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Prevention of seizure-induced up-regulation of endothelial P-glycoprotein by COX-2 inhibition

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ABSTRACT

In the epileptic brain, seizure activity induces expression of the blood–brain barrier efflux transporter, P-glycoprotein, thereby limiting brain penetration and therapeutic efficacy of antiepileptic drugs. We recently provided the first evidence that seizures drive P-glycoprotein induction through a pathway that involves glutamate-signaling through the NMDA receptor and cyclooxygenase-2 (COX-2). Based on these data, we hypothesized that selective inhibition of COX-2 could prevent seizure-induced P-glycoprotein up-regulation. In the present study, we found that the highly selective COX-2 inhibitors, NS-398 and indomethacin heptyl ester, blocked the glutamate-induced increase in P-glycoprotein expression and transport function in isolated rat brain capillaries. Importantly, consistent with this, the COX-2 inhibitor, celecoxib, blocked seizure-induced up-regulation of P-glycoprotein expression in brain capillaries of rats in vivo. To explore further the role of COX-2 in signaling P-glycoprotein induction, we analyzed COX-2 protein expression in capillary endothelial cells in brain sections from rats that had undergone pilocarpine-induced seizures and in isolated capillaries exposed to glutamate and found no change from control levels. However, in isolated rat brain capillaries, the COX-2 substrate, arachidonic acid, significantly increased P-glycoprotein transport activity and expression indicating that enhanced substrate flux to COX-2 rather than increased COX-2 expression drives P-glycoprotein up-regulation. Together, these results provide the first in vivo proof-of-principle that specific COX-2 inhibition may be used as a new therapeutic strategy to prevent seizure-induced P-glycoprotein up-regulation at the blood–brain barrier for improving pharmacotherapy of drug-resistant epilepsy.

1. Introduction

In up to 40% of epileptic patients seizure activity cannot be adequately controlled with antiepileptic drugs. In these patients, limited antiepileptic drug penetration across the blood–brain barrier into the CNS is considered to be one important contributor to therapeutic failure (Loscher and Potschka, 2005). On a molecular level, over-expression of blood–brain barrier drug efflux transporters occurs as a consequence of seizure activity, which has been associated with limited antiepileptic drug brain penetration. In this regard, recent studies have demonstrated that selective modulation of the major blood–brain barrier drug efflux transporter, P-glycoprotein, enhances brain uptake of antiepileptic drugs and improves anticonvulsant response (Brandt et al., 2006; Clinckers et al., 2005; van Vliet et al., 2006). These findings point to a specific role of seizure-induced P-glycoprotein over-expression as a limiting factor in epilepsy pharmacotherapy. Thus, elucidation of the mechanistic links that connect seizure activity to increased P-glycoprotein expression holds the promise to identify new therapeutic targets for preventing seizure-induced transporter over-expression and improving antiepileptic drug therapy.

We recently demonstrated that blood–brain barrier P-glycoprotein is up-regulated in response to exposure to glutamate, a neurotransmitter released during epileptic seizures. Experiments in isolated rat brain capillaries showed that extracellular glutamate at concentrations similar to those found in seizures signals through the NMDA receptor and cyclooxygenase-2 (COX-2) to increase P-glycoprotein (Bauer et al., 2008). This effect was blocked by the COX-2 inhibitor, celecoxib; no such up-regulation occurred in brain capillaries from COX-2 null mice. Importantly, in an in vivo

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experiment using the pilocarpine-induced status epilepticus rat model, the non-selective COX inhibitor, indomethacin, blocked seizure-induced up-regulation of P-glycoprotein in brain capillaries (Bauer et al., 2008).

The present study addresses two issues related to COX-2 regulation of P-glycoprotein expression at the blood–brain barrier. First, we extend our previous findings by demonstrating blockade by specific COX-2 inhibitors of glutamate-induction of P-glycoprotein expression and transport activity in isolated rat brain capillaries (NS-398 and indomethacin heptyl ester) and of seizure-induced induction of P-glycoprotein expression in rat brain capillaries in vivo (celecoxib). Second, we show that neither glutamate exposure of isolated brain capillaries ex vivo nor seizures in vivo increased endothelial expression of COX-2 protein, but we demonstrate that P-glycoprotein expression and transport function in brain capillaries were increased by the COX-2 substrate, arachidonic acid.

2. Materials and methods

2.1. Chemicals

Glutamate, pilocarpine, and arachidonic acid were purchased from Sigma (St. Louis, MO). Celecoxib was from LKT Laboratories (St. Paul, MN), NS-398 was from Cayman Chemicals (Ann Arbor, MI), and indomethacin heptyl ester was from Calbiochem (San Diego, CA). For the in vivo studies, celecoxib was used as Celebrex® (Pfizer, Karlsruhe, Germany). Mouse monoclonal C219 antibody to P-glycoprotein for Western blotting and brain capillary immunochemistry was purchased from Signet Laboratories (Dedham, MA), goat polyclonal antibody Mdr C-19 used for P-glycoprotein staining of brain sections was from Santa Cruz Biotechnology (Heidelberg, Germany), mouse monoclonal β-actin antibody was from Abcam (Cambridge, MA), rabbit polyclonal COX-2 antibody was from Cayman Chemical (Ann Arbor, MI) and goat polyclonal COX-2 antibody from Santa Cruz Biotechnology (Heidelberg, Germany). NBD-CSA was custom-synthesized by R. Wenger (Basel, CH) (Schramm et al., 1995). PSC833 was a kind gift from Novartis (Basel, CH). All other chemicals were of highest analytical grade and obtained from commercial sources.

2.2. Animals

For ex vivo brain capillary experiments 50 male retired breeder Sprague–Dawley rats were used (500–600 g, Taconic, Germantown, NY); for the in vivo study 75 female Wistar Unilever rats (180–200 g, Harlan-Winkelmann, Borchen, Germany, and Harlan Netherlands, Horst, The Netherlands) were used. Animals were kept under controlled environmental conditions (24–25 °C, 50–60% humidity, 12-h dark/light cycle) with free access to tap water and standard feed. Before experiments animals were allowed to adapt to the new environment for at least 1 week. All animal protocols were approved by the Institutional Animal Care and Use Committees of the University of Minnesota, The University of Munich, and NIEHS/NIH and were in compliance with the European Communities Council Directive, 86/609/EEC, the German and USDA Animal Welfare Act, and NIEHS/NIH guidelines.

2.3. Induction of status epilepticus with pilocarpine

To study the role of COX-2 in seizure-induced P-glycoprotein up-regulation we used the lithium–pilocarpine status epilepticus model. It has been reported by us and others that this model is suitable to induce a reproducible and robust increase in P-glycoprotein expression at the blood–brain barrier (Bankstahl and Loscher, in press; Bauer et al., 2008). For in vivo COX-2 inhibition, female Wistar Unilever rats received in total seven i.p. injections in 12-h intervals of either vehicle (10% DMSO) or celecoxib (Celebrex®, Pfizer, Karlsruhe, Germany; 20 mg/kg i.p.). The celecoxib dose was chosen based on previous studies reporting anti-inflammatory and neuroprotective effects in the CNS (Chu et al., 2004; Sinn et al., 2007).

To induce a status epilepticus, lithium chloride (127 mg/kg i.p., Sigma, Taufkirchen, Germany) was administered 14 h before pilocarpine and methyl-scopolamine (1 mg/kg i.p., Sigma; Taufkirchen, Germany) was administered 30 min before pilocarpine dosing. As described previously (Glien et al., 2001), pilocarpine (Sigma, Taufkirchen, Germany) was given by i.p. injection (10 mg/kg) every 30 min until the onset of ongoing generalized convulsive seizures (status epilepticus). The total number of pilocarpine injections was limited to 12 per animal; seizure activity was monitored continuously. Control rats received saline injections instead of pilocarpine and methyl-scopolamine. Seizures in pilocarpine-treated rats were terminated after 90 min by i.p. injection of diazepam (10 mg/kg) and repeated after 3 min if seizure activity continued. Only rats displaying continuous convulsive seizure activity during status epilepticus were used for further analysis.

2.4. Tissue preparation

Two days following status epilepticus, rats were decapitated. Brains were immediately removed, embedded in Tissue Freezing Medium® (Jung, Nussloch, Germany), frozen in liquid nitrogen, and stored at −80 °C. Brain tissue was cut in 14 μm slices using a cryostat (HM 560; Microm, Walldorf, Germany) and sections were mounted on HistoBond® adhesion slides (Marienfeld, Lauda-Koenigshofen, Germany).

2.5. P-glycoprotein immunohistochemistry and P-glycoprotein/COX-2 double-labeling in brain sections and isolated brain capillaries

P-glycoprotein in brain sections was stained as described previously (Bauer et al., 2008; Volk et al., 2004b) using Mdr C-19 polyclonal goat antibody (1:100). Brain sections were processed simultaneously to obtain comparable staining intensity. For P-glycoprotein/COX-2 double-labeling, sections were first washed with 0.05 M Tris-buffered saline (TBS, pH 7.6), transferred to 10 mM citrate buffer (pH 9) and boiled for 30 min at 95 °C. Sections were washed with TBS, incubated for 1 h in blocking solution containing 2% bovine serum albumin, 0.3% Triton X-100, and 5% normal donkey serum (Jackson Immunoresearch Laboratories, West Grove, PA, USA), and transferred into primary antiserum (mouse anti-P-glycoprotein (C219), 1:100, Calbiochem, Darmstadt, Germany; goat anti-COX-2, 1:50, Santa Cruz Biotechnology, Heidelberg, Germany) and incubated overnight at 4 °C. The next day, sections were washed with TBS, incubated for 1.5 h in secondary antiserum (1:500 cyanin-2 conjugated donkey anti-mouse; 1:500 biotinylated donkey anti-goat; both from Jackson Immunoresearch Laboratories, West Grove, PA, USA), rinsed in TBS and incubated for 1.5 h in horseradish peroxidase-conjugated streptavidin (1:375, Dako Cygomatics, Hamburg, Germany). After washing with TBS, the nickel-intensified diaminobenzidine (DAB) reaction (0.05% 3,3-diaminobenzidine, 0.01%, nickel ammonium sulphate; both from Sigma, Taufkirchen, Germany, and 0.01% H_2O_2) was performed. Finally, all brain sections were washed, air dried, dehydrated, and coverslipped with Entellan (Merck, Darmstadt, Germany).

Immunostaining of isolated rat brain capillaries was performed as reported previously (Bauer et al., 2008). Freshly isolated rat brain capillaries adhering to glass coverslips were fixed for 15 min with 3% paraformaldehyde/0.2% glutaraldehyde at room temperature. Capillaries were washed with PBS, permeabilized with 0.1% Triton X-100 for 30 min, washed again, and blocked with 1% BSA. Capillaries were incubated overnight at 4 °C with mouse C219 antibody to P-glycoprotein (1:25, Signet Laboratories, Dedham, MA) and rabbit polyclonal COX-2 antibody (1:250, Cayman Chemical, Ann Arbor, MI). After washing (PBS and 1% BSA), capillaries were incubated for 1 h at 37 °C with the corresponding Alexa Fluor 488- or 568-conjugated secondary IgG (all 1:1000; Molecular Probes, OR, USA). Immunofluorescence was visualized by confocal microscopy (Nikon C1 LSC microscope unit, Nikon TE2000 inverted microscope, 40× oil immersion objective, numerical aperture: 1.3, 488 nm line of an argon laser, 543 nm line of a HeNe laser). Digital confocal images were processed using ImageJ 1.41 software.

2.6. Histological evaluation and image analysis

P-glycoprotein staining of brain sections was analyzed using a computer-assisted image analysis system as described previously (Bauer et al., 2008; Volk et al., 2004b). The hardware consisted of an Olympus BH2 microscope with a Plan-Neofluar objective (Zeiss, Göttingen, Germany), a CCD color camera (Axiocam; Zeiss, Göttingen, Germany), and an AMD Athlon™ 64 processor-based computer with an image capture interface card (Axiocam MR Interface Rev. A; Zeiss, Göttingen, Germany). Brain sections were analyzed at a 400× magnification. Captured images were 1300 × 1030 pixels in dimension and were processed using KS400 image analysis software (Windows Release 3.0; Carl Zeiss Vision, Halbergmoos Germany).

Detailed image analysis methodology has been previously published (Volk et al., 2004a,b). Briefly, prior to image analysis, a spatial calibration was performed and a signal threshold value was defined to exclude background signals. This signal threshold value was used for analysis of all sections within the same experiment. Thus, data reported reflect pixel density above the threshold. P-glycoprotein immunostaining was analyzed in the hilus and the dentate gyrus of the hippocampus and in the parietal cortex. The area labeled for P-glycoprotein was evaluated using 3–10 fields of 43,434 μm^2 per subfield.

Endothelial COX-2 expression at various time points following status epilepticus was semi-quantitatively assessed by a grading system: score 0, no obvious endothelial COX-2 expression; score 1, only single COX-2 positive capillaries per field of view; score 2, few COX-2 positive capillaries; score 3, various COX-2 positive endothelial cells. In all experiments, image analysis was done in an observer-blinded fashion.

2.7. Brain capillary isolation

Rat brain capillaries were isolated according to Bauer et al. (2008). Animals were euthanized with CO_2, decapitated, and brains were collected in cold PBS (2.7 mM KCl, 1.46 mM KH_2PO4, 136.9 mM NaCl, 8.1 mM Na_2HPO_4, supplemented with 5 mM D-glucose and 1 mM Na-pyruvate, pH 7.4). Brains were cleaned from meninges,

choroid plexus and white matter, and brain cortex was homogenized in PBS. The brain homogenate was mixed with Ficoll® (final concentration 15%, Sigma, St. Louis, MO) and centrifuged at 5800g for 20 min at 4 °C. The resulting capillary pellet was suspended in 1% BSA-PBS and the capillary suspension was passed over a glass bead column. Capillaries were collected by gentle agitation in 1% BSA-PBS and washed with PBS. Freshly isolated brain capillaries were exposed to glutamate for 30 min, washed, and let incubate in buffer for 5½ h, or they were exposed to arachidonic acid for 6 h. After a total incubation time of 6 h, capillaries were used for transport experiments, or plasma membrane isolation.

2.8. P-glycoprotein transport activity

P-glycoprotein transport activity in isolated brain capillaries was assessed as described previously (Bauer et al., 2007; Hartz et al., 2004, 2006). Capillaries were incubated in confocal imaging chambers for 1 h at room temperature with 2 μM of the fluorescent P-glycoprotein substrate, NBD-cyclosporin A (NBD-CSA). For each treatment group, images of 10–15 capillaries were acquired using confocal microscopy (Zeiss LSM 510 META inverted confocal microscope, 40× water immersion objective, NA = 1.2; Zeiss LSM 410 inverted confocal microscope, 40× water immersion objective, NA = 1.2). NBD-CSA fluorescence intensity in capillary lumens was quantitated using Zeiss Image Examiner software or Scion Image software. Specific luminal NBD-CSA fluorescence was taken as the difference between total luminal fluorescence and fluorescence in the presence of the P-glycoprotein-specific inhibitor, PSC833. This difference provides a measure of specific P-glycoprotein transport activity in the capillaries (Bauer et al., 2008; Hartz et al., 2008).

2.9. Capillary membrane isolation and Western blotting

Brain capillaries were homogenized in lysis buffer (Sigma, St. Louis, MO) containing protease inhibitor (Roche, Mannheim, FRG). Lysed samples were centrifuged at 10,000g for 15 min and denucleated supernatants were subsequently centrifuged at 100,000g for 90 min. Crude plasma membrane pellets were resuspended and samples were stored at −80 until use. Western blots were performed using the Invitrogen NuPage™ electrophoresis and blotting system (Invitrogen, Carlsbad, CA). Membranes were incubated overnight with antibody to P-glycoprotein (1:100, 1 μg/ml), β-actin (1:1000, 1 μg/ml), or COX-2 (1:1000, 0.5 μg/ml). Blotting membranes were washed and incubated with the corresponding horseradish peroxidase-conjugated ImmunoPure® secondary antibody (1:15,000, Pierce, Rockford, IL). Proteins were detected using SuperSignal® West Pico Chemoluminescent Substrate (Pierce, Rockford, IL) and visualized with a BioRad Gel Doc™ XRS imaging system (BioRad, Hercules, CA).

2.10. Statistical analysis

Data are expressed as mean ± SEM. For ex vivo capillary experiments, statistical differences were analyzed by Student's *t*-test. Due to Gaussian distribution of data as tested with the Kolmogorow–Smirnov test, statistical differences between controls and treated groups of in vivo experiments were also analyzed using Student's *t*-test. Scored data of endothelial COX-2 expression in brain sections were analyzed using the Mann–Whitney *U*-test. Differences between means were considered to be statistically significant when $P < 0.05$.

3. Results

3.1. Status epilepticus model

Ninety-four percent of celecoxib-treated animals ($n = 15$ out of 16) developed a status epilepticus in response to repeated injections of the cholinomimetic, pilocarpine. In the vehicle-treated controls a status epilepticus was successfully induced in 83% of the animals ($n = 20$ out of 24). No significant difference was observed in the amount of pilocarpine required to induce a status epilepticus. In celecoxib-treated rats a mean pilocarpine dosage of 35 ± 5 mg/kg (mean ± SEM) was administered prior to onset of status epilepticus. Vehicle-treated rats required a mean dosage of 34 ± 1 mg/kg pilocarpine (mean ± SEM). Importantly, the severity of the status epilepticus in celecoxib-treated animals was not different from vehicle-treated animals. Once a second generalized seizure was observed, rats exhibited ongoing generalized seizure activity until administration of diazepam. Comparison of the mortality rate during status epilepticus revealed no significant differences between both groups (Fisher exact, $P = 0.3401$). For the study of endothelial COX-2 expression, additional 28 animals were used. A convulsive status epilepticus was successfully induced in 92% ($n = 22$ out of 24) of the rats.

3.2. Specific COX-2 inhibitors block P-glycoprotein up-regulation in brain capillaries ex vivo and in vivo

We recently described a protocol in which ex vivo isolated brain capillaries from rats were exposed for 30 min to 100 μM glutamate to mimic seizure conditions in vivo. Using this protocol, we demonstrated a doubling of P-glycoprotein expression and transport activity in brain capillary membranes 6 h after glutamate exposure (Bauer et al., 2008). Here we tested the effect of the highly selective COX-2 inhibitors, NS-398 (COX-2 IC50 0.103 μM, COX-1 IC50 ≥ 16.8 μM) and indomethacin heptyl ester (COX-2 IC50 40 nM, COX-1 IC50 ≥ 66 μM), on P-glycoprotein in brain capillaries exposed to glutamate. Fig. 1 shows that in agreement with previous studies (Bauer et al., 2008), P-glycoprotein expression and transport activity were significantly increased in capillaries exposed to glutamate. These effects were fully blocked by NS-398 and indomethacin heptyl ester (IHE), indicating COX-2 involvement in signaling P-glycoprotein up-regulation.

To confirm these results in vivo, we used immunohistochemistry to assess capillary P-glycoprotein expression in brains of rats treated with pilocarpine to induce status epilepticus. This animal model has previously been used to demonstrate seizure-induced P-glycoprotein up-regulation (Bankstahl and Loscher, in press; Bauer et al., 2008). In our experiments only animals that displayed continuous convulsive seizure activity during status epilepticus were used for histochemical P-glycoprotein analysis in brain sections. We observed P-glycoprotein immunostaining in brain microvessel endothelial cells of rats from all treatment groups (Fig. 2A–C). Note that such staining was not detectable in parenchymal brain cells, indicating that the DAB staining method used in the present experiments was specific for endothelial P-glycoprotein.

In animals that had experienced status epilepticus, P-glycoprotein immunolabeling was increased by 90–123% in brain capillary endothelial cells of the hippocampal hilus, the hippocampal granule cell layer, and in the cortex region (Fig. 2B, D–F). Importantly, treatment with the COX-2 inhibitor, celecoxib, prevented seizure-induced P-glycoprotein expression increases in all three brain regions. In celecoxib-dosed rats, P-glycoprotein expression levels were no different from controls (Fig. 2C–F). These findings suggest a crucial role for COX-2 in seizure-induced P-glycoprotein up-regulation.

3.3. P-glycoprotein up-regulation does not involve increased COX-2 expression

Based on our finding that COX-2 inhibitors blocked P-glycoprotein induction caused by seizures, we studied the role of COX-2 in more detail by looking at COX-2 protein expression in brain capillaries. COX-2 immunostaining was detected in brain parenchyma cells and in endothelial cells of the hippocampus and the cortex from rats of all treatment groups (data not shown). To confirm COX-2 expression in endothelial cells we isolated rat brain capillaries for immunostaining COX-2 and P-glycoprotein. As expected, Fig. 3A shows a distinct P-glycoprotein staining (green, left image) in the luminal membrane. COX-2 staining (red, middle image) was diffuse and extended over endothelial cytoplasm and the capillary luminal membrane. Overlaying green P-glycoprotein staining and red COX-2 staining resulted in a merged image that shows substantial co-localization (yellow, right image) of both proteins in the luminal plasma membrane. Note that while COX-2 and P-glycoprotein co-localization was limited to the luminal

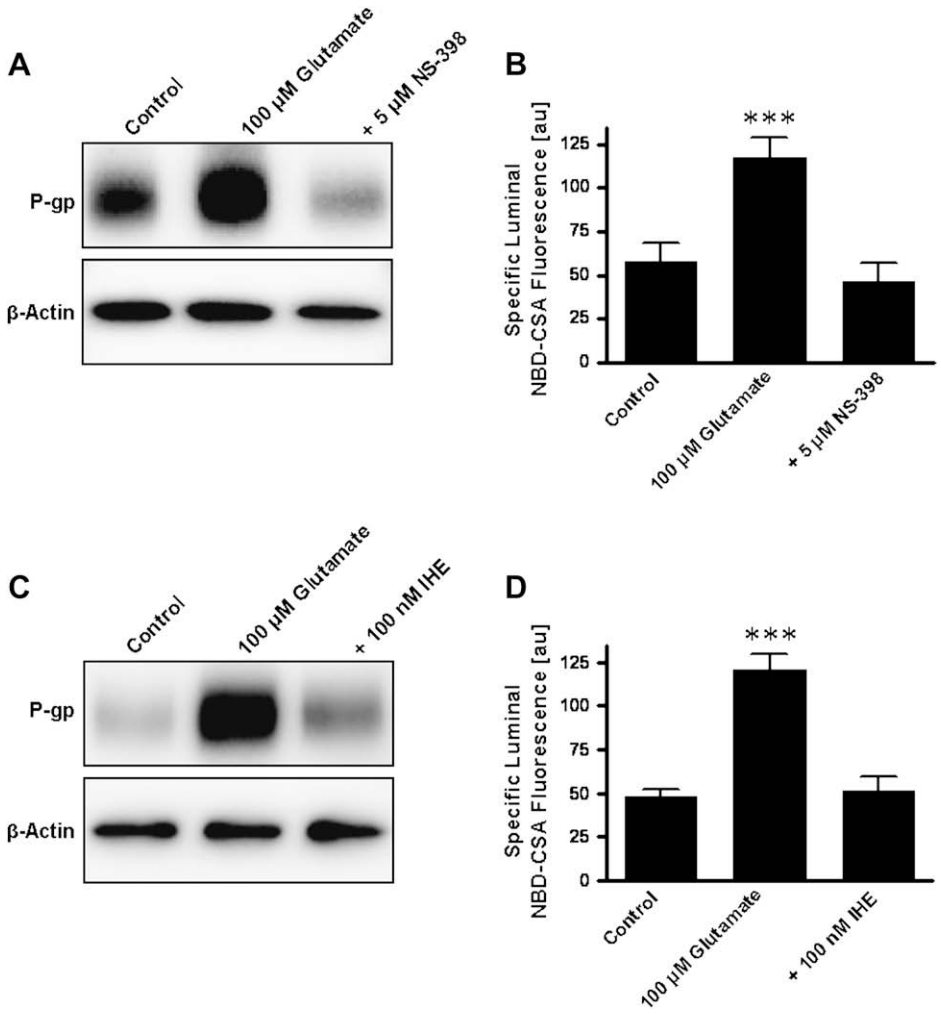

Fig. 1. Blocking COX-2 with NS-398 and indomethacin heptyl ester abolished up-regulation of P-glycoprotein in brain capillaries. (A) Western blot showing that the glutamate-induced increase of P-glycoprotein expression was blocked by NS-398, a selective COX-2 inhibitor. β-Actin was used as protein loading control. (B) NS-398 also blocked the glutamate-induced increase of P-glycoprotein transport activity (shown as a function of specific luminal NBD-CSA fluorescence). (C and D) Indomethacin heptyl ester, another COX-2-selective inhibitor, also blocked the effect of glutamate on P-glycoprotein expression and transport function. For specific luminal NBD-CSA fluorescence, data represent the mean value for 10–15 capillaries from a single preparation (pooled tissue from 10 rats); variability is given by SEM bars. Units are arbitrary fluorescence units (scale 0–255). Statistical comparison: ***significantly higher than controls, $P < 0.001$.

membrane, COX-2 staining was also present in the endothelial cytoplasm.

In addition, we quantitated COX-2 expression in brain sections from animals that experienced status epilepticus. While neuronal COX-2 expression was increased in the dentate gyrus and in the piriform cortex (data not shown), which has been described previously by other groups, endothelial COX-2 expression was not affected by status epilepticus at any of the time points investigated (Table 1). We confirmed this finding using isolated rat brain capillaries that were exposed to 50 or 100 μM glutamate. Under these conditions P-glycoprotein was up-regulated (Fig. 1 and Bauer et al., 2008), however, COX-2 expression did not increase (Fig. 4A).

Based on these findings, we hypothesized that increased flux of the COX-2 substrate, arachidonic acid, could drive up-regulation of blood–brain barrier P-glycoprotein in response to seizures. Fig. 4B and C show that exposing brain capillaries to arachidonic acid increased P-glycoprotein expression and transport function in a concentration-dependent manner. These data suggest that arachidonic acid flux to COX-2 resulting in increased enzyme activity, rather than increased COX-2 expression, drives P-glycoprotein induction in seizures.

4. Discussion

We recently demonstrated that glutamate-signaling through the NMDA receptor and COX-2 increased protein expression and transport activity of the drug efflux pump, P-glycoprotein, in isolated rat brain capillaries (Bauer et al., 2008). Using a rat seizure model we also showed that using the non-specific inhibitor, indomethacin, to block COX abolished P-glycoprotein up-regulation in brain endothelial cells in vivo. Since seizures cause excessive glutamate release (Barnes and Slevin, 2003), these findings for the first time linked seizure activity to increased endothelial P-glycoprotein expression and function at the blood–brain barrier. Our findings also pointed to COX-2 as a potential therapeutic target to prevent P-glycoprotein over-expression, which could be of high clinical relevance in treating drug-resistant epilepsy.

In the present study we continued to focus on COX-2 as a potential target and tested the hypothesis that specific COX-2 inhibition prevents seizure-induced P-glycoprotein up-regulation at the blood–brain barrier. In a first set of experiments we found that the highly selective COX-2 inhibitors, NS-398 and indomethacin heptyl ester, both blocked glutamate-induced increases of

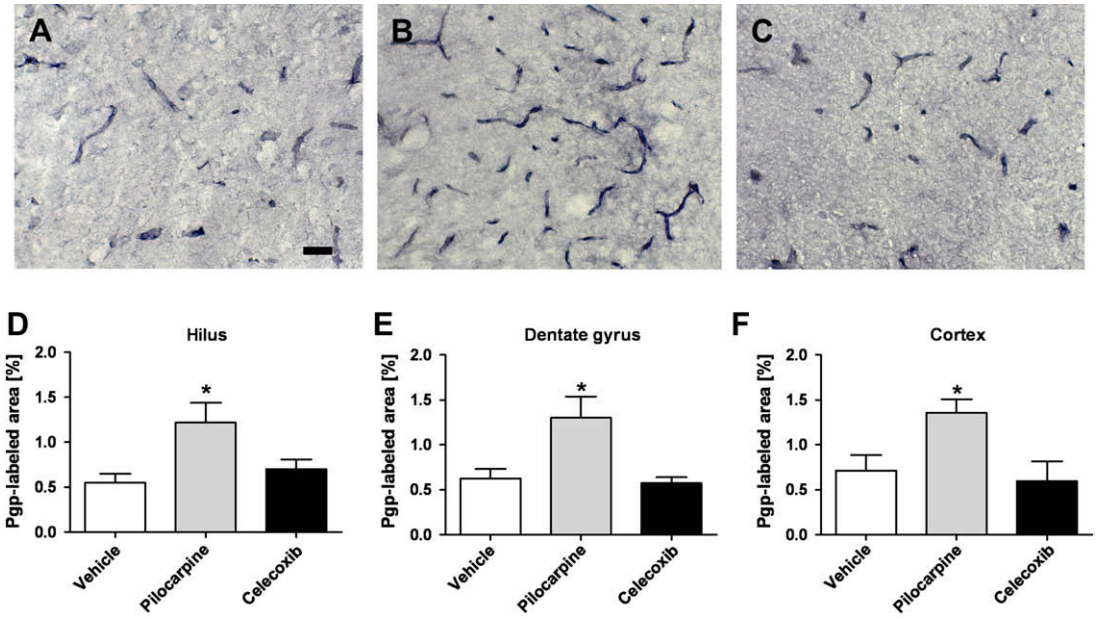

Fig. 2. Celecoxib prevents P-glycoprotein up-regulation in a status epilepticus animal model in vivo. (A) Immunostaining of P-glycoprotein in the brain cortex of a vehicle-treated rat. (B) P-glycoprotein immunostaining in a corresponding brain section from a rat after status epilepticus. Note the striking increase in endothelial P-glycoprotein expression. (C) P-glycoprotein immunostaining in the brain cortex of a celecoxib-treated rat after status epilepticus. Treatment with celecoxib prevented the status epilepticus-induced increase of capillary P-glycoprotein expression. Analysis of P-glycoprotein immunostaining in the hippocampal hilus (D), the hippocampal dentate gyrus (E), and the cortex region (F) in vehicle-treated control rats ($n = 7$), vehicle-treated rats following status epilepticus ($n = 9$) and celecoxib-treated rats following status epilepticus ($n = 7$). Computer-assisted digital image analysis revealed that seizures induced an increase of capillary P-glycoprotein expression in all three brain regions. Note that celecoxib treatment prevented seizure-mediated induction of capillary P-glycoprotein. Data are given as mean ± SEM. Statistical comparisons: *significantly higher than controls, $P < 0.05$. (A–C) Scale bar = 100 μm.

P-glycoprotein expression and transport activity in isolated rat brain capillaries ex vivo. In a second experiment using a status epilepticus rat model we demonstrated that selective COX-2 inhibition with celecoxib prevented seizure-induced up-regulation of brain endothelial P-glycoprotein. It is noteworthy that with both ex vivo and in vivo COX-2 inhibition P-glycoprotein expression and function was maintained at control levels, suggesting that COX-2 does not modulate basal P-glycoprotein expression. Thus, our data strongly support the hypothesis that seizure-induced up-regulation of blood–brain barrier P-glycoprotein can be prevented by selective COX-2 inhibition.

Three points of the study require further discussion. First, the importance of P-glycoprotein up-regulation in antiepileptic drug-resistance is still a matter of debate. At issue here is the extent to which P-glycoprotein handles antiepileptic drugs. However, numerous studies using rodent epilepsy models and epileptic tissue from pharmacoresistant patients have shown a strong correlation between seizure-induced P-glycoprotein over-expression and resistance to antiepileptic drugs (Loscher and Potschka, 2005). This is in accord with a recent PET study in pharmacoresistant epilepsy patients that indicated increased P-glycoprotein transport function in epileptic brain regions (Langer et al., 2007). Moreover, studies in animal models of pharmacoresistant epilepsy demonstrated that it is possible to overcome drug-resistance by modulating P-glycoprotein transport function (Brandt et al., 2006; Clinckers et al., 2005; van Vliet et al., 2006). Clearly, there is an increasing body of convincing evidence that several first line antiepileptic drugs are indeed P-glycoprotein substrates (Loscher and Potschka, 2005).

Second, targeting a component of the brain's inflammatory response, requires taking into consideration tolerability of COX-2 blockers. Most studies with epilepsy models have shown that non-selective COX-1/-2 inhibition as well as selective COX-2 inhibition is neuroprotective (Bauer et al., 2008). However, detrimental effects

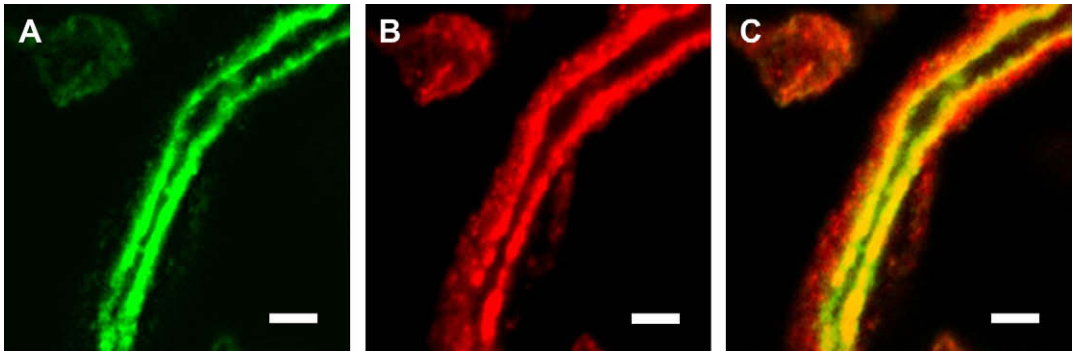

Fig. 3. Co-localization of P-glycoprotein and cyclooxygenase-2 in an isolated rat brain capillary. Representative image of a brain capillary immunostained for P-glycoprotein (green, A) and COX-2 (red, B). (A) P-glycoprotein immunostaining (green) shows transporter localization in the luminal membrane of a brain capillary. (B) COX-2 immunostaining is diffuse and extends over cytoplasm and the luminal membrane. (C) Overlay of A and B. The overlay shows a yellow merge at the capillary luminal membrane, suggesting substantial co-localization of P-glycoprotein and COX-2. Scale bar = 5 μm (for interpretation of colour in this figure, the reader is referred to the web version of this article).

Table 1
Endothelial COX-2 expression in vehicle-treated, control rats and rats 2, 4, and 8 h after status epilepticus.

Treatment	Piriform cortex				Parietal cortex			
	−2.3	−3.8	−5.8	Summary	−2.3	−3.8	−5.8	Summary
Vehicle	2.19 ± 0.45	2.06 ± 0.16	2.56 ± 0.28	2.27 ± 0.15	2.5 ± 0.29	0.75 ± 0.14	1.0 ± 0.20	1.42 ± 0.55
2 h	2.0 ± 1.0	0.88 ± 0.38	1.25 ± 0.25	1.38 ± 0.55	1.13 ± 0.88	1.0 ± 0.0	1.25 ± 0.50	1.13 ± 0.07
4 h	1.58 ± 0.30	1.69 ± 0.24	2.06 ± 0.06	1.78 ± 0.15	1.17 ± 0.44	1.94 ± 0.06	1.50 ± 0.20	1.54 ± 0.22
8 h	1.38 ± 0.38	1.75 ± 0.14	1.75 ± 0.32	1.63 ± 0.21	1.13 ± 0.52	1.88 ± 0.13	1.69 ± 0.24	1.57 ± 0.23

Endothelial COX-2 expression was scored in the piriform cortex and the parietal cortex. Analysis was performed in at least two sections that were located −2.3 mm, −3.8 mm, and −5.8 mm relative to bregma. The mean value of all sections is given in the summary. Data are given as mean ± SEM. In addition, COX-2 expression was studied in one rat 48 h after status epilepticus. In this rat, endothelial COX-2 expression was at control levels (data not shown).

including aggravation of seizures and neuronal loss have also been described (Baik et al., 1999). In this regard it is worth mentioning that in the present study seizure severity during status epilepticus was not affected by COX-2 inhibition. Nevertheless, there is an increased risk of renal, cardiovascular and cerebrovascular side effects using COX-2 inhibitors. Unwanted cardiovascular and cerebrovascular events were observed in long-term clinical trials testing selective COX-2 inhibitors, which eventually led to withdrawal of several COX-2 inhibitors from the market and resulted in a specific warning for celecoxib. Also, based on its high potential for side effects, celecoxib is excluded for use in patients who are at risk for vascular events. Thus, translating our strategy into the clinic would require careful patient selection and monitoring.

On the other hand, preventing up-regulation of P-glycoprotein transporter expression in the epileptic brain through COX-2 inhibition has the potential of improving brain penetration and efficacy of antiepileptic drugs that are P-glycoprotein substrates. This approach may allow achieving therapeutic brain levels with lower drug doses which would decrease potential adverse side effects. Such a strategy may also help to overcome pharmacoresistance in a certain subpopulation of patients not responding to treatment with antiepileptic drugs at all. Experiments in animal models are currently under way to determine whether this can be best done by targeting COX-2 or another signaling molecule in this pathway. In general, targeting signals that up-regulate transporter expression at the blood–brain barrier may have advantages over the use of specific inhibitors that directly target P-glycoprotein. As shown in previous ex vivo brain capillary experiments (Bauer et al., 2007), COX-2 inhibition leaves P-glycoprotein expression at basal levels without disrupting its protective function at the blood–brain barrier or other blood–tissue barriers as well as its clearing function in excretory organs.

Third, we analyzed COX-2 expression levels in brain sections from animals that experienced a status epilepticus. COX-2 expression was induced in the granule cell layer of the dentate gyrus and in the piriform cortex. Transcriptional activation of the gene encoding COX-2 has been reported to occur in a number of epilepsy models (Kawaguchi et al., 2005; Lee et al., 2007; Takemiya et al., 2006; Voutsinos-Porche et al., 2004) as well as in the human epileptic brain. COX-2 induction was observed in brain regions where seizures developed including the hippocampus, amygdala, and cortex. COX-2 also functions as a signaling molecule in brain capillary endothelial cells (Andras et al., 2007; Bauer et al., 2008; Mark et al., 2001; Sharp et al., 2003). Takemiya et al. (2006) reported induction of brain endothelial COX-2 expression 24 h after kainic acid injections into the hippocampus. However, the authors did not quantitate COX-2 expression. In the present study, pilocarpine-induced status epilepticus did not affect endothelial COX-2 expression at the time points we investigated. In accordance with these results, exposing isolated rat brain capillaries to glutamate also had no effect on COX-2 expression. However, incubation of isolated rat brain capillaries with the COX substrate, arachidonic acid, increased P-glycoprotein expression and transport function. From this we conclude that enhanced substrate flux to COX-2 leading to increased

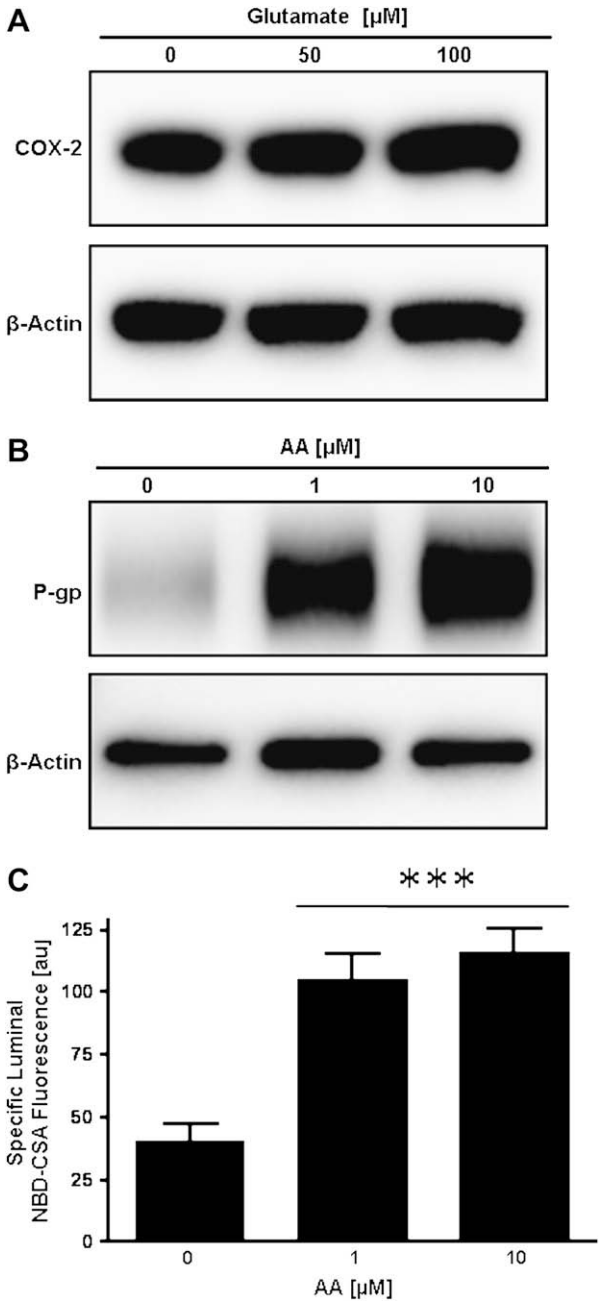

Fig. 4. (A) Western blot showing that COX-2 expression was not affected by exposing brain capillaries to glutamate. (B) Western blot showing that arachidonic acid increased P-glycoprotein expression (B) and P-glycoprotein transport function (C) in a concentration-dependent manner. For specific luminal NBD-CSA fluorescence, data represent the mean value for 10–15 capillaries from a single preparation (pooled tissue from 10 rats); variability is given by SEM bars. Units are arbitrary fluorescence units (scale 0–255). Statistical comparison: ***significantly higher than controls, $P < 0.001$.

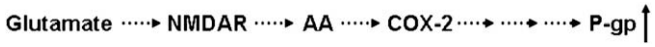

Fig. 5. Proposed signaling pathway that leads to seizure-induced P-glycoprotein up-regulation at the blood–brain barrier, based on the present results and our previously published study (Bauer et al., 2008).

COX-2 enzyme activity rather than increased COX-2 expression drives P-glycoprotein up-regulation in response to seizures.

Based on the data presented here and our previously published findings, we propose the signaling pathway shown in Fig. 5. In this pathway, glutamate released during an epileptic seizure activates the NMDA receptor leading to generation of arachidonic acid, activation of COX-2 and up-regulation of P-glycoprotein. In this regard, a series of publications has shown that NMDA receptor activation leads to release of arachidonic acid from the cell membrane (Chang et al., 2008; Lee et al., 2008; Taylor et al., 2008). This step is known to be mediated by phospholipase A2 in response to increased calcium influx via the NMDA receptor ion channel. Since arachidonic acid is the only substrate of COX-2, its release is always associated with increased COX-2 activity and generation of prostanoids such as prostaglandin E2 (PGE2) (Yang and Chen, 2008). Such signaling has been described in brain capillary endothelial cells, where arachidonic acid caused an increase in PGE2 (Yakubu and Leffler, 2005). The intermediate steps between COX-2 and P-glycoprotein are unknown at this point, but likely involve PGE2 signaling through EP receptors and activation of downstream effectors that initiate transcription of MDR1, the gene coding for P-glycoprotein. Future studies are needed to address in more detail COX-2 downstream signaling leading to up-regulation of P-glycoprotein in brain capillary endothelial cells. In addition, from our in vivo status epilepticus experiments we cannot rule out that COX-2 induction in glial cells or neurons affected signaling in endothelial cells, or whether or not such intercellular communication contributed to the celecoxib effects in endothelial cells. However, our experiments in isolated rat brain capillaries indicate that the described signaling pathway is independent from adjacent cells and that in endothelial cells COX-2 is exclusively involved in this particular pathway.

In conclusion, the present study provides first in vivo proof-of-principle that COX-2 inhibition could potentially be used as a new therapeutic strategy to prevent seizure-induced P-glycoprotein up-regulation at the blood–brain barrier. Future studies using a chronic drug-resistant rat model have to address whether this strategy can be used to overcome pharmacoresistance.

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