Activation of the Epidermal Growth Factor Receptor in Optic Nerve Astrocytes Leads to Early and Transient Induction of Cyclooxygenase-2

Xu Zhang and Arthur H. Neufeld

PURPOSE. The epidermal growth factor receptor (EGFR) appears in astrocytes after neural injury. The authors’ laboratory has reported the presence of EGFR in glaucomatous optic nerves. The activation of EGFR is often associated with induction of cyclooxygenase (COX)-2. In this study, the induction of COX-2 pathway in rat optic nerve astrocytes was investigated.

METHODS. Induction of COX-2 was determined by immunoblot and immunocytochemistry in optic nerve astrocytes stimulated with EGF. EGF-induced prostaglandin (PG)E₂ release into the culture medium was assayed by ELISA. The effects of the EGFR tyrosine kinase inhibitor, AG1478, were studied on COX-2 expression and PGE₂ synthesis. In rat optic nerve transection and a rat optic nerve explant culture model, the relationship between the expression of COX-2 and activation of EGFR was examined.

RESULTS. Activation of EGFR caused the rapid and transient induction of COX-2 in optic nerve astrocytes. The level of COX-2 was rapidly upregulated in optic nerves after axotomy and in an optic nerve explant culture model. When induced, COX-2 localized to the nuclear membrane of the astrocytes. When COX-2 was induced in response to activation of EGFR, the activated astrocytes produced and released the proinflammatory mediator, PGE₂, in a time-dependent manner. EGF-stimulated induction of COX-2 protein and synthesis of PGE₂ were abolished by the EGFR tyrosine kinase inhibitor AG1478. The stimulatory action of EGF on release of PGE₂ was inhibited by the COX-2-selective inhibitor NS398.

CONCLUSIONS. The data demonstrate that the activation of EGFR in optic nerve astrocytes leads to the induction of the immediate early gene COX-2 and subsequent signaling through the synthesis of PGE₂. This early signal of neural tissue damage may be important in setting up secondary events in the damaged tissue. (Invest Ophthalmol Vis Sci. 2005;46:2035–2041) DOI: 10.1167/iovs.04-1473

Astrocytes are the major glial cell type in the optic nerve and are responsible for providing homeostatic and metabolic support to the axons of the retinal ganglion cells (RGCs). Astrocytes form the interface between neurons, connective tissue surfaces, and surrounding blood vessels. After neural injury or in neurodegenerative diseases, quiescent, mature astrocytes become reactive astrocytes. Reactive astrocytes increase production of intermediate filaments, migrate, proliferate, synthesize extracellular matrix, and release mediators of inflammatory responses.

Cyclooxygenase (COX)-2 is the inducible isoform of COX, the key enzyme in the synthesis of prostaglandins from arachidonic acid. In many tissues, COX-2 is an immediate early gene product associated with injury. COX-2 is rapidly induced in response to cytokines, such as IL-1β, TNF-α, or growth factors. In the central nervous system (CNS), COX-2 is constitutive in certain neurons and astrocytes. Several studies have shown that COX-2 expression is altered by ischemia in neural tissue and retina. In brain ischemia, induced COX-2 contributes to ischemic brain damage. Experiments using selectively COX-2 inhibitors as well as COX-2-knockout (Cox-2−/−) mice have implicated inducible COX-2 as a proinflammatory gene in the CNS. The reaction products of COX-2, prostaglandin E₂ (PGE₂) and reactive oxygen species, may be chemotactic and cytotoxic, leading to secondary neuronal injury.

Activation of the epidermal growth factor receptor (EGFR) pathway and the induction of COX-2 are reported in a wide variety of cancers—for example, non-small-cell lung cancer and squamous cell carcinoma. Treatments of certain forms of cancers have been at least partially successful with either an inhibitor of EGFR tyrosine kinase or an inhibitor of COX-2. In cancer cells, the activation of EGFR leads to the induction of COX-2. Nevertheless, although both EGFR and COX-2 appear in astrocytes after injury, there has been no demonstration of any association between these two proteins.

The EGFR is a member of a family of plasma membrane receptor tyrosine kinases that control many important cellular functions. In the normal adult brain, EGFR is not detected in astrocytes. However, EGFR appears in glia in disorders of the CNS, ranging from injury to neurodegenerative diseases to tumor development. Activation of the EGFR pathway is perhaps responsible for the proliferation of reactive astrocytes at the site of neural injury. In gliomas, astrocytes overexpress EGFR and are transformed into malignant cells.

EGF is significantly upregulated and activated in reactive astrocytes in the human glaucomatous optic nerve head. In vitro, optic nerve astrocytes express EGFR, and activation of EGFR leads to expression of inducible iNOS. The presence of iNOS in glaucomatous optic nerve astrocytes has been hypothesized as being responsible for making neurotoxic quantities of nitric oxide in glaucomatous optic neuropathy. Using primary astrocyte cultures prepared from rat optic nerves, we found that activation of EGFR causes the rapid induction of COX-2. We also demonstrated that EGFR-dependent induction of COX-2 occurred in astrocytes of the optic nerve after injury. These findings demonstrate a novel regulatory function of EGFR in optic nerve astrocytes, which may be relevant to the regulation of responses of astrocytes after axonal injury.
Materials and Methods

Human EGF was purchased from Sigma-Aldrich (St. Louis, MO); NS398 and rabbit polyclonal COX-2 antibody from Cayman Chemical Co. (Ann Arbor, MI); AG1478 from Calbiochem (La Jolla, CA); GFAP, HLA-DR, α-smooth muscle actin, and von Willebrand factor (VWF) antibodies from Santa Cruz Biotechnology (Santa Cruz, CA); Dulbecco’s modified Eagle’s medium (DMEM)/Ham’s F-12 and fetal bovine serum (FBS) from Washington University Medical School Tissue Culture Support Center (St. Louis, MO); and PSFM (penicillin, streptomycin, and amphotericin B) from Invitrogen-Gibco (Gaithersburg, MD). Chemicals not listed were of the highest grade available from Sigma-Aldrich.

Primary Astrocyte Culture

Primary astrocyte cultures were prepared from the anterior portions of rat optic nerves of Wistar rats, aged 3 months (Charles River, Wilmington, MA), modified from the procedure previously described. Briefly, explants from the anterior optic nerve were carefully dissected, placed in T-25 flasks, and maintained in DMEM/F-12 with 10% FBS and PSFM. The first passage cells were characterized by immunostaining of glial fibrillary acidic protein (GFAP), neural cell adhesion molecule, and other cellular markers (HLA-DR, α-smooth muscle actin, and von Willebrand factor) to distinguish them from other cell types in this region. The primary cell cultures were purified for astrocytes by growing the cells for one week in modified astrocyte-defined, serum-free medium and serum-starved in ADM for 3 to 7 days before being used in the experiments.

EGF Treatment

Astrocyte cultures were incubated with EGF (0, 0.1, 1, 5, 10, and 25 ng/mL) in the serum-free medium at 37°C for various lengths of time. To determine PGE₂ production, the conditioned medium was collected under sterile conditions and stored at −80°C until PGE₂ assays were performed.

Inhibitor Treatment

NS398 and AG1478 were dissolved in dimethylsulfoxide (DMSO) and all other substances were dissolved in distilled water. Chemicals were further diluted in incubation medium to obtain working solutions. Astrocytes were treated with vehicle, NS398 (20 μM) or AG1478 (3 or 30 nM) 30 minutes later, cultures were treated with 10 ng/mL EGF for 24 hours.

Optic Nerve Explant Culture

Optic nerve explants were obtained from Wistar rats. All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. After rats were anesthetized, the connective tissue and muscles surrounding the eye were cut to access the intraorbital portion of the optic nerve, which was then transsected at 2 mm away from the posterior scleral wall of the eyeball with scissors. Isolated optic nerves were collected by cutting at the scleral wall and the explants cultured in ADM. The cultures were maintained at 37°C in 5% CO₂/air in an incubator for different lengths of time and in the presence of different drugs, as described earlier.

Optic Nerve Transection

Rats were anesthetized, and a skin incision was made close to the superior orbital rim. The orbit was opened, leaving the supraorbital vein intact. The superior extraocular muscles were spread with a small retractor. The optic nerve was exposed by longitudinal incision of the eye retractor muscle and the perineurium. The optic nerve was cut 3 to 4 mm behind the globe. Special care was taken to avoid damaging the central retinal artery. The left optic nerve was cut in each animal, and the right eye served as the sham-operation control, in which the surgery was performed without cutting the optic nerve. Animals were killed 1 day and 2 days after the surgery.

Western Blot Analyses

Cells were washed twice with ice-cold PBS and then lysed in the extraction buffer (20 mM HEPES [pH 7.0], 10 mM KCl, 2 mM MgCl₂, 0.5% NP-40, 1 mM Na₃VO₄, 1 mM PMSF, and 0.15 U/mL aprotinin). Rat optic nerves were homogenized in the same extraction buffer. Protein concentrations were determined with the Bradford colorimetric assay. Aliquots containing an equal amount of protein (30 μg) were analyzed by SDS-polyacrylamide gel electrophoresis on 10% gels and were transferred to nitrocellulose membranes. The blots were then blocked with 5% nonfat milk in Tris-buffered saline with Tween 20 (25 mM Tris-HCl, 150 mM NaCl, and 0.05% Tween 20) and incubated with the appropriate primary antibodies followed by incubation with peroxidase-conjugated secondary antibodies. Finally, proteins on membranes were detected with an ECL chemiluminescence kit (Amersham Life Sciences, Arlington Heights, IL).

PGE₂ Production Assay

Supernatants of the astrocyte cultures were collected at different times. The concentration of PGE₂ was determined by enzyme-linked immunoassay according to the manufacturer’s manual (Cayman Chemical Co.). Total protein concentration of each culture sample was determined with the Bradford colorimetric assay. Determinations were performed in triplicate. PGE₂ values were expressed as picograms of PGE₂ per milligrams of cell protein.

Immunocytochemistry and Immunohistochemical Analyses

Cells grown on coverslips were fixed in 4% paraformaldehyde at 4°C for 30 minutes, washed in PBS, and treated with 0.5% FBS/0.2% Triton X-100/0.5% glycine in PBS for 20 minutes. The coverslips were incubated with specific primary antibodies: rabbit polyclonal antibody against COX-2 (diluted 1:50; Cayman Chemical Co.) and mouse monoclonal antibody against GFAP (1:25; Sigma-Aldrich). Because COX-2 is located in the macula densa of the kidney, sections of rat and mouse kidney were used as the positive control. After washing several times with PBS, the coverslips were incubated with the appropriate secondary antibody. For double immunofluorescent staining, the coverslips were sequentially incubated with the second primary antibody and the second appropriate secondary antibody. After the coverslips were washed several times with PBS, the coverslips were mounted (Pro-Long: Molecular Probes, Eugene, OR). Secondary antibodies: goat anti-mouse Oregon green (1:400) and goat anti-rabbit rhodamine red-X conjugated IgG (1:1000) were purchased from Molecular Probes. For immunohistochemical analysis of the optic nerve tissue, the eyes or optic nerves (for optic nerve explant culture) were fixed in 4% paraformaldehyde and embedded, sectioned, and deparaffinized. The sections were stained with goat polyclonal antibody against COX-2 or pEGFR (diluted 1:100, Santa Cruz Biotechnology) and biotinylated rabbit anti goat IgG (Dako Corp., Glostrup, Denmark). The reaction was developed with diaminobenzidine, as previously described.

Evaluation of Cell Morphology

To evaluate the cell morphologic changes in response to EGF (10 ng/mL), we used toluidine blue staining. The astrocytes were plated on coverslips at 5000/cells dish. The cells were cultured in serum-free medium for 1 week and then treated with EGF and/or inhibitors for 1 day or 2 days. Coverslips were immersed in toluidine blue for 10 minutes, washed several times with distilled water, and mounted on slides in acrylic medium (Cytoseal; Stephens Scientific, Cornwall, NJ).

Statistical Analysis

All experiments were repeated at least three times, using cultured cells that were obtained from different rat optic nerve head tissue. Data are expressed as the mean ± SEM of four replicate samples. The results
were analyzed by Student’s unpaired t-test, to determine the significant difference between means, or by two-way ANOVA followed by a least-significance procedure, to determine the significance of the response. P < 0.05 was considered significant.

RESULTS

Effect of EGF on COX-2 Expression in Primary Rat Optic Nerve Astrocytes

Treatment of astrocytes with EGF resulted in significantly increased levels of COX-2 expression in a time- and dose-dependent manner (Fig. 1). The induction of COX-2, as indicated by the appearance of COX-2 protein, was apparent as early as 3 hours after exposure to 10 ng/mL EGF, peaked between 6 and 24 hours, and returned to basal levels in 4 days in the continuous presence of EGF. The induction of COX-2 expression was apparent 24 hours after exposure to 1 ng/mL EGF and reached a maximum level at 10 ng/mL EGF.

We determined the intracellular location of EGF-induced COX-2 in optic nerve astrocytes by immunofluorescence microscopy. Figure 2A shows that, in cells not exposed to EGF, there was no positive labeling for COX-2 expression, whereas GFAP was present in the cytoplasm. Exposure of these cells to EGF (10 ng/mL) for 24 hours led to positive labeling for COX-2, localized primarily to the nuclear membrane and perinuclear areas (Fig. 2B). The shape of rat optic nerve astrocytes changed significantly after EGF treatment. The astrocytes became elongated with very long and thin processes. The morphologic changes of the astrocytes in response to EGF were clearly visible in the labeling for GFAP, which appeared to increase after exposure to EGF (Fig. 2B). In astrocytes exposed to EGF, COX-2 colocalized with GFAP in the perinuclear area (Fig. 2B). These results demonstrate that the activation of astrocytes induces the increased expression of COX-2 in EGF-treated primary rat optic nerve astrocytes.

EGF-Induced PGE2 Production in Primary Rat Optic Nerve Astrocytes

Because COX-2 catalyzes the biosynthesis of PGs, we examined whether there was EGF-induced PGE2 production by rat optic nerve astrocytes. As shown in Figure 3, COX-2 protein expression induced by EGF was accompanied by an increased accumulation in the culture medium of PGE2 in a time-dependent manner. PGE2 production reached its maximum level at 24 hours. The results indicate that EGFR activation can lead to COX-2 protein expression and subsequently PGE2 biosynthesis by rat optic nerve astrocytes.

Effect of EGFR Inhibition and COX-2 Inhibition on EGF-Induced PGE2 Release

To demonstrate further that EGF-induced synthesis of active COX-2 protein in rat optic nerve astrocytes is through activation of the EGFR, we tested the effect of a specific inhibitor for EGFR tyrosine kinase, AG1478. In the presence of AG1478 (3 and 30 nM), the EGF-induced synthesis of COX-2 protein was completely inhibited (Fig. 4A). The lower concentration of inhibitor (3 nM AG1478) also caused complete inhibition of PGE2 release (Fig. 4B). By using a selective inhibitor of COX-2, NS398, we also demonstrate that the PGE2 produced was by COX-2. At 20 μM NS398, there was complete inhibition of EGF-induced PGE2 production (Fig 4B). These data suggest a direct relationship between activation of EGFR, induction of COX-2, and synthesis and release of PGE2 by rat optic nerve astrocytes.
Shape Change in Astrocytes in Response to EGF

As previously demonstrated, exposure of primary rat and human optic nerve astrocytes to EGF results in a marked shape change in the astrocytes and the formation of cribriform structures. AG1478, an EGFR tyrosine kinase inhibitor, completely blocked the shape change and formation of the cribriform structures by rat optic nerve astrocytes in response to EGFR activation by the ligand EGF. To determine whether the synthesis of PGE2 by COX-2 is an intermediary in the morphologic changes, we treated astrocytes with NS398 during their exposure to EGF. Inhibition of the activity of COX-2 by NS398 did not block the EGF-induced change in shape of the astrocytes or the formation of the cribriform structures (Fig. 5). We also examined cell proliferation 3 days after EGF and inhibitor treatment. In EGF-treated cultures, there was a significant increase in the number of cells. However, there was no change in the number with EGF + NS398 compared with EGF alone (data not shown). These results demonstrate that the EGFR-mediated shape change or increase in cell numbers is not dependent on COX-2 activity.

COX-2 Expression in Optic Nerve Explant Culture

As a possible model for the study of responses of astrocytes to axonal injury, we transected rat optic nerves and observed them in vitro for up to 3 days. To establish the presence of COX-2 in explanted optic nerve tissue, extracts of optic nerve explants, cultured in ADM media for 1, 2, and 3 days were prepared for Western blot analyses. COX-2 was not detected in optic nerve extracts made fresh from the animal. However, COX-2 did appear within 24 hours in the explanted tissue and then was reduced to lower levels by 2 and 3 days of culture (Fig. 6A). During the 3 days of optic nerve explant culture, there was no obvious loss or death of astrocytes (data not shown). To explore further whether the COX-2 expression in this explanted culture system was dependent on the activity of EGFR, rat optic nerve explants were cultured for 1 day in the presence or absence of AG1478. Explants treated with 3 nM AG1478 exhibited a much lower level of COX-2 than the explants not treated with the inhibitor of EGFR tyrosine kinase.
COX-2 expression in rat optic nerve explant cultures. (A) Optic nerve explants were cultured for 1, 2, and 3 days in ADM. Total protein lysates from explants were prepared, and COX-2 immunoblot analyses were performed. (B) Optic nerve explants were cultured 24 hours in the absence or presence of AG1478 or NS398. COX-2 expression was determined by immunoblot analyses. (C) Immunohistochemistry for COX-2 and pEGFR in fresh optic nerve (control) and after 1 day in culture as explants. Arrows: COX-2- and pEGFR-positive immunostaining. RatONEX and RONEX: rat optic nerve explant; NB, nerve bundle. Original magnification, ×1000.

FIGURE 6. COX-2 expression in rat optic nerve explant cultures. (A) Optic nerve explants were cultured for 1, 2, and 3 days in ADM. Total protein lysates from explants were prepared, and COX-2 immunoblot analyses were performed. (B) Optic nerve explants were cultured 24 hours in the absence or presence of AG1478 or NS398. COX-2 expression was determined by immunoblot analyses. (C) Immunohistochemistry for COX-2 and pEGFR in fresh optic nerve (control) and after 1 day in culture as explants. Arrows: COX-2- and pEGFR-positive immunostaining. RatONEX and RONEX: rat optic nerve explant; NB, nerve bundle. Original magnification, ×1000.

There was also a slightly lower expression of COX-2 in the presence of NS398 treatment (Fig. 6B). These data suggest that in response to axonal injury, optic nerve astrocytes activate an EGFR pathway that causes the transient induction of COX-2.

By immunohistochemistry, neither the active form of EGFR (pEGFR) nor COX-2 was present in freshly excised optic nerve tissue, but both pEGFR and COX-2 were localized to astrocytes in rat optic nerve explants cultured for 1 day. pEGFR localization was throughout the astrocyte; whereas COX-2 localization was nuclear and perinuclear (Fig. 6C).

COX-2 Protein Expression and Localization in Optic Nerve after Axotomy

Optic nerve transection (axotomy) is widely used in experimental neuropathology, both to define the process of RGC degeneration on a molecular level and to establish therapeutic approaches to the neuroprotection and regeneration of injured RGCs. We explored the involvement of COX-2 in a rat model of optic nerve axotomy. After axotomy, COX-2 was significantly induced and reached a maximum at 1 day in the rat optic nerve. COX-2 was not present in normal, nontransected optic nerves (Fig. 7A). Immunohistochemical localization for COX-2 in the optic nerve 1 day after axotomy indicated that the increased levels of immunoreactive COX-2 protein were localized to the nucleus and cytoplasm of astrocytes (Fig. 7B). Two days after axotomy, only a very weak staining of COX-2 expression was observed in the optic nerve, which was consistent with COX-2 immunoblot data (data not shown). These findings suggest that there is a link between the transient induction of COX-2 and the reactive astrocytes in the optic nerve after injury. Experiments in which AG1478 was administered orally before and after optic nerve transection did not result in blocking the induction of COX-2 (data not shown). Whether oral administration of AG1478 can reach therapeutic concentrations in the optic nerve is unknown.

DISCUSSION

In the current study, activation of EGFR caused the rapid and transient induction of COX-2 in rat optic nerve head astrocytes. EGF stimulation caused induction of COX-2 protein, as shown by immunoblot analysis, and induction of COX-2 was abolished by the EGFR tyrosine kinase inhibitor AG1478. 33 When COX-2 is induced in response to activation of EGFR, astrocytes produce and release the proinflammatory mediator PGE2 in a time-dependent manner. The stimulatory action of EGF on PGE2 release was abolished by the COX-2 selective inhibitor NS398, 34 demonstrating that EGF-mediated PGE2 release is a consequence of COX-2 activity. Our findings were consistent in optic nerve astrocytes in vitro, optic nerve explant cultures and after optic nerve transection in vivo. We note that, although we used EGF as the ligand to activate EGFR in vitro, we do not know whether EGFR activation in vivo is due to another EGF ligand or to transactivation.

The expression of COX-2 and the synthesis of PGE2 in response to activation of EGFR in optic nerve astrocytes is a novel finding. The importance of this finding may be related to the appearance of EGFR in optic nerve astrocytes in glaucomatous optic neuropathy. In optic nerve astrocytes, activation of EGFR also leads to the induction of iNOS, which is implicated in the destruction of optic nerve axons. EGFR appears in astrocytes in other regions of the CNS after neural injury and may cause a similar induction of COX-2, particularly in white matter tracts.

The appearance and subsequent activation of EGFR in adult astrocytes after neural injury may allow this membrane-bound initiator of intracellular signaling pathways to alter the phenotype of the quiescent astrocyte. After injury to the CNS, as a result of trauma, genetic disorders, or chemical insults, quiescent astrocytes become reactive astrocytes and respond in a stereotypical manner termed astrogliosis, 39 which is characterized by astrocyte proliferation and functional changes. 38 Activation of EGFR affects many cell processes in astrocytes. Ligands of EGFR stimulate proliferation and differentiation of astrocytes, 39-41 cause a shape change and extended astrocytic process formation 32 and enhance the mobility of astrocytes in vitro. 41 Activation of EGFR causes astrocytes to express iNOS, 27 the glutamate aspartate transporter (GLAST), 40 and the interleukin-1 receptor. 41 We now add to this list the EGFR-dependent induction of COX-2. The signaling of such a variety of responses by activation of EGFR in astrocytes after neural injury may phenotypically change a quiescent astrocyte into what is referred to in the literature as a "reactive astrocyte." 36

By immunocytochemistry we showed that COX-2 is induced rapidly in the nuclear and perinuclear regions of astrocytes after treatment with EGF. COX-2 immunoreactivity and histofluorescence have been detected in a nuclear distribution. 42-43 The precise role of nuclear COX-2 is uncertain but may be related to regulation of the expression of certain genes. Nevertheless, our data provide evidence that EGF-induced nu-
clear COX-2 is capable of synthesis and extracellular release of PGE2 on astrocyte activation.

In this study, we found that induction of COX-2 and synthesis of PGE2 are not necessary for the change in cell shape and number in astrocytes activated by EGF. The selective COX-2 inhibitor NS398 failed to attenuate EGF-induced astrocyte differentiation into stellar shaped cells.

Our laboratory has shown that COX-2 is induced early in the rat retina after retinal ischemia and that hematogenous cells containing iNOS invade the retina and cause neurodegeneration. PGE2, synthesized by induced COX-2 in the retina is apparently chemotactic and causes the hematogenous cells to invade the retina and destroy the tissue. The induction of COX-2 in optic nerve astrocytes, and the subsequent synthesis of PGE2, may be responsible for the invasion of hematogenous cells into the optic nerve after transection.

In summary, in optic nerve astrocytes after injury, the activation of EGFR increases COX-2 expression and PGE2 synthesis and release. Preventing induction of COX-2 by inhibiting activation of EGFR may alter secondary events in acute neurodegeneration in the optic nerve and perhaps the CNS. The present study demonstrates a novel regulatory system, EGFR-COX-2/PGE2, of astrocyte functions which may contribute to secondary damage in neurodegenerations.

Acknowledgments

The authors thank Smita Vora and Belinda K. McMahan for technical assistance.

References


