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Neuronal and Nonneuronal COX-2 Expression Confers Neurotoxic and Neuroprotective Phenotypes in Response to Excitotoxin Challenge

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Abstract

Treating acute brain injuries with COX-2 inhibitors can produce both neuroprotective and neurotoxic effects. This study investigated the role of COX-2 in modulating acute brain injury induced by excitotoxic neural damage. Intrastriatal injection of excitotoxin (*RS*)-(tetrazole-5yl) glycine elicited COX-2 expression in two distinct groups of cells. cortical neurons surrounding the lesion and vascular cells in the lesion core. The vascular COX-2 was expressed in two cell types, endothelial cells and monocytes. Selective deletion of COX-2 in vascular cells in Tie2Cre Cox-2^{flox/flox} mice did not affect the induction of COX-2 in neurons after the excitotoxin injection but resulted in increased lesion volume, indicating a neuroprotective role for the COX-2 expressed in the vascular cells. Selective deletion of monocyte COX-2 in LysMCre Cox-2^{flox/flox} mice did not reduce COX-2-dependent neuroprotection, suggesting that endothelial COX-2 is sufficient to confer neuroprotection. Pharmacological inhibition of COX-2 activity in Tie2Cre Cox-2^{flox/flox} mice reduced lesion volume, indicating a neurotoxic role for the COX-2 expressed in neurons. Furthermore, COX-2-dependent neurotoxicity was mediated, at least in part, via the activation of the EP1 receptor. These results show that Cox-2 expression induced in different cell types can confer opposite effects.

Keywords

injury; inflammation; knockout mouse

Increased COX-2 expression in the brain occurs in numerous neurodegenerative conditions, including Alzheimer's disease (Hoozemans et al., 2001), Down syndrome (Strauss, 2008), cerebral ischemia (Iadecola et al., 1999), and amyotrophic lateral sclerosis (ALS; Yasojima et al., 2001). In experimental animals, increased brain COX-2 expression has been

demonstrated in cerebral ischemia (Hara et al., 1998), traumatic brain injury (Strauss et al., 2000), spreading depression (Koistinaho and Chan, 2000), and excitotoxic brain injury (Adams et al., 1996; Kaufmann et al., 1996). Thus, induction of brain COX-2 expression is a common component of many acute and chronic neurodegenerative conditions.

In addition, brain COX-2 expression has been associated with functional deficits after neurotrauma (Gopez et al., 2005) and deleterious outcomes in various forms of dementias (Oka and Takashima, 1997; Jellinger, 2004). The duration and level of COX-2 induction have been correlated with the severity of brain injury (Strauss, 2008). Furthermore, selective blockade of COX-2 activity can ameliorate neural damage (Iadecola et al., 2001; Candelario-Jalil, 2008; Ahmad et al., 2009), preserve blood–brain barrier permeability, and reduce brain edema (Hakan et al., 2010). These findings have spurred advocacy for the use of COX-2 inhibitors as a therapeutic treatment for acquired brain injury (Strauss, 2010).

However, other studies have shown that blocking COX-2 activity is not always beneficial in neurodegenerative contexts. For example, the use of COX-2 inhibitors failed to show clinical benefits in human clinical trials for both ALS and cerebral ischemia (Cudkowicz et al., 2006; Mangoni et al., 2010) and in a mouse model of ALS (Azari et al., 2005). The use of COX-2 inhibitors was also unable to retard cognitive decline in Alzheimer's patients (Sainati, 2000). From a swine head-injury model, Friess et al. (2012) showed that pretreatment with COX-2 inhibitor meloxicam actually increased mortality. Likewise, *Cox-2* deletion enhanced N-methyl-D-aspartate (NMDA)-induced seizure intensity, and pretreatment with COX-2 inhibitors exacerbated kainic acid-induced seizure (Kim et al., 2008). An unexpected neuroprotective, rather than neurotoxic, role for COX-2 has also been observed in spinal cord injury (Lopez-Vales et al., 2006). These observations, and those described in the preceding paragraph, suggest that COX-2 expression can be either neurotoxic or neuroprotective, depending on the nature of the neural injury.

Two distinct patterns of COX-2 induction in the brain during neural injury have been observed. COX-2 is rapidly induced to very high levels in neurons (Adams et al., 1996; Miettinen et al., 1997; Resnick et al., 1998; Koistinaho et al., 1999; Koistinaho and Chan, 2000), caused by activation of glutamate receptors (Adams et al., 1996; Koistinaho et al., 1999). On the other hand, brain tissue damage and neuroinflammation can cause non-neuronal COX-2 expression, often in association with brain blood vessels (Quan et al., 1998; Proescholdt et al., 2002; Lopez-Vales et al., 2004). It seems likely that the opposite effects of COX-2 during neural injury are related to the COX-2 expressed in different cell types.

The present study was designed to dissect the role of COX-2 expressed in different cell types in a mouse model of excitotoxic neural injury by using cell-type-specific *Cox-2* knockout mice. The results revealed a previously unrecognized mechanism by which COX-2 expression in injured brain provided significant neuroprotection.

MATERIALS AND METHODS

Animals

Tie2Cre *Cox-2*^{flox/flox} mice were generated by cross-breeding Tie2Cre;*Cox-2*^{+/+} transgenic mice (Jackson Laboratories, Bar Harbor, ME; stock No. 004128) with *Cox-2*^{flox/flox} mice. LysMCre *Cox-2*^{flox/flox} mice were generously provided by Dr. Reddy (Department of Medicine, UCLA). In the Tie2-Cre;*Cox-2*^{+/+} mouse, the Tie2 promoter restricts Cre recombinase expression in endothelial cells and hematopoietic cells during embryogenesis and adulthood (Constien et al., 2001). Therefore, the *Cox-2* gene is selectively deleted in endothelial cells and in hematopoietic cells in Tie2Cre *Cox-2*^{flox/flox} mice. In LysMCre;*Cox-2*^{+/+} mice, transgenic expression of Cre recombinase is restricted to myeloid-lineage cells; consequently, *Cox-2* is deleted specifically in myeloid cells in LysMCre *Cox-2*^{flox/flox} mice (Narasimha et al., 2010). Results in Tie2Cre *Cox-2*^{flox/flox} mice and LysMCre *Cox-2*^{flox/flox} mice were compared with their Cre-negative *Cox-2*^{flox/flox} littermates. Mice 10–16 weeks of age, with body weights of 25–30 g, were used in experimental procedures. All the procedures were approved by The Ohio State University Animal Care and Use Committee. No overt phenotype was observed in Tie2Cre *Cox-2*^{flox/flox}, Tie2Cre;*Cox-2*^{+/+}, LysMCre *Cox-2*^{flox/flox}, or *Cox-2*^{flox/flox} mice. All these lines are fertile and viable. The growth rates of these lines are not different from control nontransgenic animals, and no obvious differences were observed between litter-mate controls and mice carrying the altered genotypes.

Genotyping

Genomic DNA was purified from mouse tail tissue. Briefly, tail samples were frozen for at least 15 min at -80°C . Each sample was incubated with 500 μl lysis buffer for 2 hr at 56°C with repeated agitation. The lysis buffer contained 10 mM Tris-HCl, pH 8.0; 100 mM EDTA; 0.5% SDS; 0.2 mg/ml ribonuclease A (Invitrogen, Carlsbad, CA); and 1 mg/ml proteinase K (Invitrogen). Samples were then centrifuged at 13,000 rpm for 10 min to remove tissue residue from the lysate. Genomic DNA was precipitated by adding 500 μl isopropanol and washed with 1 ml ice-cold 70% ethanol. DNA pellets were dissolved in 50 μl of 5 mM Tris-HCl buffer (pH 8.5) by incubation at 65°C for 10 min.

To detect the presence of Cre recombinase by PCR, the following primer set was used for the generation of a 300-bp amplicon: Cre300F 5'-CGATGCAACGAGTGATGAGG-3' and Cre300R 5'-CGCATAACCACTGAAACAGC-3'. To detect the knockout *Cox-2* alleles, the following primer set was used: COX-2E3F1 5'-AATTACTGCTGAAGCCCACC-3' and COX-2I5R1 5'-GAATCTCCTAGAACTGACTGG-3'. The *Cox-2* floxed allele amplicon is 2,670 bp, and the same primer set detects the *Cox-2* deleted allele as a 1,054-bp amplicon.

Reagents

(*RS*)-(tetrazol-5-yl)glycine (TZG; Tocris Bioscience, Ellisville, MO), a potent NMDA agonist, was dissolved in phosphate-buffered saline (PBS; pH 7.4; Invitrogen). NS-398 (Cayman Chemicals, Ann Arbor, MI), celecoxib (Sigma, St. Louis, MO), and SC-51089 (Cayman Chemicals) were dissolved in dimethylsulfoxide (DMSO) to concentrations of 10, 2, or 1 mg/ml. These concentrations were generated to achieve injection doses of 10, 2, or 1

mg/kg of drugs, respectively. For an animal weighing 30 g, 30 μ l of one of these drugs in DMSO or 30 μ l DMSO (vehicle control) was injected intraperitoneally. Antibodies used for immunohistochemistry were rabbit anti-mouse COX-2 (Cayman Chemicals), rat anti-mouse CD206 (Santa Cruz Biotechnology, Santa Cruz, CA), rat anti-mouse CD45 (BD Pharmingen, Franklin Lakes, NJ), goat anti-mouse PECAM (Santa Cruz Biotechnology), rat anti-mouse F4/80 (ABD Serotec, Raleigh, NC), and NeuN (EMD Millipore, Billerica, MA).

Intrastriatal Injection of NMDA Agonist

Mice were anesthetized by intraperitoneal (IP) injection of 100 mg/kg Nembutal (Abbott Laboratories, North Chicago, IL) and then securely fastened onto a small-animal stereotaxic frame with a mouse head holder (David Kopf Instruments, Tujunga, CA). A burr hole was drilled and an injection cannula (28 gauge) was lowered into the center of the right striatum (anterior 0.5 mm, lateral 2.0 mm, ventral 4.0 mm to bregma, according to the mouse brain atlas of Paxinos and Franklin [2004]). The precise position of the injection cannula is shown in Figure 1A. Ten microliters of TZG (5 μ M) or sterile PBS was injected at rate of 0.8 μ l/min, using a microinjection system (KDS Scientific, Holliston, MA). Needles were left unmoved for an additional 5 min after fluid injection, to prevent backflow. Wounds were then sutured, and animals were allowed to recover from anesthesia. Animals were sacrificed and brain samples were collected at 4 hr, 8 hr, 12 hr, or 24 hr after the intrastriatal injection.

Hematoxylin and Eosin Staining

Animals were killed by cervical dislocation followed by decapitation. Brains were harvested and immediately frozen in cold isopentane (-20°C) before brain sections were generated by cryosection. Fresh-frozen brain sections were allowed to air dry for at least 15 min before fixing in 95% alcohol for 5 min. Sections were then briefly rinsed in water and stained in Mayer's hematoxylin solution (Sigma-Aldrich) for 15 min. After rinsing in water five times, slides were stained in 1% eosin Y solution (Fisher Scientific, Pittsburgh, PA) for 3 min. Slides were then dipped five to 10 times in 95% and 100% alcohol prior to attaching coverslips with Permount (Fisher Scientific).

Determination of Lesion Volumes

Brains were sectioned on a cryostat to generate 20- μ m sections throughout the entire striatum. Every third coronal section was collected and stained as described above. Lesion areas on each section were quantified in ImageJ (National Institute of Health). All the lesion areas throughout the extent of the lesion were integrated to determine the lesion volume. All lesion sizes were quantified by a person blind to the treatment groups.

Immunohistochemistry

Fresh-frozen coronal brain sections were fixed in an acetone/alcohol mixture (3:1) for 5 min. The sections were treated with glucose oxidase and sodium azide to reduce background interference. They were then incubated with anti-COX-2 antibody (1:200), followed by an anti-rabbit secondary antibody (1:200). The labeling was amplified with the ABC solution (Vector Laboratories, Burlingame, CA) and visualized with a diaminobenzidine (DAB)

peroxidase substrate kit (Vector Laboratories). To identify the type of cells expressing COX-2, double-label immunohistochemistry (IHC) was performed.

COX-2-staining cells were double labeled with antibodies directed against the cellular protein markers anti-NeuN (neurons; 1:5,000), anti-PECAM (endothelial cells; 1:200), anti-CD206 (perivascular macrophages; 1:200), or anti-F4/80 (monocytes; 1:600). The labeling of these cell type markers was visualized with Cy2-streptavidin (1:400) or Cy3-streptavidin (1:400; Jackson ImmunoResearch, West Grove, PA).

Semiquantitative Analysis of COX-2 Immunoreactivity

Relative values of COX-2 immunoreactivity (ir) are given as estimates of the density of immunohistochemical staining. Four coronal sections (two 1.3 mm anterior to bregma and two 0.5 mm anterior to bregma) per animal for three animals per group were analyzed (Table I). For each brain section, COX-2 ir induced by TZG injection in different regions was quantified. Animals injected with saline from both groups were used as controls. A five-point scale was used to rate the data: +++ (5) high density of cellular signals, ++ (4) moderate density, + (3) low density, ± (2) single cellular signals in some cases, – (1) no signals. The values for two sections per animal were averaged, and the means of these averages, for the three animals per group, are presented. Quantification of the ir data was performed by an investigator blind to the treatment groups.

Statistical Analysis

Data were analyzed, as appropriate, by one-way or two-way ANOVA, followed by post hoc student *t*-tests when appropriate.

RESULTS

Excitotoxin release is a common pathogenic mechanism in many brain injury paradigms (Hardingham, 2009). This study used an excitotoxic injury model to determine the role of COX-2, expressed in different cell types, in mediating both neurotoxicity and neuroprotection. To induce excitotoxin damage, 10 µl of an NMDA receptor agonist, TZG (5 µM), was delivered by microinjection into the center of the right striatum of experimental mice (Fig. 1A). COX-2 expression induced by the TZG injection was detected by immunohistochemical (IHC) labeling. A representative brain section (Fig. 1B) shows COX-2 labeling in the wild-type (Cox-2^{flox/flox}) mouse 24 hr after excitotoxin injection. COX-2 expression was induced in two distinct patterns (neurons and nonneuronal cells), primarily on the ipsilateral side of the injection. COX-2 was induced in cells with neuronal morphology in the somatosensory cortex surrounding the affected striatal region (Fig. 1B, arrows). These COX-2-expressing cells were confirmed to be neurons by the IHC double labeling with antibodies to COX-2 and NeuN (a neuronal protein marker; see Fig. 2A–C). All COX-2-ir cells in the cortex were also NeuN immunoreactive.

COX-2 was also found in nonneuronal cells associated with vasculature in or near the injured striatum (arrowheads in Fig. 1B). The inset in Figure 1B shows a high-magnification micrograph of the vasculature-associated COX-2 labeling. COX-2-ir cells appear to be lining the blood vessel wall (red arrowheads) and are also present in the blood vessel lumina

(blue arrows). To illustrate the distribution pattern of induced COX-2 expression, locations of COX-2-ir cells are plotted in Figure 1D,E. Anterior to the injection site (for example, at 1.3 mm anterior to bregma; Fig. 1D), neuronal COX-2-ir cells (red dots) were seen in the cingulate cortex (Cg), motor-sensory cortex (M-S), and claustrum (Cl). Near the level of the injection site (for example, at 0.5 mm anterior to bregma; Fig. 1E), neuronal COX-2 was also observed in cells of the piriform cortex (Pir). Nonneuronal, vasculature-associated COX-2-ir staining cells (blue dots) were also found in the corpus callosum, external capsule, injured striatum, and (more scarcely) cortex (Fig. 1E). Because the nonneuronal COX-2-expressing cells were confined to blood vessels and did not display microglia or astrocyte morphology, they are likely to be endothelial cells and/or hematopoietic cells.

Results from the semiquantitative analysis of the density of COX-2-ir following TZG injection into *Cox-2^{flox/flox}* and *Tie2Cre Cox-2^{flox/flox}* mice are summarized in Table I. The density of COX-2-ir in *Cox-2^{flox/flox}* and *Tie2Cre Cox-2^{flox/flox}* mice is indistinguishable after saline injections. Consequently, the data from these two groups are combined for the data for COX-2-ir control values. Increased COX-2-ir was induced in cingulate cortex, in motor-sensory cortex, and in claustrum in coronal sections at 1.3 mm anterior to bregma in both *Cox-2^{flox/flox}* and *Tie2Cre Cox-2^{flox/flox}* mice (Table IA). At 0.5 mm anterior to bregma, COX-2 ir was also induced in the Pir and striatum of *Cox-2^{flox/flox}* mice, but not in the striatum of *Tie2Cre Cox-2^{flox/flox}* mice (Table IB). Double-label IHC showed that the nonneuronal COX-2 ir was present in the region of the blood vessels in the lesion core 24 hr after excitotoxin injection. COX-2 staining was colocalized mostly with PECAM staining (an endothelial cell marker; Fig. 2D – F) or F4/80 staining (a monocyte marker; Fig. 2G–I) but not with CD45 (a pan-leukocyte marker; Fig. 2J–L) or CD206 (a perivascular macrophage marker; Fig. 2M–O) staining. In the striatum, 61% of the COX-2-ir cells colocalized with PECAM, and 31% of COX-ir cells colocalized with F4/80. Figure 2J–O shows a lack of colocalization, even when adjacent cells are stained for COX-2 and either for CD45 or for CD206. These data indicate that endothelial cells and/or monocytes are likely to be the nonneuronal cells that produce COX-2 in response to excitotoxin administration.

To begin to dissect the functions of COX-2 expressed in neurons vs. nonneuronal cells, a conditional knockout mouse line, *Tie2Cre Cox-2^{flox/flox}* mice, was generated to delete *Cox-2* gene expression specifically in endothelial and myeloid cells. TZG-induced COX-2 expression in neurons (arrows) was retained in *Tie2Cre Cox-2^{flox/flox}* mice (Fig. 1C). However, COX-2 expression in the nonneuronal cells was abrogated.

Lesion volumes in *Tie2Cre Cox-2^{flox/flox}* mice and their wild-type (*Cox-2^{flox/flox}*) littermates were compared. Figure 3A–F shows representative micrographs of H&E-stained brain sections at the level of the injection needle. Four hours after TZG injection, the size of the injury was not significantly different between the wild-type mice and the *Tie2Cre Cox-2^{flox/flox}* mice (Fig. 3A,D,G). At 8 and 24 hr postinjection, however, the lesion sizes were significantly larger in the *Tie2Cre Cox-2^{flox/flox}* mice than in the *Cox-2^{flox/flox}* mice (Fig. 3B,E,C,F, and G). Eight and twenty-four hours postinjection, the lesion volumes in *Tie2Cre Cox-2^{flox/flox}* mice were approximately twice as large as those of their wild-type

littermates. The data suggest that endothelial and/or myeloid cell COX-2 expression plays a role in limiting lesion size in this excitotoxin injury model.

COX-2-dependent neurotoxicity can be mediated by prostaglandin E₂ (PGE₂), working via the EP1 receptor (Kawano et al., 2006). To determine whether PGE₂ is involved in mediating neurotoxicity in this model, mice received an IP injection of the EP1 receptor antagonist SC-51089 (10 mg/kg) 1 hr prior to the TZG injection. Blockade of EP1 activation reduced the lesion volume in both TZG-treated Cox-2^{flox/flox} mice and TZG-treated Tie2Cre Cox-2^{flox/flox} mice (Fig. 4A). (Note that the lesions in the control Tie2Cre Cox-2^{flox/flox} mice that did not receive SC-51089 are approximately twice the size of the lesions in the control Cox-2^{flox/flox} mice that did not receive SC-51089, as previously observed [Fig. 3]). These data suggest 1) that TZG induced neurotoxicity results, at least in part, from neuronal COX-2-dependent PGE₂ production and is mediated by a PGE₂/EP1 pathway and 2) that this neuronal COX-2-dependent PGE₂/EP1-mediated neurotoxicity can be ameliorated by a nonneuronal (either myeloid or endothelial) COX-2-dependent process.

The role of COX-2 activity in neuroprotection and neurotoxicity in response to excitotoxin administration was also studied using the selective COX-2 inhibitors NS-398 and celecoxib. NS-398 was administered to wild-type Cox-2^{flox/flox} mice and to Tie2Cre Cox-2^{flox/flox} mice 1 hr before TZG injection. Pretreatment with a high dose of NS-398 (10 mg/kg) reduced the TZG-induced lesion volumes in both Cox-2^{flox/flox} mice and Tie2Cre Cox-2^{flox/flox} mice (Fig. 4B) compared with their vehicle controls. In contrast, instead of reducing neural damage, pretreatment with a low dose of NS-398 (2 mg/kg) increased the lesion volume in Cox-2^{flox/flox} mice but had no effect on lesion volumes in Tie2Cre Cox-2^{flox/flox} mice (Fig. 4B). Recall that neuronal COX-2 expression, but not nonneuronal (endothelial and/or myeloid) COX-2 expression, can be induced by TZG injection in Tie2Cre Cox-2^{flox/flox} mice (Figs. (1 and 2)). Perhaps the most parsimonious explanation for these results is that this low-dose NS-398 effect may be caused by the blockade of COX-2 activity in the nonneuronal cells exerting neuroprotective effects following neurotoxin injection.

To substantiate this conclusion, the effects of low and high doses of a second COX-2-selective inhibitor, celecoxib, were tested in the wild-type mice. As with the low-dose NS-398 experiment, pretreatment with low-dose celecoxib significantly increased lesion volume (Fig. 4C). In contrast, high-dose celecoxib, like high-dose NS-398, reduced lesion volume (Fig. 4C). Thus, two different COX-2-selective inhibitors, NS-398 and celecoxib, show the same alternative modulatory effects on responses to excitotoxin administration at low and high doses.

It is possible that the increased lesion volume in the Tie2Cre Cox-2^{flox/flox} animals resulted from an artifact of the presence of Cre recombinase alone; artifacts of this nature have been observed in other targeted deletion experiments (Loonstra et al., 2001). To eliminate this possibility in our experiments, we compared lesion volumes in Cox-2^{+/+} mice and Tie2Cre;Cox-2^{+/+} mice after the TZG injection. The presence of Tie2-driven Cre recombinase did not change TZG-induced lesion volume in Cox-2^{+/+} mice (Fig. 4D).

Collectively, these data suggest that TZG injection induces 1) neuronal COX-2-dependent PGE₂ production, which then elicits an EP1-dependent neurotoxic response, and 2) nonneuronal (endothelial and/or myeloid) COX-2 induction, whose eicosanoid product(s) evoke a neuroprotective effect.

LysMCre expression deletes “floxed” DNA sequences in both macrophages and neutrophils (Takeda et al., 1999). To evaluate further the cell type(s) responsible for the production of the neuroprotective COX-2 following excitotoxin administration, we examined the consequences of TZG injection into LysMCre Cox-2^{flox/flox} mice. TZG injection induced COX-2 expression both in cortical neurons and in cells associated with blood vessels in LysMCre Cox-2^{flox/flox} mice (Fig. 5A). To ascertain that there was no leaky monocyte COX-2 expression following Cox-2^{flox/flox} deletion in these mice, COX-2 and F4/80 (a macrophage marker) double labeling was performed; F4/80 (red) and COX-2 (green) do not colocalize in LysMCre Cox-2^{flox/flox} mice (Fig. 5A, inset), as expected. TZG injection results in similar lesion volumes in LysM-Cre Cox-2^{flox/flox} mice compared with their wild-type littermates. Lesion volume in LysMCre Cox-2^{flox/flox} mice following TZG administration is similar to lesion volume in control Cox-2^{flox/flox} mice (Fig. 5B), in contrast to the increased lesion volume observed in Tie2Cre Cox-2^{flox/flox} mice (Fig. 3). Low dose NS-398 treatment increases the size of TZG induced lesions in LysMCre Cox-2^{flox/flox} mice (Fig. 5C), just as it does in wild-type mice, presumably as a result of inhibiting endothelial cell COX-2-dependent production of an eicosanoid subsequently requiring the manifestation of the neuroprotective effect.

DISCUSSION

COX-2-selective inhibitors are used for the treatment of cancer, pain, and arthritis (Chan et al., 2010; Ashok et al., 2011). Consequently, COX-2-dependent neuroprotective and neurotoxic effects are important not only for patients seeking treatment for acquired neural injury but also for patients who use COX-2 inhibitors to treat cancer, pain, and arthritis, because their susceptibility to acquired neural injury may be significantly altered by these drugs.

Elevated neuronal COX-2 expression has been widely reported in various models of acute brain injury, including cerebral ischemia (Hara et al., 1998), traumatic brain injury (Strauss et al., 2000), spreading depression (Koistinaho and Chan, 2000), and excitotoxic brain injury (Adams et al., 1996; Kaufmann et al., 1996). The present results show that, in addition to neuronal COX-2 induction, nonneuronal COX-2 expression was induced in scattered blood-vessel-associated cells throughout the injured striatum in response to excitotoxin administration. These results are consistent with reports that vasculature-associated, nonneuronal COX-2 induction can be found in the injured brain (Busija et al., 1996; Lopez-Vales et al., 2004; Wu et al., 2011), including human samples (Iadecola et al., 1999).

Previous studies have suggested that neuronal COX-2 could mediate neurotoxic effects during acute brain injury. Dore et al. (2003) showed that transgenic neuronal COX-2 overexpression exacerbated ischemic brain damage. However, this approach does not demonstrate whether endogenously expressed COX-2 is neurotoxic. Kawano et al. (2006)

showed that COX-2 mediates neurotoxicity through the activation of the EP1 receptor; ligand binding to this receptor augments excitotoxin-induced neuronal Ca^{2+} influx. However, the COX-2-expressing cell type responsible for this neurotoxicity was not identified. In the present study, COX-2-dependent neurotoxic effects were reduced by an EP1 antagonist in both wild-type and Tie2Cre *Cox-2^{flox/flox}* mice. Because COX-2 induction could be observed only in neurons in the Tie2Cre *Cox-2^{flox/flox}* mice following excitotoxin injection, our results suggest that endogenous neuronal COX-2 expression, in cortical regions known to project to striatum (Arikuni and Kubota, 1986), during excitotoxic injury exerts a neurotoxic effect via the EP1 receptor.

The COX-2-dependent neuroprotective effect was revealed by two observations in the present study. First, cell-type-specific *Cox-2* gene deletion in the nonneuronal cells of Tie2Cre *Cox-2^{flox/flox}* mice resulted in increased neural damage in response to TZG injection. Thus, COX-2 expression in this nonneuronal (presumably endothelial or myeloid) cell population must play an essential role in neuroprotection in response to excitotoxin challenge. Because this effect was not evident in Tie2Cre *Cox-2^{+/+}* mice, the exacerbation of neural damage observed in Tie2Cre *Cox-2^{flox/flox}* mice was not caused by the presence/activity of Cre recombinase alone. Second, pretreatment with low doses of two selective COX-2 inhibitors, NS-398 and celecoxib, in wild-type mice, like Tie2Cre-mediated *Cox-2* targeted deletion, increased neural damage in response to TZG challenge. The most parsimonious explanation for the enhancement of excitotoxin-induced neural damage in response to low-dose COX-2-selective inhibitors is that the COX-2 inhibitor is likely to access the nonneuronal COX-2-expressing cells nearest the circulating blood supply and, consequently, preferentially inhibit a neuroprotective effect of COX-2 from these cells. The suggestion that those cells that express Tie2 (and in which, as a consequence, the *Cox-2* gene is conditionally deleted in Tie2-Cre *Cox-2^{flox/flox}* mice) and those cells in which low-dose COX-2 inhibitors elicit neuroprotection are the same cells is substantiated by the observation that low-dose NS-398 administration cannot further increase the exacerbated neural damage observed in Tie2Cre *COX-2^{flox/flox}* mice in response to TZG administration, presumably because the *Cox-2* gene responsible for neuroprotective COX-2 expression has already been deleted in the critical cell type in Tie2Cre *Cox-2^{flox/flox}* mice treated with low-dose NS-398. In contrast, high-dose NS-398 pretreatment reduced TZG-induced lesion volume in both wild-type and Tie2Cre *Cox-2^{flox/flox}* mice, presumably as a result of selective inhibition of COX-2 expressed in neurons in both mouse strains in response to the excitotoxin following administration of the COX-2 inhibitor at the higher dose. The common results observed with two structurally distinct COX-2-selective inhibitors strongly suggest that COX-2-derived prostanoids mediate the effects observed here on excitotoxin-induced neural damage. Current literature favors the view that COX-2 expression during acquired acute brain injury is generally neurotoxic and that COX-2 inhibitors should be considered as an effective drug treatment (Strauss, 2010). This conclusion has been reached primarily as a result of the use of either global *Cox-2* knockout mice (Iadecola et al., 2001) or mice treated with COX-2-selective inhibitors. Both approaches, which eliminate COX-2-dependent prostanoid production in all cells, generally produced beneficial outcomes (Candelario-Jalil, 2008; Ahmad et al., 2009; Hakan et al., 2010). Only a minority of reports showed the use of COX-2 inhibitors can be detrimental and exacerbate neural injury (Friess

et al., 2012). It should be noted that studies showing neuroprotective effects of COX-2 inhibitors have typically used high doses of COX-2 inhibitors, which produced outcomes similar to those that we observed with high-dose COX-2-selective inhibitor amelioration of TZG-induced neural damage. However, the detrimental effects of the low-dose COX-2 inhibitor uncovered in the present study should not be dismissed, both in understanding the alternative role(s) of COX-2 in response to brain injury and because of the practical aspect that there may be a limit to the dose of COX-2 inhibitors human patients can be given without adverse effects. For example, the use of high-dose COX-2-selective inhibitors is known to be associated with cardiovascular-induced morbidity (Peura, 2002).

In this study, neuronal COX-2 expression was observed in cells surrounding the lesion core following TZG injection. In contrast, the nonneuronal COX-2 expression often occurred in the lesion core. Because COX-2 eicosanoid products are highly unstable, they generally exert their influence within a short distance from the point of origin, either as autocrine or as paracrine effectors. Thus, it is likely that, after TZG exposure, the prostanoids produced by the neuronal and the non-neuronal COX-2-expressing cells in different locations have different targets. Thus, the TZG-induced neuronal COX-2 may cause neurotoxicity by prostaglandins (PGs) released from synapses linking the COX-2-producing cells in the cortex to the lesion core, whereas PGs produced by the neuroprotective nonneuronal COX-2 might target primarily cells that have access to the blood vessels.

The most likely vasculature-associated cells that might produce COX-2 in response to excitotoxin insult are the endothelial cell and cells of the myeloid lineage (macrophages/monocytes). Under various pathophysiological conditions, microglia cells (Anrather et al., 2011; Straccia et al., 2013) and perivascular macrophages (Schiltz and Sawchenko, 2002) are known to contribute to COX-2-mediated PG production. The present study did not show any COX-2-labeled cells with microglial morphology, and COX-2 expression was not found in perivascular macrophages. Therefore, the observed phenomena related to the COX-2 deletion likely are not mediated by these two cell types. The abrogation of neuroprotection in Tie2Cre *Cox-2^{flox/flox}* mice suggests that the endothelial cell is likely to be responsible for this COX-2-dependent neuroprotection. In contrast, the lack of any effect in LysMCre *Cox-2^{flox/flox}* mice suggests that monocytes/macrophages in the vascular domain do not play a role in COX-2-dependent neuroprotection.

In summary, the dual role of COX-2 expression in the context of excitotoxic neural injury can be dissected on the basis of the two distinct patterns of COX-2 induction: expression of COX-2 in neurons surrounding the lesion core augments neural damage via an EP1 receptor-mediated mechanism. In contrast, nonneuronal COX-2 expression in the lesion core leads to neuroprotection.

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Fig. 1.

COX-2 was induced in two distinct patterns 24 hr after TZG injection into the mouse striatum. **A:** Site of TZG injection in the right striatum (described in Materials and Methods). **B:** Twenty-four hours after TZG injection, COX-2 labeling in wild-type $Cox-2^{flox/flox}$ mice is present in cells with neuronal morphology in the cortex (arrows) and in nonneuronal cells (arrowheads) associated with blood vessels. **Inset** shows a high-magnification photograph of a COX-2-labeled blood vessel. COX-2-immunoreactive cells appear to be lining the blood vessel wall (red arrowheads) and are present within the blood vessel lumina (blue arrows). **C:** COX-2 was expressed only in neurons (arrows) in Tie2Cre $Cox-2^{flox/flox}$ mice. **D,E:** Distribution patterns of induced COX-2 in neurons (red dots) and nonneuronal cells (blue dots) in $Cox-2^{flox/flox}$ mice; coronal section of mouse brain at 1.3 mm anterior to bregma (D) and coronal section at 0.5 mm anterior to bregma (E). **F:** In Tie2Cre $Cox-2^{flox/flox}$ mice, COX-2 was induced only in neurons in the cortex 12 hr after TZG injection; the section is 0.5 mm anterior to bregma. Black lines indicate needle tracks. Cg, cingulate cortex; Cl, claustrum; M-S, motor and somato-sensory cortex; Pir, piriform cortex. Scale bars 500 μ m in B,C; 100 μ m in inset.

Fig. 2. Identification, by IHC, of COX-2-expressing cell types in the mouse 24 hr after TZG injection. **A–C:** In the cortex, COX-2 was colocalized with NeuN. **D–I:** In the injured striatum, COX-2 was colocalized with PECAM and F4/80. **J–O:** COX-2 was not colocalized with either CD45 or CD206. Scale bars 5 25 μm.

Fig. 3. Lesion sizes in Cox-2^{flox/flox} mice and Tie2Cre Cox-2^{flox/flox} mice. **A–C:** Representative micrographs show H&E-stained neural damage in striatum 4, 8, and 24 hr after TZG injection in Cox-2^{flox/flox} mice. **D–F:** H&E stained neural damage in Tie2Cre Cox-2^{flox/flox} mice. Dotted lines show the margins of the lesion. **G:** Comparison of lesion volumes at different times after excitotoxin injection in Cox-2^{flox/flox} mice (open bars) and Tie2Cre Cox-2^{flox/flox} mice (solid bars). Data were analyzed by two-way ANOVA followed by post hoc *t*-test. **P* < 0.001 relative to the lesion volumes in Cox-2^{flox/flox} mice at the same time

point; $^{\#}P < 0.001$ relative to the lesion volumes in Tie2Cre Cox-2^{flox/flox} at 8 hr. N = 5 in all experimental groups. Scale bar = 300 μm in A (applies to A–C); 400 μm for D–F.

Fig. 4.

Modulation of TZG-induced lesion volume by an EP1 antagonist and by two COX-2-selective inhibitors. **A:** Lesion volume was reduced by pretreatment with the EP1 receptor antagonist (SC-51089) in both Cox-2^{flox/flox} and Tie2Cre Cox-2^{flox/flox} mice. * $P < 0.01$ relative to vehicle-treated controls. **B:** In Cox-2^{flox/flox} mice, lesion volume was increased by pretreatment with low-dose COX-2 inhibitor (NS-398, 2 mg/kg) and reduced by high-dose NS-398 (10 mg/kg); * $P < 0.05$ relative to both low-dose and high-dose NS-398-treated groups. Low-dose NS-398 had no effect on lesion volume in Tie2Cre Cox-2^{flox/flox} mice; lesion volume was reduced by high-dose NS-398; * $P < 0.01$ relative to vehicle-treated controls. **C:** In Cox-2^{flox/flox} mice, low-dose celecoxib (2 mg/kg) increased, whereas high-dose celecoxib (10 mg/kg) decreased, TZG-induced lesion volume; * $P < 0.05$ relative to both low-dose and high-dose celecoxib-treated groups. **D:** Lesion volumes in Tie2Cre;Cox-2^{+/+} mice and Cox-2^{flox/flox} mice after TZG injection. No difference was detected between these two groups. $N = 5$ in all experimental groups. Data in A–C were analyzed by two-way ANOVA followed by post hoc *t*-test; data in D were analyzed by Student's *t*-test.

Fig. 5.

Deletion of the Cox-2 gene in myeloid cells does not alter TZG-induced neurotoxicity. **A:** COX-2 staining is present in neurons and endothelial cells in LysMCre Cox-2^{flox/flox} mice. The **inset** shows that COX-2 (green) is not colocalized with the macrophage F4/80 marker (red). **B:** TZG-induced lesion volumes were not significantly different between Cox-2^{flox/flox} and LysMCre Cox-2^{flox/flox} mice. **C:** TZG lesion volume was increased by 2 mg/kg NS-398 treatment 1 hr before TZG injection **P* < 0.01 relative to 2 mg/kg NS-398 treatment. *N* = 5 in all experimental groups. Data in B and C were analyzed by Student's *t*-test. Scale bars = 150 μm in A; 25 μm in inset.

TABLE I

Semiquantitative Analysis of TZG-Induced COX-2 Immunoreactivity *

Regions	Cox-2 ^{flox/flox}	Tie2Cre Cox-2 ^{flox/flox}	Control (saline)
A. 1.3 mm anterior to bregma			
Striatum	±	–	–
Cg	++	++	–
M-S	++	++	±
Cl	++	++	±
Pir	–	–	–
B. 0.5 mm anterior to bregma			
Striatum	++	–	–
Cg	+++	+++	–
M-S	+++	+++	±
Cl	+++	+++	±
Pir	+++	+++	–

* +++ (5) high density of cellular signals; ++ (4) moderate density; + (3) low density; ± (2) single cellular signals in some cases; – (1) no signals.
Cg, cingulate cortex; Cl, claustrum; M-S, motor and somatosensory cortex; Pir, piriform cortex.