of which have opposing physiological activity. Moreover, there are several other related biomolecules (e.g., docosahexenoic acid and docosanoids, the endocannabinoids anandamide and 2-arachidonoyl glycerol, etc.) that

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further expand the scope of influence of COX2 in neurophysiological functions.

Inhibition of COX2 after pathological insult has been shown to benefit recovery in the brain and spinal cord (Nagayama et al., 1999; Resnick et al., 1998). However, the mechanisms of COX2 in neuropathology are not well described. Our working hypothesis is that *prolonged* COX2 expression in the brain interferes with intrinsic neuroprotective mechanisms, contributing to the establishment of a “vicious cycle” in which cell death, rather than survival pathways dominate; and tissue damage is made worse by propagation of oxidative damage and chemotactic signals. Thus, we propose that COX2 inhibition blocks delayed cell death and neuroinflammation.

That COX2 inhibitors may function in the brain by shunting arachidonic acid down alternate metabolic pathways has been alluded to by Christie et al. (Christie et al., 1999) in a model of opioid-NSAID synergy, who “speculated that blockade of cyclooxygenase and/or 5-lipoxygenase might lead to shunting of arachidonic acid metabolism... [and] enhanced formation of 12-LOX metabolites, thereby enhancing the efficacy of opioids” in the periaqueductal gray. Arachidonic acid can be oxidized to many biologically and chemically active derivatives, the most prevalent being prostaglandins. Thus, under conditions where COX2 activity increases, proportionately more arachidonic acid is converted to prostanoids and less to other metabolites. Conversely, when COX2 activity is inhibited, arachidonic acid, that would otherwise be converted to prostanoids, accumulates or is converted to other eicosanoids (Figure 2, arachidonic acid shunting). Both these conditions are especially germane under conditions where phospholipases are activated, with the resultant increase in free arachidonic acid. The succeeding review examines some observations of the reactions of COX2 to brain injuries, its association with cell death and neuroinflammation, and its response to COX2 inhibitor treatments.

COX2 expression in the brain

Normally, COX2 is rare or absent in most organs of the body. However, significant levels of COX2 mRNA are expressed in mammalian brain (Feng et al., 1993; Seibert et al., 1994). Prostaglandin products of COX2 have also been found in moderate concentrations in postmortem human brain (Ogorochi et al., 1984). Normal brain COX2 protein is found primarily in neuronal cell bodies and dendrites (Breder et al., 1992; Kaufmann et al., 1996; Li et al., 1993b; Strauss et al., 2000a; Tsubokura et al., 1991). Low steady state levels of COX2 are found in neurons of the cerebral cortex, hippocampus and cerebellum, but not in glia or endothelial cells. Localization of gene expression in rat brain by *in situ* hybridization histochemistry has shown high levels of COX2 mRNA in pyramidal and granule cells of the hippocampus and cerebellar granule cells. Moderate levels are found in the pyramidal cells of piriform cortex, cellular layers of cortex, central n. of amygdala, and several hypothalamic nuclei. Cyclooxygenase-1 is also expressed at low levels in microglia throughout the brain and in subsets of granule neurons (O'Neill and Ford-Hutchinson, 1993; Yermakova et al., 1999), however this isoform is not readily induced by neural perturbations, as is COX2.

Neuronal COX2 is glutamatergically regulated

Steady-state COX2 appears to be regulated by normal glutamatergic synaptic activity in the adult brain (Adams et al., 1996; Chen et al., 1995; Kaufmann et al., 1996; Marcheselli and Bazan, 1996; Yamagata et al., 1993). Several recent *in vivo* studies have demonstrated specificity of glutamate-mediated COX2 gene regulation in the brain (Candelario-Jalil et al., 2002; Hirst et al., 1999; Koistinaho et al., 1999; Miettinen et al., 1997). Basal COX2 expression in cultured neurons could be suppressed using a variety of glutamatergic receptor antagonists (Strauss and Marini, 2002). Interestingly, only *toxic* concentrations of glutamate, kainate, and N-methyl-D-aspartate (NMDA) induced overexpression of COX2 mRNA in cultured rat neurons (Hewett et al., 2000; Strauss and Marini, 2002). In contrast, COX2 gene induction in non-neural tissues (endothelial cells, immune cells, fibroblasts, etc.) has been shown to be controlled by glucocorticoids, interleukin-1, or protein kinase C, via several DNA-binding activities (for reviews, see Herschman et al., 1995; Smith and Dewitt, 1996). Immunofluorescence colocalization studies revealed prolonged COX2 expression in neurons and glia of the injured brain (Strauss et al., 2000a), not in vascular endothelial or other lining cells as seen after peripheral inflammatory challenges (Cao et al., 2001; Minghetti, 2004; Parfenova et al., 1997; Sanz et al., 1997; Tzeng et al., 2005). Thus, inflammatory and brain lining cells regulate COX2 via interleukin-1-dependent mechanisms, while neurons and astrocytes do not (Nadjar et al., 2005; Strauss et al., 2000a). COX2 induction in these neural-derived cells appears to be via excitotoxic and oxidative mechanisms.

COX2 overexpression in the brain

Neuronal COX2 overexpression is induced by excitotoxicity *in vivo* (Adams et al., 1996; Chen et al., 1995; Kaufmann et al., 1996; Koistinaho et al., 1999; Marcheselli and Bazan, 1996; Yamagata et al., 1993) and *in vitro* (Hewett et al., 2000; Strauss and Marini, 2002; Tocco et al., 1997). *In vitro*, overactivation of kainate or NMDA receptors results in cell death, killing most cultured neurons (Marini and Paul, 1992; Novelli et al., 1988; Schramm et al., 1990). Kainate strongly induces neuronal COX2 overexpression. The NMDA receptor antagonist, MK-801, completely blocks glutamate and NMDA toxicity. MK-801 also attenuates COX2 mRNA induction in glutamate-treated neural cultures (Hewett et al., 2000; Strauss and Marini, 2002). In contrast, alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) only weakly induces cell death, and metabotropic receptor group 1 agonists are not excitotoxic at all. Neither type of glutamatergic receptor agonist cause increases in COX2 mRNA levels.

Excessive glutamate receptor activation and pathological COX2 induction have been associated with delayed cell death in brain regions specifically associated with functional deficits that occur following brain injuries (Caggiano et al., 1996; Collaco-Moraes et al., 1996; Hewett et al., 2000; Kunz and Oliw, 2001; Strauss et al., 2000a; Strauss and Marini, 2002; Wallace et al., 1998). Molecular findings from our lab indicate that excitotoxic levels of glutamate receptor agonists (kainate $\gg$ NMDA $\gg$ AMPA) increase neuronal COX2 expression, implicating COX2 as an effector of neurological deficits. As we shall see, COX2 activity is intimately linked to excitotoxic neuronal death.

Oxidant stress is also an important determinant of COX2 gene expression. The role of oxidative stress in functional outcome after brain injuries has been abundantly demonstrated. Antioxidants such as dimethyl sulfoxide (DMSO), tetramethylthiourea (TMU) (Feng et al., 1995), pyrrolidine dithiocarbamate (PDTC) and rotenone (Tetsuka et al., 1996) block interleukin-1 β induction of COX2 protein *in vitro*. In both studies COX2 protein production was diminished, however, DMSO and TMU inhibited interleukin-1 induction of COX2 mRNA, while PDTC and rotenone did not. This implies a post-transcriptional regulation of COX2 by reactive oxygen species. A byproduct of COX2 activity is reactive oxygen species, adding support to the idea that aberrant COX2 activity results in a vicious cycle of overexpression that contributes to cell death and neuroinflammation.

COX2 and brain injuries

Brain injuries activate multiple cellular pathways; some of these appear to be adaptive, others are pathological, and still others are initially adaptive but persist and become harmful. The response of COX2 to neural injury appears to belong in the latter category. For example, we have found that COX2 levels increase in proximal and distant brain regions following many types of acquired brain injury. Neural COX2 activity and expression are rapidly induced and may remain elevated for many days. Interestingly, COX2 expression is increased for prolonged periods in brain regions specifically associated with functional deficits after neurotrauma (Gopez et al., 2005; Strauss et al., 2000a). COX2 has been associated with worse outcomes after brain injury, as well as early onset dementia (Jantzen et al., 2002; Jellinger, 2004; Oka and Takashima, 1997). Further, COX2 appears at abnormally high levels in the (post mortem) hippocampus of Alzheimer's disease patients (Ho et al., 1999). The discrete localization and distinct regulation of COX2 gene expression in the brain provide a rational basis to suspect that perturbation of COX2 and arachidonic acid metabolism play a significant role in normal and pathological responses.

The severity of neural injury appears to correlate to the degree and duration of COX2 overexpression; mild injuries yield shorter elevations (≤ 24 h) of COX2 mRNA and prostaglandin production, while moderate to severe injuries yield extended elevations (≥ 3 days) in brain cells. This may be due to a "vicious cycle" (Figure 2), in which secondary injury cascades promulgate COX2 gene expression. In the brain, COX2 appears to propagate the central inflammatory response, in part by modulation of complement component C1qB, which is involved in neurodegenerative processes (Spielman et al., 2002). Increased COX2 expression has been observed with head trauma, cerebral ischemia, spreading depression, and seizures, as well as in several progressive neurodegenerative conditions, e.g., Alzheimer's disease, Parkinson's disease, Huntington's disease, Down's syndrome, etc. (Caggiano et al., 1996; Collaco-Moraes et al., 1996; Dash et al., 2000; Graham et al., 1996; Nakayama et al., 1997; Nogawa et al., 1997; Pasinetti and Aisen, 1998; Sanz et al., 1997; Strauss et al., 2000a; Walton et al., 1997). Overexpression of brain COX2 in these disorders may reflect its role in chronic neuroinflammation and neural cell death.

Early on after moderate brain injuries, neurons show increased COX2 levels that may persist for 1-3 days. At later time points (i.e. ≥ 3 days), COX2 remains elevated and (at least in rat models of brain injury) appears to be

primarily expressed in glial cells proximal to the site of injury. This is in contrast to the neural cell populations that have been found to produce COX2 after a peripherally induced inflammatory challenge (Strauss et al., 2000a).

Interestingly, we have observed a temporal “wave” of COX2 mRNA starting in CA1-2 neurons at 2h to 6h after neurotrauma and appearing in CA3 and dentate gyrus neurons by 6h to 24h postinjury in the rat. In contrast, the induction of COX2 expression following transient global ischemia in the rat appeared to “move” in the opposite direction (dentate gyrus → CA3 → CA2-1) over the same time course (Koistinaho et al., 1999; Nakayama et al., 1998). Whether this reflects on the findings that mainly CA1 neurons are lost to apoptotic cell death after ischemic insult, while mostly CA3 neurons are lost after unilateral traumatic brain injury, remains to be determined.

Eicosanoids and brain injuries

Injuries that result in cell membrane damage activate phospholipases, releasing arachidonic acid from phospholipid membranes (Dumuis et al., 1988; Gardiner et al., 1981). Increases of intracellular free Ca^{++} also mediate activation of phospholipases that release arachidonic acid from membrane stores (Dhillon et al., 1997; Lewen and Hillered, 1998; Marklund et al., 1997; Prasad et al., 1994; Sevanian and Kim, 1985). Arachidonic acid is converted to eicosanoids producing reactive oxygen species in the process. This can result in lipid peroxidation via formation of highly reactive OONO^- , O_2^- , and OH^- free radicals that damage neural membranes (including the blood-brain barrier) and initiate a central inflammatory cascade that includes COX2 upregulation.

Prostaglandins products of COX2 mediate many brain-specific functions, e.g., temperature regulation (Saper and Breder, 1992) and hippocampal long term potentiation (Nishizaki et al., 1999b). Normally, prostaglandin (PG) D2 levels are highest in the brain, however brain injuries induce the production of large amounts of other prostanoids. Prostaglandins, particularly PGE_2 levels, rise dramatically after traumatic brain injury (Dewitt et al., 1988; Ellis et al., 1989; Ellis et al., 1981; Shohami et al., 1987). We measured PGE_2 changes in the hippocampus and parietal cortex ipsilateral and contralateral to injury. Tissue levels rose by 2h and continued increasing to a maximum by 24h, then returned to baseline over the next several days (Strauss et al. 2000b, 2005). Treatment with the COX2-specific inhibitor DFU (5,5-dimethyl-3(3-fluorophenyl)-4(4-methylsulphonyl) phenyl-2(^5H)-furanone, Merck-Frosst, 1 mg/kg, intraperitoneal injections, twice daily for 3 days) attenuated the injury-induced rise in PGE_2 at 24 h postinjury by more than 60%.

The induction of COX2 after brain injuries results in increased prostaglandin E_2 (PGE_2) levels. Surprisingly, the proinflammatory role of prostaglandins in the brain has met with much debate. PGE_2 has been reported to suppress proinflammatory tumor necrosis factor (TNF) release, and a few studies support the view that PGE_2 facilitates cell survival. However, neural cells are isolated from the periphery by the blood brain barrier, use limited substrates for energy, and have unique anatomical and electrophysiological traits. Consequently, many genes expressed in the central nervous system (CNS) function differently than in the periphery. As we shall see, COX2 is such an effector gene that has come under intense investigation.

The effects of PGE₂ on TNF is different in peripheral organs than in the CNS. In many tissues, increased PGE₂ levels suppress the intrinsic release of TNF, resulting in immunosuppression (Bertrand et al., 1998; Fennekohl et al., 2002; Flohe et al., 2000; Goncalves de Moraes et al., 1996; Ishaque et al., 2003; Vassiliou et al., 2003). In contrast, PGE₂ does not reduce TNF signaling in neural tissues (Quadros et al., 2003; Sacco et al., 1998). Interestingly, unlike primary peritoneal macrophages, cultured RAW cells do not show a decreased TNF response to PGE₂ after LPS treatment (Rouzer et al., 2005). The presence of high basal levels of PGD₂ (and its receptors) in RAW cells, as in the brain, may help to explain this difference. In fact, this could be the basis for establishment of a vicious cycle of COX2 expression in the brain; the absence of the TNF reduction response to increased PGE₂ levels may propagate neural tissue damage.

On the other hand, some of our rodent traumatic brain injury studies indicate acute COX2 activity may be beneficial (Gopez et al., 2005). The immediate production of thromboxane (0.5 to 2h, (Strauss et al., 2000b)), for example, could help in hemostasis and reduce the brain's exposure to toxic blood products. The peak in brain PGE₂ levels (12 to 24h) may also be adaptive, perhaps helping to increase blood flow to recovering neural tissue, while minimizing the premature activation of monocytes and infiltration by macrophages (Westcott et al., 1987; Zhang and Rivest, 2001). However, we propose it is prolonged COX2 overexpression and activity that causes harm to the injured brain.

Eicosanoids activate G-protein coupled receptors (Abramovitz et al., 2000), and may act directly as chemokines and neuromodulators. Prostaglandins are vasoactive (constriction, dilatation), alter platelet aggregation, vascular permeability, and act as chemokines (Cao et al., 1995), among numerous other activities (Ellis et al., 1989; Ellis et al., 1979; Kontos et al., 1980a; Kontos et al., 1980b; Wei et al., 1981). Leukotriene products are inflammatory mediators, alter vascular permeability, and also function in the hippocampus and pineal (Manev et al., 2000; Uz and Manev, 1998). HPETEs can alter neuronal potassium currents (Ba-insensitive) (Vaughan et al., 1997). Formation of reactive oxygen species in these reactions and spontaneous oxidation to isoprostanes can promote inflammation in the brain. HETEs and EETs are potently vasoactive and may have proinflammatory properties. However, cytochrome P450 eicosanoids and arachidonic acid derivatives appear to have potentially neuroprotective properties, as well.

Node et al. (Node et al., 1999) demonstrated that certain EETs had unique antiinflammatory properties in a cardiac muscle system. Hampson and Grimaldi (Hampson and Grimaldi, 2002) provided evidence that certain HETEs ameliorate AMPA-type excitotoxic cell death in cultured neurons. And Panikashvili et al. (Panikashvili et al., 2001) clearly demonstrated that 2-arachidonoyl glycerol (2-AG), systemically administered to mice after closed head injury, was neuroprotective and improved functional outcomes. Most recently, Alkayed et al. (Alkayed et al., 2002; Koerner et al., 2005) have presented evidence of cerebral protection mediated by the arachidonic acid epoxygenase metabolites, 11,12- EET and 14,15-EET. They demonstrated that cytochrome P450 (CYP) arachidonate epoxygenase CYP2C11 (Liu and Alkayed, 2005), and its major product 14,15-EET (Koerner et al., unpublished data) mediate neuroprotection in rodent models of stroke and preconditioning.

Moreover, this group has furthered our understanding of how neural tissue may utilize short-acting eicosanoids to regulate local blood flow “on demand” (Ilyff et al., 2007).

Arachidonic acid shunting

Many studies on the role of COX2 in neuropathology have focused on the specificity of the third generation, highly specific COX2 inhibitors in reducing prostanoid production. A newer hypothesis that we are testing proposes that COX2 inhibitors' beneficial effects may be mediated, at least in part, by the increased formation of potentially neuroprotective and antiinflammatory eicosanoid metabolites, so called “arachidonic acid shunting.” Although the formation of proinflammatory eicosanoids is also a possibility, the net effect of COX2 inhibition after CNS injury has been generally beneficial, or at least not detrimental, to neurological recovery (Dash et al., 2000; Resnick et al., 1998; Shapira et al., 1988; Shohami et al., 1990).

Arachidonic acid shunting may be especially relevant after acute insults that activate phospholipase A2. Massive amounts of arachidonic acid are released from membrane stores (Dumuis et al., 1988; Gardiner et al., 1981). Free arachidonic acid can be converted by cyclooxygenases and lipoxygenases to proinflammatory prostanoids and leukotrienes. When COX2 activity increases after brain injury, proportionately more arachidonic acid is converted to prostanoids and less to other metabolites. Conversely, under conditions of COX2 “deficiency” after brain injuries, arachidonic acid is more effectively metabolized to other eicosanoids.

An alternate pathway for arachidonate metabolism produces epoxyeicosatrienoic acids (EETs), and hydroxyeicosatetraenoic acids (HETEs). These epoxyeicosanoids are synthesized by the cytochrome P450 (CYP) epoxygenases. These mitochondrial enzymes catalyze the monooxygenation of arachidonic acid, producing specific epoxide regioisomers (5,6-EET; 8,9-EET; 11,12-EET; and 14,15-EET). EETs are metabolized by epoxide hydrolase to the corresponding dihydroeicosatrienoic acids (DiHETEs). Allylic oxidation of arachidonic acid is also catalyzed to form HETEs (5-, 8-, 9-, 11-, 12-, 15-, 19-, and 20-HETE). Certain HETEs (e.g., 5-, and 12-HETE) can also be formed via lipoxygenase action from hydroperoxyeicosatetraenoic acid (HPETE) precursors.

The presence of COX2 inhibitors may result in shunting free arachidonic acid down alternate metabolic pathways to potentially neuroprotective eicosanoids. For example, DFU treatments resulted in increased EET and HETE levels in injured rat brain (Gopez et al. 2005, K. Strauss, manuscript in preparation). We began to explore this idea in an effort to reconcile seemingly contradictory results from our laboratory. Administration of either a dehydroepiandrosterone analog (DHEF) or a COX2-specific inhibitor (DFU) significantly improved functional recovery in a rat TBI model. However, DHEF augmented the observed rise in total prostaglandin levels in brain tissue following injury, while DFU potently inhibited this rise. We wondered whether altered arachidonic acid metabolism might be more important to functional recovery than altered prostanoid formation per se.

As described above, several eicosanoids may have beneficial effects in the brain and/or periphery. In fact, arachidonic acid (a small dose, 5 μ M) itself reproducibly protected cultured cerebellar neurons against glutamate-mediated excitotoxicity (A. Marini and K. Strauss, unpublished data). Thus, the enzymes necessary to shunt arachidonic acid metabolism toward protective compounds are likely expressed in neurons. Interestingly, NMDA administration induces neuronal arachidonic acid release (Dumuis et al., 1988; Lazarewicz et al., 1990; Lazarewicz et al., 1988; Sanfeliu, 1990); and stimulates prostaglandin D₂ production *in vivo* (Lazarewicz et al., 2000). This link between the glutamate and the arachidonic acid pathways may indicate parallel mechanisms of toxicity and/or neuroprotection (e.g., via the phenomenon of preconditioning).

Induction of the CYP450 epoxygenase pathway has been identified in the normal and pathological brain (Alkayed et al., 1996; Ellis et al., 1991; Harder et al., 1995; Schilter et al., 2000; Schilter and Omiecinski, 1993), as well as in the endovascularity of the heart, kidney (Campbell, 2000; Makita et al., 1994; Node et al., 1999; Yu et al., 2000a), liver and other organs (Capdevila et al., 1988; Capdevila et al., 1982; Ellis et al., 1991; Harder et al., 1995; McGiff and Carroll, 1991). P450 epoxygenase activity has been implicated in the physiology of the febrile response, pancreatic glucagon and insulin release, stimulation of hypothalamic somatostatin release, pituitary vasopressin, oxytocin and luteinizing hormone release, inhibition of arachidonic acid-induced platelet aggregation, inhibition of the activity of Na⁺K⁺-ATPase in the nephron and corneal epithelium, regulation of blood pressure, vasodilation of local microcirculation in the kidney, intestine, heart and brain (Fitzpatrick and Murphy, 1988; Kozak et al., 2000b; Makita et al., 1996; McGiff and Carroll, 1991). Many of the CYP enzymes are induced during inflammatory challenge, however recent evidence suggests that the EETs and HETEs may also function in diminishing inflammation (Campbell, 2000; Chen et al., 2001; Kozak et al., 2000b; Node et al., 1999).

COX2 inhibitors and brain injuries

Several COX2-specific inhibitors have been employed to treat brain injuries (e.g., NS398, nimesulide, celecoxib, DFU). Their efficacy, when administered at various doses and times before or after neurological insult has not been entirely consistent, perhaps because of widely different partition coefficients across the blood-brain barrier. However, the overwhelming preponderance of evidence clearly shows that protracted brain COX2 activity mediates a toxic response that worsens functional and neuroanatomical deficits after brain and spinal cord injuries (Candelario-Jalil et al., 2002; Candelario-Jalil et al., 2003; Candelario-Jalil et al., 2005; Dash et al., 2000; Gopez et al., 2005; Resnick et al., 1998; Shapira et al., 1988; Shohami et al., 1990). Further, proof of principle is provided in a study of ischemic stroke using genetically modified COX2 null mutant mice (Iadecola et al., 2001). Compared to wild type littermates, COX2 deficient mice had reduced lesion volumes, attenuation of glutamate neurotoxicity and postischemic inflammation after middle cerebral artery occlusion. Thus, COX2 inhibitors that benefit the injured brain likely produce their effects primarily by reducing COX2 activity rather than by suppressing free radical-mediated brain damage or other non-specific mechanisms.

COX2 inhibitors are potent neuroprotectants *in vitro* and *in vivo*. Work from Hewett's group clearly showed that inhibition of COX2 protected neurons in mixed cultures against NMDA excitotoxicity (Hewett et al., 2000). Significantly, COX2-specific inhibition (NS398, 3–30 μ M) blocked neuronal cell death, whereas COX1-specific inhibition (valeryl salicylate, 10–100 μ M) did not (Hewett et al., 2000). In addition, newly synthesized COX2 appeared to be responsible; irreversible cyclooxygenase inhibition with aspirin pretreatment (100 μ M, 1.5h) did not block subsequent excitotoxic cell death. Our results concur with these; in nearly homogeneous cultured cerebellar granule neurons, DFU (1nM to 1 μ M) protected from glutamate-mediated apoptosis (Strauss and Marini, 2002). In fact, DFU (10nM) protected 100% of vulnerable neurons from glutamate (100 μ M) mediated excitotoxicity (vital dye staining at 24h). A DNA fragmentation assay yielded identical results; COX2 inhibition attenuated apoptotic cell death in cultured neurons.

In vivo, systemic COX2 inhibitor administration reduces lesion size, neuroinflammation, and cell death in various rat brain injury models. As further proof of principle, in a genetic model, COX2^{-/-} null mutant mice exposed to either ischemia or neurotoxic cerebral NMDA injection showed ~40% less neural damage 1–4 days after insult compared to wild-type littermates (Iadecola et al., 2001; Sasaki et al., 2003).

COX2 inhibitor treatments reduced apoptosis *in vivo* as well. The activation of caspase-3 is a good marker of cellular commitment to apoptotic cell death. A biphasic response in the appearance of activated caspase-3 immunoreactive (AC3-ir) cells was observed in injured cortex and hippocampus after lateral cortical impact traumatic brain injury (Gopez et al., 2005). At 6 h postinjury, large numbers of AC3-ir cells were found in the injured cortex and the CA1–2 hippocampal regions, where COX2 levels were also maximal. By 24 h postinjury, AC3-ir cell counts were lower than at 6h or 72h in the injured cortex, ipsilateral CA2 and CA3 hippocampus, and contralateral perirhinal and piriform cortices, but at least double the control numbers. AC3-ir cell counts decreased to sham control levels in all other brain regions. As expected from delayed cell death profiles, of the 3 time points examined, the injured cortex and hippocampal CA3 region exhibited the highest number of AC3-ir cells at 72 h postinjury. DFU treatment attenuated the number of AC3-ir cells at 6 h and 72h postinjury in all cortical regions where an increase was observed. In the hippocampus at 6 h postinjury, DFU did not appreciably reduce AC3-ir cell number in the ipsilateral hippocampus. At 24h and 72h postinjury, when COX2 levels in the most vulnerable hippocampal regions DFU diminished the number of AC3-ir cells in the entire Ammon's horn. The protective effects of DFU in cortex, and particularly in hippocampus, coincided with preestablished profiles of maximal COX2 expression for these areas at the later times. Thus, COX2 “deficiency” is important in acute neurotoxicity and chronic neuroinflammation.

COX2 inhibition improves behavioral recovery

Recently, several groups have demonstrated improved functional recovery with systemic COX2 inhibitor treatments. Nimesulide improved memory (Barnes maze) and motor (rotarod) performance after neurotrauma (Cernak et al., 2001, 2002). Candalario-Jalil and colleagues also observed improved functional recovery (neurological severity score, rotarod) with this COX2

inhibitor in a rat model of cerebral ischemia (Candelario-Jalil et al., 2004; Candelario-Jalil et al., 2005).

DFU treatments effectively improved functional recovery, compared with vehicle-treated injured control rats (Gopez et al., 2005). Neurological reflexes (as measured by observing forelimb flexion, hindlimb extension, and lateral pulsion at 3 days postinjury) improved significantly for both low dose and high dose groups (1 mg/kg, 10 mg/kg) whether the first dose of DFU was administered 30 min, 2 or 6 hours after the injury. Significantly, working spatial memory was also improved after neurotrauma by DFU treatments. Memory performance in the Morris water maze was improved only in the high dose treatment groups and nearly achieved sham levels of performance. In our hands, DFU improved memory and neurological recovery in rats treated up to 6 hours after the injury. No injury-induced changes in swim speed or exploratory behavior were observed between any of the groups.

DFU and nimesulide improved behavioral recovery when administered hours after brain injury (Candelario-Jalil et al., 2002, 2003; Gopez et al., 2005). These findings provide evidence that COX2 inhibitors have an extended window of opportunity to protect vulnerable neurons from secondary damage after neurotrauma. This also correlated with reduced inflammatory proteins and cell death in the cortex and hippocampus. We have set the stage for acute COX2 inhibitor treatment clinical trials in brain injury patients. Notwithstanding their potential adverse cardiovascular side-effects, these findings have compelling implications for the use of COX2 inhibitors in chronic neurodegenerative disorders, as well.

COX2 inhibition attenuates COX2 expression in the injured brain

DFU apparently crossed the blood brain barrier and improved behavioral recovery, so we hypothesized that COX2-specific inhibition might affect COX2 expression to break the proposed “vicious cycle” that results in prolonged upregulation. Immunohistochemical analyses revealed that DFU attenuated both the number and intensity of COX2-immunoreactive (COX2-ir) staining in the cortex and hippocampus after traumatic brain injury, compared to vehicle-treated controls (Gopez et al. 2005). Specifically, there was observably less COX2-ir in the ipsilateral cortex, including cells of the cingulate, injured and adjacent parietal, perirhinal and piriform regions. The COX2-ir decreases observed at 6 h and 24 h persisted through 72 h postinjury, and the decrease in the injured cortex at this time could be demonstrated by immunoblot analysis. COX2 inhibition produced no observable effects on basal levels of COX2 either *in vitro* or *in vivo* (Gopez et al., 2005; Strauss and Marini, 2002)

DFU treatment also reduced COX2-ir in the ipsilateral hippocampus at 6 h, 24 h, and 72 h postinjury. At 6 h postinjury, the attenuation occurred primarily in the CA1, CA2 and dentate gyrus regions. In the CA3 region, most vulnerable to delayed cell death, DFU attenuated COX2-ir at 6 h postinjury, and in the dentate gyrus at 24 h postinjury, compared to vehicle-treated controls.

These observations support the “vicious cycle” hypothesis (Figure 2), that brain COX2 activity promotes further COX2 induction and eventual tissue damage. Thus, COX2 inhibitors not only block harmful enzymatic activity,

but may also turn down the gain on dysregulated inflammatory gene transcription.

Antiinflammatory and neuroprotective effects of COX2 inhibition

Central inflammation following traumatic brain injuries includes the proliferation of activated astrocytes and microglia proximal to the injury site (Csuka et al., 2000; Morganti-Kossmann et al., 1997) that may exacerbate tissue damage after injury (Yakovlev et al., 1997). Cytokines affect inflammatory cell proliferation and infiltration. Proinflammatory cytokines such as interleukin-1 and interleukin-6 initiate inflammation after TBI, and antiinflammatory interleukin-10 reduces this activity (Knoblauch and Faden, 1998; Yakovlev et al., 1997). Interleukin-1 induces COX2 in endothelial, inflammatory, and lining cells of the brain. COX2 may promote inflammatory cell proliferation and infiltration into the CNS after injury (Amruthesh et al., 1993; Bezzi et al., 1998; Blom et al., 1997; Hirst et al., 1999; Scali et al., 2003; Scali et al., 2000), but the role of eicosanoids in this process has not been well described. Thus, prolonged COX2 activity may contribute to brain infiltration by macrophages and leukocytes, implicated in the secondary processes that produce edema (Holmin and Mathiesen, 2000; Mayhan, 2000), cavity and scar formation (Fitch et al., 1999; Fitch and Silver, 1997) at delayed times postinjury (Whalen et al., 1999).

The EP3 receptor is the predominant PGE₂ receptor in neurons and is distributed widely in the rat, mouse, and pig brain (Ericsson et al., 1995; Li et al., 1993a; Sugimoto et al., 1994). It has the highest binding affinity to PGE₂ among the four EP receptors (Kiriya et al., 1997). EP3 receptors are strictly confined to IL-1 β -responsive neurons in the rat preoptic anterior hypothalamus, suggesting that EP3 receptors may be involved in the cytokine-induced inflammatory response of the CNS (Ericsson et al., 1995).

Our studies showed COX2 inhibition reduced COX2 expression in the cortex and hippocampus 72h after TBI (Gopez et al., 2005). This treatment also reduced interleukin-1 β (IL-1 β), a proinflammatory cytokine, in the injured brain at the acute 12h time point (S. Lewis and K. Strauss, manuscript in preparation). IL-1 β is proteolytically activated by caspase-1 (ICE); thus it is not surprising that its biphasic appearance closely follows that of AC3 (Gopez et al., 2005). Intracerebral microinjections of IL-1 β in rats increased inflammatory cells, neuronal death, and vasogenic edema (Holmin and Mathiesen, 2000). Histochemical analyses also indicate a reduction in vascular endothelial adhesion molecule expression (K. Strauss, manuscript in preparation), similar to that described in cardiac vascular endothelial cells (Node et al., 1999).

Cellular adhesion molecules (I-CAM, V-CAM, E-selectin) facilitate the adherence of peripheral inflammatory cells to the cerebrovascular endothelium, the first step in extravasation into the brain. Infiltration and proliferation of peripheral blood cells, e.g., neutrophils and macrophages (Kubo et al., 2000; Weaver et al., 2000) exacerbate brain injury (Bank et al., 1999; Rimpilainen et al., 2000). In a pig model of ischemic brain injury, leukocyte depletion lowered mortality, improved behavioral recovery and neuropathology scores at day 7 postinjury (Rimpilainen et al., 2000). In addition, vasogenic edema is the result of changes in the blood-brain barrier, emanating from the interactions of astrocytic end-feet and the cerebral

vascular endothelium. If astrocyte and endothelial cell metabolism is stabilized by P450 eicosanoids, their ability to withstand the injury and retain an intact blood brain barrier may be preserved (Campbell, 2000; Fitch et al., 1999; Medhora and Harder, 1998; Medhora et al., 2001; Zeldin and Liao, 2000).

Activation and/or inhibition of transcription factor NF- κ B is a likely candidate mechanism for COX2 inhibitor-mediated neuroprotection. Activated NF- κ B increases transcription of COX2 in neural tissue (Kaltschmidt et al., 2002; Lukiw and Bazan, 1998). Its inhibitor, I κ B can be inactivated via phosphorylation or by direct oxidation by free radicals (Barr et al., 2007; Heck et al., 1999; Nakai et al., 2000; Post et al., 1998; Vollgraf et al., 1999; Zou and Crews, 2006). By reducing prostaglandin and ROS production, COX2 inhibition may reduce NF- κ B activation thus affecting its own transcription, and that of other apoptosis-related genes. In addition, other eicosanoid activities might involve stabilization of NF- κ B (Acarin et al., 2001; Acarin, 2002; Hernandez et al., 2001; Lukiw and Bazan, 1998; Nurmi et al., 2004; Yoshida et al., 2003), or its inhibitor I κ B (Grilli and Memo, 1999; Node et al., 1999; Richter et al., 2001; Tamatani et al., 2000). Thus, increased brain eicosanoid production and COX2 “deficiency” reduce peripheral inflammatory infiltration (Node et al., 1999), glial proliferation (Stephenson et al., 1996) and scar formation (Fitch et al., 1999; Sasaki et al., 2003).

Some alternative viewpoints

Most studies have found that activation of NF- κ B and phosphorylation of its inhibitor I κ B, correspond to a proinflammatory stimulus for gene expression. There are contradictory findings in the literature regarding the role of NF- κ B in central inflammation. One study, using glutamate excitotoxicity, showed activated NF- κ B was associated with cell death, and was reversed by high concentration aspirin (Grilli et al., 1996); while another study showed a rise in activated NF- κ B during NMDA-mediated neuroprotection (Lipsky et al., 2001). NMDA-mediated neuroprotection induced the phosphorylation of I κ B (Lipsky et al., 2001), whereas peripherally, antiinflammatory agents are known to stabilize I κ B (Campbell, 2000). Interestingly, EETs have been shown to block (indirectly) the TNF- α mediated activation of I κ B kinase (IKK) in endothelial cells (Campbell, 2000; Node et al., 1999; Stuhlmeier et al., 1997). However, it remains to be determined if EETs exert similar inhibitory effects in astrocytes, microglia, macrophages, and neutrophils in the injured CNS.

A few studies indicate prostaglandins may be protective, but the preponderance of evidence show the opposite. Moreover, the studies in which prostanoids (or their EP2 receptors) are characterized as neuroprotective have never shown improvements in functional outcomes. In our brain injury studies, some data indicate *acute* COX2 activity may be beneficial (Gopez et al., 2005). However, it is prolonged COX2 overexpression and activity that we propose may cause harm to the injured brain. The “neuroprotective” effects of prostaglandins (PGE₂) in neural excitotoxicity models are limited to subacute reductions in propidium iodide staining at 24 h (no protection was seen after 48 h (Liu et al., 2005)). McCullough et al. (2004) show the effects of PGE₂ on neuronal viability differ significantly in dispersed neuronal cultures and organotypic cultures, likely because of

the preservation of astrocytic/neuronal interactions in organotypic cultures. PGE₂, likely via the EP2 receptor, mediates reduced neuroinflammatory responses in cultured neurons, but not in the presence of glia (McCullough et al., 2004). Astrocytic/ neuronal interactions likely account for these differences. For example, high levels of PGE₂ stimulate astrocytic glutamate release (Bezzi et al., 1998; Sanzgiri et al., 1999), thus exacerbating excitotoxicity. Interestingly, one recent study has provided evidence that COX2 prostaglandins rather than reactive oxygen species are responsible for COX2-mediated neurotoxicity (Manabe et al., 2004).

COX2 and cognitive function

The contribution of brain COX2 in cognitive function has recently become a topic of intense investigation. Many studies have demonstrated the benefit of COX2-selective inhibitors in improving memory function after brain injuries (see above). Treatments that induce COX2 overexpression in the brain interfere with cognitive function. For example, IL-1 β , injected bilaterally into the dorsal hippocampus significantly impaired working memory, and this impairment was attenuated by pretreatment with diclofenac, a partially selective COX2 inhibitor (Matsumoto et al., 2004). Working memory was also impaired in rats administered a bilateral intrahippocampal injection of prostaglandin E₂, in a dose-dependent manner. Interestingly, non-selective cyclooxygenase inhibitor treatments resulted in sustained deficits in spatial learning in the Morris watermaze (Shaw et al., 2003). In hippocampal slice preparations, oxygen-glucose deprivation causes a rapid and persistent tissue depolarization within 5 min. These challenges also induce rapid increases in COX2 (Kim et al., 2007). Pretreatment with cyclooxygenase or lipoxygenase inhibitors as well as free radical scavengers improved the latency of postischemic recovery from depolarization, while 14,15-EET or 20-HETE did not (Tanaka et al., 2003). These results suggest that the activation of the arachidonic acid cascade via phospholipase A2 and the free radicals produced by arachidonic acid metabolism contribute to the irreversible depolarization produced by in vitro ischemia. Other findings in hippocampal slices showed selective inhibition of COX-2 (but not COX1) decreased excitatory responses (Slanina and Schweitzer, 2005). Furthermore, the interactions between gene regulation and activation of brain COX2 and phospholipases under basal and pathological conditions is just beginning to be investigated in neural tissues (Sapirstein et al., 2005; Strokin et al., 2004).

Arachidonic acid release via phospholipase A2 has direct effects on brain cholinergic activity (Almeida et al., 1999; Kjome et al., 1998; Nishizaki et al., 1999a), and in the presence of COX2 inhibitors may also affect neurotransmission via catalysis down alternative metabolic pathways. Its eicosanoid metabolites are numerous, possessing a variety of neurophysiological activities. These include energy modulation (5-HPETE (Foley, 1997)), cannabinoid receptor binding (anandamide and 2-arachidonoyl glycerol (Devane et al., 1992; Mechoulam et al., 1995)), lipoxygenase-mediated analgesia (Christie et al., 1999; Mechoulam et al., 1998), circadian modulation (Uz and Manev, 1998) and antiinflammatory effects ([11,12]-EET (Node et al., 1999)), and neuroprotection from glutamate excitotoxicity (12-HETE (Hampson and Grimaldi, 2002)). Shohami's group (Panikashvili et al., 2001) clearly demonstrated that the endocannabinoid 2-arachidonoyl glycerol (2-

AG), systemically administered to mice, was neuroprotective, improving functional outcome after closed head injury.

As a final point, COX2 overexpression in the brain may act as an arachidonic acid sink. Increased levels of free arachidonic acid are observed after tissue damage, and COX2 may serve to “sop it up.” While this may be adaptive in the acute time frame (e.g., leading to rapid local thromboxane synthesis and hemostasis), prolonged COX2 activity appears to harm the injured brain, perhaps by diverting arachidonic acid metabolism away from the synthesis of potentially beneficial metabolites and via free radical production. Furthermore, COX2 activity may inactivate neuroprotective eicosanoids, particularly in an environment of increasing COX2 levels, as seen after TBI. Support for this supposition includes work by Kozak and colleagues, and results from our laboratory. For example, COX2 can metabolize arachidonate, docosahexanoate, and other long chain fatty acid derivatives including 2-AG (Kozak et al., 2004; Kozak et al., 2000a). Indeed, neurotrauma reduced brain 2-AG levels in the rat, and the COX2 inhibitor DFU reversed this decrease within 24 hours after injury (Gopez et al. 2005). Moreover, dynamic changes in brain levels of potentially neuroprotective EETs and HETEs after brain injury are enhanced by COX2 inhibition (Gopez et al. 2005, K. Strauss, manuscript in preparation).

Together, these studies implicate a unique role for brain COX2 and its inhibitors in cognition and dementia. Agerelated or pathologically induced COX2 overexpression may play a key role in promoting premature cognitive decline (K. Strauss, manuscript in preparation, Casolini et al., 2002). Thus, COX2 inhibition in the brain has therapeutic utility beyond the suppression of prostaglandin and free radical formation.

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Figure 1.

Arachidonic acid metabolism. Cell damage and phospholipase activation release arachidonic acid with subsequent oxidation to a variety of eicosanoids. Arachidonic acid is converted to highly labile prostanoids and leukotrienes by COXs and lipoxygenases, respectively, producing reactive oxygen free radicals in the process. Alternatively, arachidonic acid can be monooxygenated by cytochrome P450 epoxygenases, producing highly labile epoxide regioisomers (5,6-; 8,9-; 11,12-; or 14,15-EET)(Chacos et al., 1982; Oliw et al., 1982). Allylic oxidation is also catalyzed to form HETEs (5-, 8-, 9-, 11-, 12-, 15-, 19-, or 20-HETE)(Capdevila et al., 1982; Oliw et al., 1982). Certain HETEs (e.g., 5-, or 12-HETE) can also be formed via lipoxygenase action from hydroperoxyeicosatetraenoic acid (HPETE) precursors. EETs are metabolized by epoxide hydrolase to the corresponding dihydroxyeicosatrienoic acids (DHETs)(Chacos et al., 1983; Oliw et al., 1982; Yu et al., 2000b; Zeldin et al., 1995). Interestingly, EETs and HETEs are often incorporated in membrane phospholipid, enabling phospholipase-mediated release of these activities (Brezinski and Serhan, 1990; Capdevila et al., 1987; Karara et al., 1991).

Antiapoptosis effects of specific HETEs, EETs *in vitro* Neuroprotection by CYP2C11 *in vivo*
 Hampson & Grimaldi 2001, Chen et al. 2001 Liu et al. 2005
 Antiinflammatory effects of specific EETs *in vitro* Neuroprotection by 2-AG *in vivo*
 Node et al. 1999 Panikashvili et al. 2001

Antiinflammatory, Neuroprotective Eicosanoids

Figure 2.

Arachidonic acid shunting. The action of COX2 inhibitors decreases synthesis of prostanoids and free radicals. However, because it is the dominant metabolic reaction, COX2 inhibition causes arachidonic acid shunting down alternate enzymatic pathways (e.g., cytochrome P450 epoxygenases), resulting in the synthesis of potentially neuroprotective eicosanoids. COX2 and prostanoid levels rise acutely after brain injuries, and remain elevated for days. The extent of COX2 expression may correlate to the severity of the insult. This may be due to a “vicious cycle”, in which secondary injury cascades promulgate COX2 gene expression. Prolonged elevation contributes to inflammation, programmed cell death, free radical-mediated tissue damage, and alterations in cellular metabolism. These, in turn, cause secondary injuries that worsen clinical outcomes. Thus, COX2

inhibitors' beneficial actions are mediated both by reducing prostanoid formation and by arachidonic acid shunting to form antiinflammatory, neuroprotective eicosanoids.