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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,