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Targeting Prostaglandin E_2 EP1 Receptors Prevents Seizure-Associated P-glycoprotein Up-Regulation

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ABSTRACT

Up-regulation of the blood-brain barrier efflux transporter P-glycoprotein in central nervous system disorders results in restricted brain access and limited efficacy of therapeutic drugs. In epilepsies, seizure activity strongly triggers expression of P-glycoprotein. Here, we identified the prostaglandin E2 receptor, EP1, as a key factor in the signaling pathway that mediates seizure-induced up-regulation of P-glycoprotein at the blood-brain barrier. In the rat pilocarpine model, status epilepticus significantly increased P-glycoprotein expression by 92 to 197% in the hippocampal hilus and granule cell layer as well as the piriform cortex. The EP1 receptor antagonist 8-chlorodibenz[*b*,*f*][1,4]oxazepine-10(11*H*)-carboxylic acid, 2-[1-oxo-3-(4-pyridinyl)propyl]hydrazide hydrochloride (SC-51089) abolished seizure-induced P-glycoprotein up-regulation and retained its expression at the control level. The control of P-glycoprotein expression despite prolonged seizure activity suggests that EP1 receptor antagonism will also improve antiepileptic drug efficacy. Preliminary evidence for this concept has been obtained using a massive kindling paradigm during which animals received a subchronic SC-51089 treatment. After withdrawal of the EP1 receptor antagonist, a low dose of the P-glycoprotein substrate phenobarbital resulted in an anticonvulsant effect in this pretreated group, whereas the same dosage of phenobarbital did not exert a significant effect in the respective control group. In conclusion, our data demonstrate that EP1 is a key signaling factor in the regulatory pathway that drives P-glycoprotein up-regulation during seizures. These findings suggest new intriguing possibilities to prevent and interrupt P-glycoprotein overexpression in epilepsy. Future studies are necessary to further evaluate the appropriateness of the strategy to enhance the efficacy of antiepileptic drugs.

Blood-brain barrier efflux transporters restrict the access of various central nervous system therapeutics to the target tissue (Löscher and Potschka, 2005). Whereas basal expression levels can already constitute a limiting factor, the situation worsens when pathophysiology-associated transcriptional activation of efflux transporters further strengthens the barrier function in different central nervous system diseases. In epilepsy, seizures strongly induce expression of the efflux transporter P-glycoprotein (Löscher and Potschka, 2005). This overexpression has been shown to correlate with reduced brain penetration of antiepileptic drugs and a reduction or even complete loss of pharmacosensitivity (Rizzi et al., 2002; van Vliet et al., 2007; Wen et al., 2008). Based on these data, P-glycoprotein overexpression is discussed as one factor accounting for the high rate of failure in epilepsy therapy with lack of satisfactory seizure control in up to 40% of patients (Kwan and Brodie, 2006).

An obvious strategy to overcome transporter-mediated pharmacoresistance is interference with efflux transport by competitive or noncompetitive transporter inhibitors. On an experimental level, add-on treatment with a P-glycoprotein inhibitor conclusively resulted in almost complete seizure control in rats with pharmacoresistance to the antiepileptic drug phenobarbital (Brandt et al., 2006). However, direct transporter inhibition also affects basal transporter function throughout the body, thereby limiting the protective function of efflux transport and increasing exposure of sensitive tissues to harmful xenobiotics (Fromm, 2004). Taking these caveats into consideration, targeting the regulatory path-

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ABBREVIATIONS: NMDA, *N*-methyl-D-aspartic acid; COX, cyclooxygenase; SC-51089, 8-chlorodibenz[*b*,*f*][1,4]oxazepine-10(11*H*)-carboxylic acid, 2-[1-oxo-3-(4-pyridinyl)propyl]hydrazide hydrochloride; NF-κB, nuclear factor-κB; SE, status epilepticus.

ways that drive efflux transporter overexpression during disease could be a highly promising alternative approach. Thus, elucidation of the mechanistic links that connect seizure activity to increased P-glycoprotein expression holds promise to identify new therapeutic targets for preventing seizure-induced transporter overexpression and for improving antiepileptic drug therapy. Recently, we reported that excessive glutamate release during epileptic seizures acts as an initial trigger of a signaling cascade in brain capillary endothelial cells that drives P-glycoprotein up-regulation (Bauer et al., 2008). We demonstrated that glutamate signaling through the NMDA receptor and cyclooxygenase (COX)-2 leads to P-glycoprotein overexpression in response to seizures (Bauer et al., 2008). It is important that inhibition of COX-2 abolished the glutamate effect on P-glycoprotein expression, suggesting that COX-2 inhibition could be used to improve delivery of antiepileptic drugs to the target sites in epileptogenic brain regions and to restore pharmacosensitivity. However, using this approach, one needs to bear in mind potential COX-2 inhibitor side effects, including increased risk for cardio- and cerebrovascular events. Therefore, it is of interest to identify downstream signaling proteins that might be alternative targets to prevent P-glycoprotein up-regulation in the epileptic brain.

Prostaglandin E_2 is the major product of COX-2 signaling in the brain. It acts on four different G protein-coupled receptors (EP1, EP2, EP3, and EP4), each of which has a very distinct signal transduction profile and often opposing cellular actions (Hata and Breyer, 2004). Whereas blocking EP2, EP3, and EP4 can aggravate neurodegeneration (Bilak et al., 2004; McCullough et al., 2004; Ahmad et al., 2005), blocking EP1 has neuroprotective effects (Suganami et al., 2003; Kawano et al., 2006). Thus, EP1 represents the most interesting target on which we focused our experimental efforts. Using rodent epilepsy models, we tested our hypothesis that EP1 receptors play a key role in signaling events that drive P-glycoprotein expression in the epileptic brain and that antagonism of EP1 receptors prevents P-glycoprotein induction.

Materials and Methods

Animals. Seventy-three female Wistar Unilever rats (200–220 g; Harlan-Winkelmann, Netherlands, Horst, The Netherlands) were used for the pilocarpine model. To study whether SC-51089 pretreatment increases the efficacy of phenobarbital on seizure thresholds by controlling P-glycoprotein expression, 54 male NMRI mice were used (Harlan-Winkelmann, Netherlands). 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 and the University of Munich and were in compliance with the European Communities Council Directive (86/609/EEC), the German and U.S. Department of Agriculture animal welfare acts, as well as guidelines by the German Research Foundation (Deutsche Forschungsgemeinschaft) and the National Institutes of Health (Bethesda, MD).

Induction of a Status Epilepticus by Pilocarpine. To study the role of the EP1 receptor in seizure-induced P-glycoprotein up-regulation, we used the lithium-pilocarpine status epilepticus model. Female Wistar Unilever rats received in total four intraperitoneal injections in 12-h intervals of either vehicle (aqua ad injectabilia) or SC-51089 (BIOMOL Research Laboratories, Plymouth Meeting, PA; 10 mg/kg i.p.) starting 30 min before the first pilocarpine injection. To induce a status epilepticus, lithium chloride (Sigma, Taufkirchen, Germany; 127 mg/kg i.p.) was administered 14 h and methyl-scopolamine (Sigma; 1 mg/kg i.p.) was administered 30 min before pilocarpine dosing. As described previously (Glien et al., 2001), pilocarpine (Sigma) was given by intraperitoneal 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 a maximum of 12 per animal. Seizure activity was continuously monitored after pilocarpine administration. Control rats received saline injections instead of pilocarpine and methylscopolamine. Seizures were terminated 90 min after onset of continuous generalized seizure activity with diazepam (10 mg/kg i.p.); diazepam dosing was repeated after 3 min if seizure activity continued. Only rats that displayed continuous convulsive seizure activity during status epilepticus were used for further analysis.

Tissue Preparation and P-glycoprotein Immunohistochemistry. Rats were decapitated 48 h after induction of status epilepticus. 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- and 40-μm serial slices using a cryostat (HM 560; Microm, Walldorf, Germany), and sections were mounted on HistoBond adhesion slides (Marienfeld, Lauda-Koenigshofen, Germany). Serial brain sections (40 μm) were used for Nissl staining with thionin to visualize neurodegeneration in the hippocampus. P-glycoprotein was stained in 14-μm brain sections by overnight incubation with C219 monoclonal mouse antibody (Calbiochem, Darmstadt, Germany; 1:100) at 4°C as described previously (Volk et al., 2004b; Bauer et al., 2008). The next day, brain tissue sections were washed and incubated for 1.5 h in antiserum containing biotinylated donkey anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA; 1:200). Sections were washed and subsequently incubated for 1.5 h in horseradish peroxidase-conjugated streptavidin (Dako Cygomatics, Hamburg, Germany; 1:375). Brain tissue sections were rinsed again before nickel-intensified diaminobenzidine staining [0.05% 3,3-diaminobenzidine, 0.01%, nickel ammonium sulfate (both from Sigma), and 0.01% H_2O_2 (Lukasiuk et al., 2006)]. Finally, brain sections were washed, air-dried, dehydrated, and coverslipped with Entellan (Merck, Darmstadt, Germany).

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; Pekcec et al., 2009). The hardware consisted of a BH2 microscope (Olympus , Tokyo, Japan) with a Plan-Neofluar objective (Carl Zeiss, Göttingen, Germany), a charge-coupled device color camera (Axiocam; Carl Zeiss), and an AMD Athlon 64 processor-based computer with an image capture interface card (Axiocam MR Interface Rev.A; Carl Zeiss). 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 published previously (Volk et al., 2004a,b). In brief, before image analysis, a spatial calibration was performed and a signal threshold value was defined to exclude background signals. Data reported reflect pixel density above this threshold, which was used to analyze all sections within the same experiment. P-glycoprotein immunostaining was analyzed in the hilus, dentate gyrus, and CA-3 region of the hippocampus, and the parietal and piriform cortex. The area labeled for P-glycoprotein was evaluated using either three fields (in the hilus) or 10 fields (dentate gyrus, CA-3 region, parietal, and piriform cortex) of 43,434 μm^2/subfield.

Neurodegeneration was evaluated in the following hippocampal subregions of Nissl-stained brain sections: CA1, CA2, CA3a, CA3c/CA4, and dentate gyrus. Neuronal damage was assessed by a previously described semiquantitative grading system (Pekcec et al.,

2008): score 0, no obvious damage; score 1, lesions involving 20 to 50% of neurons; and score 2, lesions involving >50% of neurons (note that neuronal loss must exceed 15–20% before it is reproducible by visual inspection; Fujikawa et al., 2000). Visual inspections were carried out in an observer-blinded manner.

Compared with the extent of cell loss occurring after an SE in CA3 and CA1, which can be easily scored by microscopic examination, loss of neurons in the dentate hilus is more difficult to assess without cell counting. Therefore, in a second step, polymorphic neurons (i.e., mossy cells and interneurons) were counted in the dentate hilus of the hippocampal formation. Neuronal loss in the hilus was quantified with the computer-assisted imaging system StereoInvestigator 6.0 (Microbrightfield Europe, Magdeburg, Germany). The hardware consists of a DMLB microscope (Leica, Bensheim, Germany), a Plan-Neofluar lens (Leica), a single chip charge-coupled device color camera (CX9000; Microbrightfield Europe), and an Athlon (tm) 64 processor (AMD, Sunnyvale, CA). An experimenter blinded to the treatment conditions traced the extent of the hippocampal hilus and performed the counting of cells by using the optical fractionator method. In slide-mounted serial sections, the area of the dentate hilus was traced and within each traced contour a step grid was placed. Counting frames were automatically and randomly placed along the grid. The thickness of the counting frame was equal to the thickness of the section (minus guard zones from the top and bottom of the section). Only cells that occurred within the counting frame and came into focus were counted. The number of counted cells was used to estimate the total number of hilar cells of the dentate hilus (West et al., 1991).

Electrode Implantation and Kindling Procedure in Mice. Mice were anesthetized with chlorohydrate (400 mg/kg i.p.) before stereotactic implantation of a Teflon-isolated bipolar stainless steel electrode into the right amygdala (stereotaxic coordinates in millimeters relative to bregma: AP, −1.0; L, −3.2; and DV, −5.3. One screw, placed above the left parietal cortex, served as the indifferent reference electrode. Anchor screws were fixed to the skull to secure all mounted material, and the basolateral amygdala electrode was embedded in dental acrylic cement. After surgery, the animals were allowed to recover for a period of at least 2 weeks.

Kindling of mice and determination of initial kindling after-discharge thresholds by using an ascending stair-step procedure were performed as described previously for rats (Potschka et al., 2004; Pekcec et al., 2007). In 1-min intervals, the initial current of 8 μA was increased in steps of approximately 20% of the previous current until after-discharges were elicited. Twenty-four hours later, kindling was started using an individual stimulation current that was 20% above the determined initial after-discharge threshold (1-ms, monophasic square-wave pulses; 50 Hz for 1 s). Electrical stimulation of the amygdala was performed once daily and five times per week. After each amygdala stimulation, seizure severity was scored according to the Racine scale (Racine, 1972); 1, immobility, eye closure, ear twitching, twitching of vibrissae, sniffing, facial clonus; 2, head nodding associated with more severe facial clonus; 3, clonus of one forelimb; 3.5, bilateral clonus without rearing; 4, bilateral clonus accompanied by rearing; 4.5, generalized clonic seizures without rearing and falling (e.g., because of direct loss of balance); and 5, rearing and falling accompanied by generalized clonic seizures. In addition to seizure severity, seizure duration and after-discharge duration were recorded after each amygdala stimulation. After 10 fully developed generalized seizures mice were considered to be fully kindled and kindling was discontinued.

Anticonvulsive Efficacy of Phenobarbital. Kindling induced seizures are known to increase P-glycoprotein expression at the blood-brain barrier (Volk et al., 2004b). To test whether prevention of seizure-induced P-glycoprotein up-regulation by EP1 receptor antagonism might enhance the anticonvulsant efficacy of an antiepileptic drug, we used a massive kindling protocol with frequent induction of kindled seizures to induce a pronounced endothelial P-glycoprotein expression. Therefore, fully kindled mice were stimulated five times a day with an interval of 1 h over nine consecutive days in total. In a subgroup of animals without treatment, we determined the degree of P-glycoprotein induction by massive kindling ($n = 10$) in comparison with animals that were fully kindled but were not elicited to a subsequent massive kindling paradigm ($n = 9$).

To test for the effect of SC-51089, the animals received repeated twice daily injections of the EP1 receptor antagonist SC-51089 (10 mg/kp i.p.; $n = 7$) or the respective vehicle solution (aqua ad injectabilia, 10 ml/kg i.p.; $n = 4$) during the stimulation paradigm (first to ninth experimental day). Injections were done 30 min before the first daily stimulation and directly after the fifth kindling stimulation of the day. To test whether this pretreatment with an EP1 receptor antagonist during a phase with repeated seizures is suitable to increase the efficacy of an anticonvulsive compound, the effect of the P-glycoprotein substrate phenobarbital was studied on subsequent days. On the 10th experimental day, all mice received an injection of vehicle solution (saline, 10 ml/kg i.p.) 30 min before evaluation of the generalized seizure threshold. Seizure thresholds were determined using the ascending stair-step procedure as described above. On the next day (11th experimental day), all mice were treated with a low dose of phenobarbital (6 mg/kg i.p.) 30 min before evaluation of seizure thresholds. The phenobarbital dose was selected based on pretests with different dosages of phenobarbital in kindled mice that had not received a pretreatment. The dosage was chosen because it did not exert an anticonvulsive effect on seizure thresholds in kindled mice and is thus suitable to evaluate increased efficacy by blood brain-barrier transporter modulation.

Although we evaluated phenobarbital efficacy after SC-51089 withdrawal with this protocol, we were keen to exclude any acute interaction of both drugs. All mice received phenobarbital (6 mg/kg i.p.) 30 min before evaluation of the generalized seizure thresholds. To test whether SC-51089 acutely enhances phenobarbital efficacy, the animals received an injection of SC-51089 (10 mg/kg i.p.; $n = 5$) or of the corresponding vehicle solution (aqua ad injectabilia; $n = 5$) immediately before phenobarbital administration. Threshold data obtained after drug administration were compared with data from a vehicle control experiment performed the day before. To prevent bias of the results by interindividual differences, this experiment was repeated after several weeks in the same animals in a crossover design.

Tissue Preparation and EP1 Western Blotting. The data obtained with the EP1 receptor antagonist in the epilepsy models raised the question whether the effects are due to an interference with signaling events in brain capillaries. Recent data demonstrated that glutamate/NMDA-receptor/COX-2 signaling in brain capillaries contributes to the transcriptional activation. The present in vivo data suggest EP1 receptors as downstream effectors in these endothelial signaling events. However, this hypothesis is based on the assumption that EP1 receptors are expressed in brain capillaries. To test for endothelial EP1 receptor expression, rat brain capillaries were isolated as described previously (Bauer et al., 2008; Zibell et al., 2009). Brain capillaries were homogenized in lysis buffer containing protease inhibitor; differential centrifugation was used to obtain crude plasma membranes from capillary lysates. Western blots of brain capillary membranes were performed using the NuPage electrophoresis and blotting system (Invitrogen, Carlsbad, CA). Blotted membranes were incubated overnight with EP1 antibody (Cayman Chemical, Ann Arbor, MI; 1:1000, 1 μg/ml). Blotting membranes were washed and incubated with the corresponding horseradish peroxidase-conjugated ImmunoPure secondary antibody (Pierce Chemical, Rockford, IL; 1:15,000). Proteins were detected using SuperSignal West Pico Chemoluminescent Substrate (Pierce Chemical) and visualized with a Gel Doc XRS imaging system (Bio-Rad Laboratories, Hercules, CA).

For determining P-glycoprotein expression the parahippocampal cortex was dissected. Tissue samples were homogenized in lysis buffer containing (per 20 ml) 200 μl of 1 M Tris, pH 8.0, 1 ml of 3 M NaCl, 2 ml of 10% Nonidet P40, 4 ml of 50% glycerol, 800 μl of

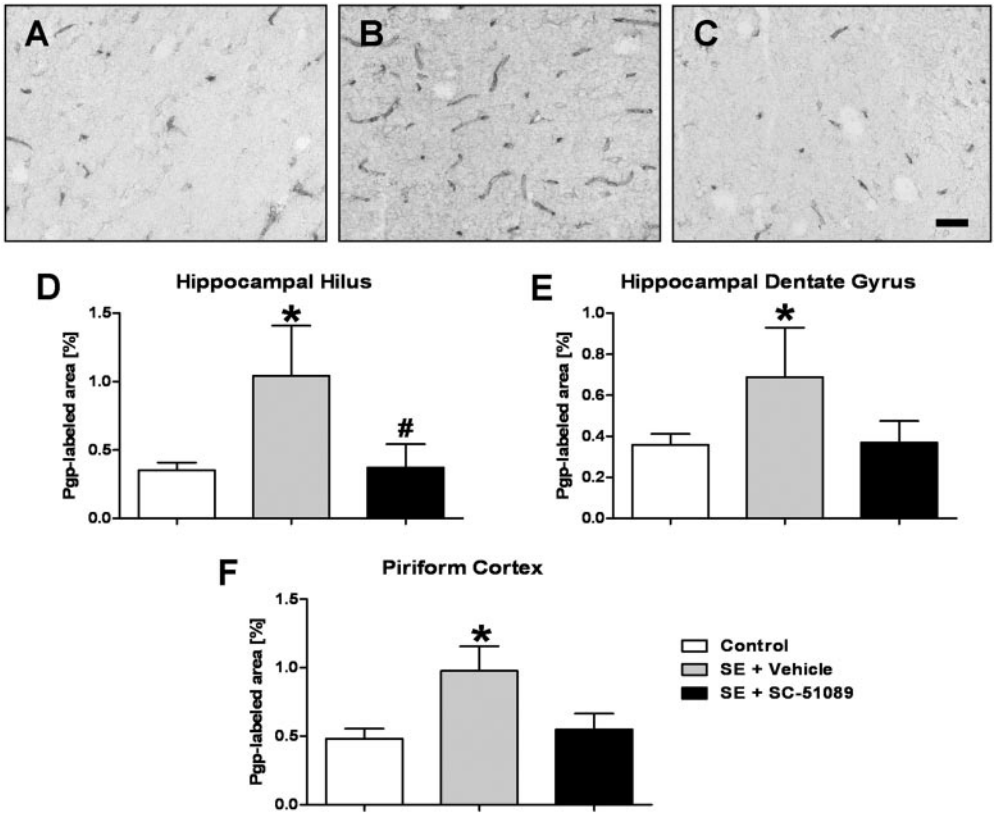

Fig. 1. SC-51089 prevents seizure-induced P-glycoprotein up-regulation. A, representative immunostaining of P-glycoprotein in the hippocampal hilus of a vehicle-treated control rat. B, P-glycoprotein immunostaining in the hilus of a rat after pilocarpine-induced status epilepticus. Note the striking increase in endothelial P-glycoprotein expression. C, P-glycoprotein immunostaining in the hippocampal hilus of a SC-51089-treated rat after pilocarpine-induced status epilepticus. Note the decrease in P-glycoprotein immunoreactivity in capillaries compared with vehicle treatment after pilocarpine-induced seizures. Quantitative analysis of P-glycoprotein immunostaining in the hippocampal hilus (D), the hippocampal dentate gyrus (E), and the piriform cortex (F). Data are given as mean ± S.E.M. Statistical comparisons: *, $p < 0.05$, significantly higher than controls; #, $p < 0.05$, significantly lower than SE + vehicle. Scale bar, 50 μm.

sodium-orthovanadate (10 mg/ml), 200 μl of 0.5 M EDTA, pH 8.0, 400 μl of protease inhibitors, 200 μl of 0.5 M NaF, and 11.2 ml of H_2O. Fifty micrograms of total protein per lane was separated by SDS-polyacrylamide gel electrophoresis (7.5% acrylamide) and transferred to nitrocellulose by electroblotting (Transblot SD; Bio-Rad Laboratories). Blots were incubated with primary antibodies (C219, 1:500; Alexis Laboratories, San Diego, CA); mouse anti-β-actin, clone AC-15 (1:50,000; Sigma); and the secondary antibody anti-mouse labeled with horseradish peroxidase (1:2500; Dako Denmark A/S, Glostrup, Denmark). Immunoreactivity was visualized with Lumi-Light PLUS Western blotting substrate (Roche Diagnostics, Mannheim, Germany), and the blots were digitized using an LAS-3000 luminescent image analyzer (Fujifilm, Tokyo, Japan). The optical density of each sample was measured using Scion Image release β3b software (Scion Corporation, Frederick, MD). For each sample, the optical density of the P-glycoprotein was calculated relative to the optical density of β-actin.

Statistical Analysis. Data are given as mean ± S.E.M. Statistical differences in P-glycoprotein expression between controls and treated groups were analyzed using the Mann-Whitney *U* test. Data of the phenobarbital experiments with kindled mice were analyzed by the Wilcoxon matched pairs test. Fisher's exact test was used to compare the number of animals that developed a status epilepticus in the different treatment groups as well as mortality rates between groups. Differences in seizure parameters during the massive kindling procedure and Western blot analysis were calculated using the Student's *t* test. All statistical tests were performed two-tailed. Differences between means were considered to be statistically significant when $p < 0.05$.

Results

Lack of an Effect of EP1 Receptor Antagonism on Status Epilepticus. Depending on the experimental conditions, COX-2 antagonism can result not only in anticonvulsant effects (Kim and Jang, 2006) but also in seizure aggravation (Baik et al., 1999). These data raise concern whether seizure susceptibility might also be affected when targeting downstream events in the COX-2-associated signaling cascades. Therefore, we carefully analyzed whether EP1 receptor antagonism exerts effects on the development of a status epilepticus in the fractionated pilocarpine model. Forty-eight percent of SC-51089-treated animals ($n = 13$ of 27) developed a status epilepticus in response to repeated injections of the cholinomimetic pilocarpine. In vehicle-treated controls, a status epilepticus was successfully induced in 46% of the animals ($n = 17$ of 37). No significant difference between both groups was observed in the amount of pilocarpine required to induce a status epilepticus. SC-51089-treated rats received 6.18 ± 0.75 pilocarpine applications (each 10 mg/kg) before onset of status epilepticus. Vehicle-treated rats required 5.69 ± 0.75 pilocarpine applications. It is important that the severity of status epilepticus in SC-51089-treated animals was not different from that of 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 ($p = 0.3414$) between the SC-51089-treated group ($n = 4$ of 13) and the vehicle-treated group ($n = 11$ of 17).

EP1 Receptor Antagonism Prevents the Induction of P-glycoprotein by Status Epilepticus. Immunolocalization of the P-glycoprotein antibody was observed in microvessel endothelial cells of all rats regardless of the treatment paradigm (Fig. 1, A–C). The 3,3′-diaminobenzidine staining method was chosen for the present experiments because it exclusively labels endothelial P-glycoprotein. Therefore, the P-glycoprotein-labeled areas determined by computer-as-

sisted analysis can be completely attributed to endothelial P-glycoprotein expression (Volk et al., 2005).

As demonstrated previously, prolonged seizure activity induced by pilocarpine represents a very strong trigger for transcriptional activation of P-glycoprotein expression. In line with these data, pilocarpine-induced status epilepticus significantly increased P-glycoprotein labeling in the hippocampal hilus, the hippocampal granule cell layer, and in the piriform cortex by 91.67 to 197.14% (Fig. 1, B, D–F). In all three brain regions, treatment with the EP1 receptor antagonist SC-51089 efficaciously prevented the seizure-associated induction of endothelial P-glycoprotein expression and kept P-glycoprotein expression at control level (Fig. 1, D–F).

Subchronic EP1 Receptor Blockade in a Massive Kindling Paradigm Gives First Evidence That Prevention of P-glycoprotein Induction Might Increase Phenobarbital Efficacy. Based on the efficacious control of P-glycoprotein expression despite prolonged seizure activity, we hypothesized that EP1 receptor antagonism may improve antiepileptic drug efficacy. Phenobarbital has been repeatedly described to be a substrate of P-glycoprotein (Potschka et al., 2002; Brandt et al., 2006; Luna-Tortós et al., 2008). The kindling model renders an elegant tool for controlled and repeated induction of seizure activity as well as for the precise assessment of anticonvulsant efficacy. Moreover, it has been repeatedly described that kindled seizures induce P-glycoprotein expression in a significant manner (Volk et al., 2004b). Therefore, we used fully kindled mice to obtain first data whether a subchronic treatment with the EP1 receptor antagonist SC-51089 during a phase with repeated elicitation of epileptic seizures (massive stimulation paradigm with 45 stimulations during the 9-day treatment phase) enhances the anticonvulsant efficacy of phenobarbital during a subsequent seizure threshold experiment. In this kindling paradigm, massive kindling proved to result in an increase of P-glycoprotein expression in the parahippocampal cortex by 49% as assessed by Western blot analysis (Fig. 2A).

In pretests, different phenobarbital doses (range, 4–8 mg/kg) were tested in a separate set of kindled mice to select a dose that does not exert a significant effect when given without pretreatment. A dose of 6 mg/kg i.p. was chosen based on these experiments.

During the massive stimulation paradigm both groups responded to repeated stimulations in a comparable manner with no significant differences in the seizure parameters. The mean seizure severity reached 4.05 ± 0.18 in vehicle-treated mice and 4.31 ± 0.18 in SC-51089-treated mice; vehicle-treated mice developed 39.0 ± 4.0 and SC-51089-treated mice 42.7 ± 1.6 (mean ± S.E.M.) generalized seizures. Cumulative duration of motor seizures during the stimulation phase amounted to 1459 ± 64 s in the vehicle-treated group and 1513 ± 84 s (mean ± S.E.M.) in the SC-51089-treated group. Cumulative duration of after discharge duration during the stimulation phase amounted to 942 ± 44 s in the vehicle-treated group and 1020 ± 72 s (mean ± S.E.M.) in the SC-51089-treated group. Subchronic SC-51089 treatment was well tolerated by all animals and was not associated with any adverse effects. After withdrawal of SC-51089, seizure thresholds proved to be comparable in both groups amounting to 240 ± 60 μA in SC-51089-pretreated mice and 260 ± 12 μA in vehicle-pretreated mice. In line with the data from the rat pilocarpine model, treatment with the EP1 receptor

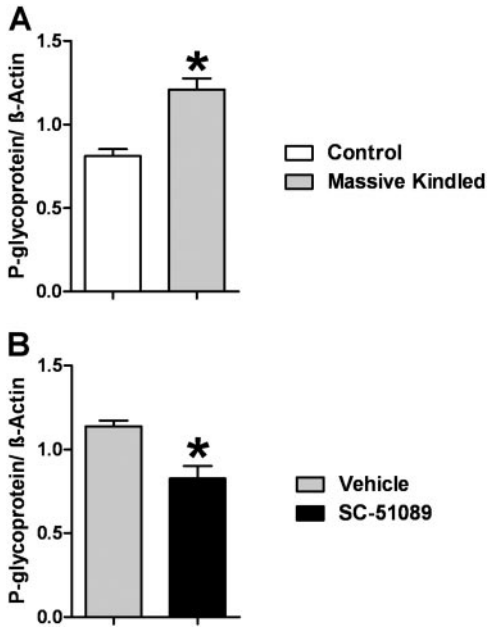

Fig. 2. Induction of P-glycoprotein expression by massive kindling and prevention of massive kindling-induced P-glycoprotein up-regulation by SC-51089. A, mean ratio (± S.E.M.) of the optical density of P-glycoprotein relative to β-actin expression measured on the Western blot for control mice and mice after massive kindling are given. Note the increase of P-glycoprotein expression after massive kindling. Statistical comparison: ∗, $p < 0.05$, significantly higher than control. B, mean ratio (± S.E.M.) of the optical density of P-glycoprotein relative to β-actin expression measured on the Western blot for vehicle-treated and SC-51089-treated massive kindled mice is given. Note that treatment with SC-51089 prevents seizure-induced up-regulation of P-glycoprotein expression. Statistical comparison: ∗, $p < 0.05$, significantly lower than vehicle-treated mice.

antagonist SC-51089 efficaciously counteracted the seizure-associated induction of P-glycoprotein expression in the parahippocampal cortex (Fig. 2B).

Phenobarbital administration resulted in an anticonvulsant effect in SC-51089-pretreated animals, in that the seizure threshold was significantly increased by 161.01% (Fig. 3A). In line with the pretests, phenobarbital did not exert a significant effect on seizure thresholds in mice that received vehicle injections during the massive stimulation paradigm. The dosage of 6 mg/kg phenobarbital did not result in behavioral side effects, such as ataxia, in vehicle-treated or in SC-51089-treated mice.

In contrast to SC-51089 pretreatment, acute SC-51089 coadministration did not potentiate the anticonvulsant effect of phenobarbital (Fig. 3B). In the acute experiments, SC-51089 did not affect the duration of motor seizures [vehicle-treated group, 35.7 ± 2.26 s (mean ± S.E.M.); SC-51089-treated group, 35.9 ± 2.81 s (mean ± S.E.M.)] or the after discharge duration [vehicle-treated group, 33.7 ± 1.48 s (mean ± S.E.M.); SC-51089-treated group, 34 ± 2.92 s (mean ± S.E.M.)].

EP1 Receptor Blockade Exerts No Effect on Seizure-Induced Neurodegeneration. EP1 receptors have been identified as downstream effectors of COX-2 neurotoxicity in a model of ischemic stroke (Kawano et al., 2006). Because COX-2 has also been implicated in the neurotoxicity resulting from epileptic seizure activity (Takemiya et al., 2006; Bauer et al., 2008), we hypothesized that targeting EP1 receptors may exert neuroprotective effects in the pilocarpine

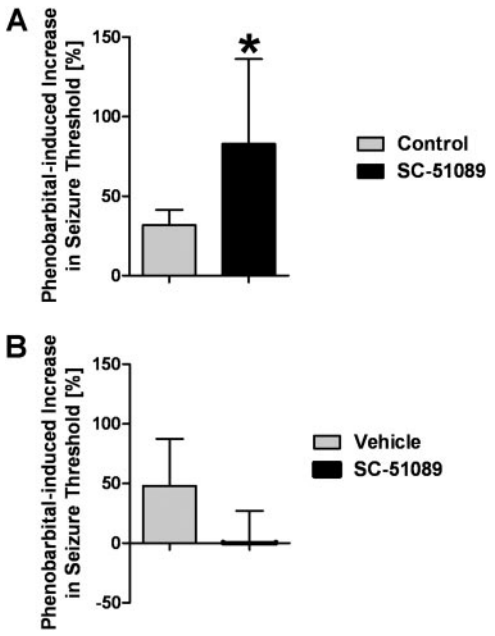

Fig. 3. Generalized seizure thresholds after phenobarbital injection in kindled mice. A, phenobarbital-induced increase of the generalized seizure threshold after a 9-day massive kindling paradigm with repeated SC-51089 treatment or respective vehicle treatment. In the vehicle-treated group of mice, phenobarbital increased the generalized seizure threshold by 31%. In contrast, SC-51089 treatment resulted in an increase of the generalized seizure threshold by 82% and thereby exceeded the anticonvulsive efficacy of phenobarbital by 161% compared with the vehicle-treated group. Data are given as mean ± S.E.M. Statistical comparison: *, $p < 0.05$, significantly higher than controls. B, lack of any acute effects of SC-51089 on phenobarbital efficacy. To exclude any acute interaction of SC-51089 and phenobarbital with subsequent enhanced phenobarbital efficacy, mice received phenobarbital as well as SC-51089 or vehicle, respectively. Threshold data obtained after phenobarbital administration were compared with data from a vehicle control experiment performed the day before. To prevent bias of the results by inter-individual differences, this experiment was repeated after several weeks in the same animals in a crossover design. Note that SC-51089 neither increases nor decreases phenobarbital efficacy, indicating a lack of any acute drug interactions.

model. A pronounced and significant decrease ($p = 0.0002$) in the neuronal density of the hippocampal hilar formation was identified in vehicle-treated rats after pilocarpine-induced status epilepticus (4768 ± 772) compared with vehicle-treated control rats (13605 ± 1328) (Fig. 4). SC-51089 treatment did not prevent status epilepticus-associated neurodegeneration in this hippocampal subregion (4859.08 ± 519.8) (Fig. 4).

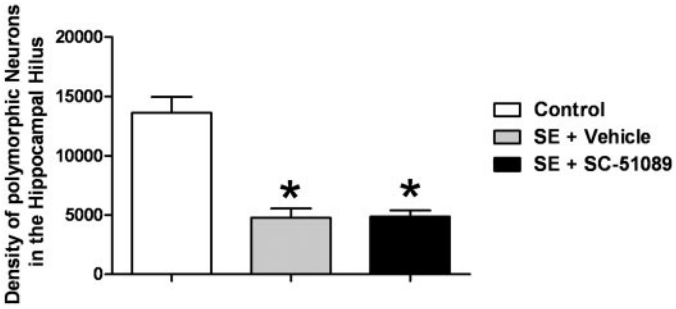

Fig. 4. SC-51089 has no effect on seizure-induced neurodegeneration. Status epilepticus significantly decreased the density of polymorphic neurons in the hippocampal hilus compared with animals that did not experience a status epilepticus. SC-51089 treatment did not prevent seizure-induced damage of hilar neurons. Data are given as mean ± S.E.M. Statistical comparison: *, $p < 0.05$, significantly different from means.

EP1 Receptor Expression in Rat Brain Capillaries. To demonstrate EP1 receptor expression in brain tissue and isolated brain capillaries, Western blot experiments were performed. Blot analysis indicated expression of the EP1 receptor in rat brain homogenates (Fig. 5). EP1 protein expression was moreover detected in isolated rat brain capillaries and the membrane fraction of isolated brain capillaries (Fig. 5).

Discussion

As an adaptation to changing requirements in tissue protection, the expression of the blood-brain barrier efflux transporter P-glycoprotein is regulated in a highly dynamic manner (Miller et al., 2008). Epileptic seizures represent a very strong trigger of P-glycoprotein transcriptional activation, resulting in a pronounced overexpression in the epileptic brain (Löscher and Potschka, 2005). It is intensely discussed that enhanced blood-brain barrier efflux might negatively affect the efficacy of antiepileptic drugs. Increased efflux may limit brain penetration of antiepileptic drugs, thereby lowering the anticonvulsant effect and contributing to pharmacoresistance (Löscher and Potschka, 2005; Kwan and Brodie, 2006). Recently, we reported the first evidence that an endothelial glutamate/COX-2 signaling pathway drives P-glycoprotein expression in response to seizure activity (Bauer et al., 2008). Here, we identified EP1 receptors as a key effector in the respective cascade up-regulating P-glycoprotein expression in epilepsies. As demonstrated in the present study, the involvement of EP1 receptors in transcriptional activation of P-glycoprotein offers new intriguing possibilities to control P-glycoprotein expression in the diseased brain and to enhance the efficacy of pharmacotherapy.

Glutamate released during seizure activity and signaling via endothelial NMDA receptors proved to function as the initial trigger that activates the intracellular cascade of events affecting P-glycoprotein expression (Bauer et al., 2008). With the regional raise in extracellular glutamate concentrations during seizures, these data also rendered an explanation for the localized overexpression of P-glycoprotein in brain regions involved in seizure generation and spread. The signaling pathway proved to involve COX-2 as a downstream effector (Bauer et al., 2008). In isolated brain capillaries, both the COX-2 inhibitor celecoxib as well as genetic deficiency of COX-2 abolished the effect of glutamate exposure on P-glycoprotein. NMDA receptor activation results in Ca^{2+} influx, which is known to activate phospholipase A2 (Rao et al., 2007). Phospholipase A2 then releases arachi-

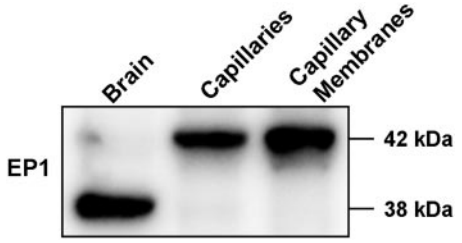

Fig. 5. EP1 is expressed in rat brain capillaries. Western blot showing EP1 expression in rat brain homogenate at approximately 38 kDa and in brain capillaries and capillary membranes at approximately 42 kDa.

donic acid from cell membranes, thereby delivering the substrate to the COX-2 enzyme. Prostaglandin E_2 represents the major product derived from processing of arachidonic acid by COX-2 in the brain (Balboa et al., 2002; Kawano et al., 2006; Yang and Chen, 2008). Prostaglandin E_2 can exert its effect via four different G protein-coupled receptors (EP1, EP2, EP3, and EP4) (Hata and Breyer, 2004). In view of our aim to identify alternate targets for control of P-glycoprotein expression in the epileptic brain, we considered tolerability issues when selecting a target candidate among these receptors. Because EP2, EP3, and EP4 receptors mediate neuroprotective effects (Bilak et al., 2004; McCullough et al., 2004; Ahmad et al., 2005), antagonism of these receptor subtypes cannot be considered as an applicable therapeutic approach. In apparent contrast, EP1 receptors are critically involved in the neurotoxicity associated with activation of NMDA receptors in the diseased brain (Bilak et al., 2004; McCullough et al., 2004; Ahmad et al., 2005). In accordance with this role, EP1 receptor antagonism exhibits neuroprotection (Suganami et al., 2003; Kawano et al., 2006). Therefore, we hypothesized that EP1 receptor antagonism may even result in dual benefit including control of endothelial P-glycoprotein as well as neuroprotection.

SC-51089 is a selective and potent antagonist of EP1 (Malmberg et al., 1994; Hallinan et al., 1996). In the present study, SC-51089 treatment blocked status epilepticus-mediated induction of P-glycoprotein expression. It is interesting that P-glycoprotein expression levels were kept at basal level in SC-51089-treated animals with a status epilepticus. These data suggest a key role for EP1 in the signaling events that drive P-glycoprotein expression in seizures. Moreover, they also suggest that glutamate signaling through the NMDA receptor, COX-2, and EP1 is an exclusive pathway up-regulating P-glycoprotein in seizures. This hypothesis is further supported by the finding that EP1 is expressed in isolated brain capillaries and brain capillary membranes. However, signaling pathways in neighboring cells that might contribute to up-regulation of endothelial P-glycoprotein cannot be ruled out at this point. Further research is necessary regarding the downstream events that mediate the effects of EP1 receptor activation on P-glycoprotein expression. The transcription factor NF-κB represents a probable candidate as EP receptor signaling can result in activation of NF-κB, and NF-κB in turn can affect P-glycoprotein expression rates (Yu et al., 2008).

At the first glance, recent data that reported an increase in COX-2 expression levels and a decrease in efflux transporter gene expression levels 2 or 24 h after a pilocarpine-induced status epilepticus might argue against our hypothesis of glutamate/NMDA receptor/COX-2/EP1 receptor signaling driving P-glycoprotein expression (Kuteykin-Teplyakov et al., 2009). However, in this study homogenates of different brain regions were analyzed without cellular separation, thus not allowing conclusions about an association between inflammatory enzymes and transporter expression in endothelial cells. Moreover, the study was limited to RNA levels and did not explore protein levels, which might not develop in parallel due to posttranscriptional regulatory mechanisms. In previous studies, we clearly demonstrated that COX-2 inhibition or its genetic deficiency efficaciously counteracts glutamate or seizure-associated increases in brain capillary P-glycoprotein in vitro and in vivo (Bauer et al., 2008; Zibell et al., 2009). Recent data demonstrated that enhanced COX-2 signaling after seizures is not due to an increase in endothelial COX-2 expression but to enhanced release of the COX-2 substrate arachidonic acid and enhanced substrate feeding of COX-2 (Zibell et al., 2009). In contrast, neuronal COX-2 expression was strongly up-regulated in neuronal cells after status epilepticus (Zibell et al., 2009). Thus, the increase in COX-2 RNA levels reported by Kuteykin-Teplyakov et al. (2009) is probably solely due to transcriptional activation in neurons.

Prevention of P-glycoprotein overexpression in the model used is especially promising because a status epilepticus induced by the cholinomimetic pilocarpine acts as an extremely strong trigger of P-glycoprotein expression (Hoffmann et al., 2006; Bauer et al., 2008). Based on data from epileptic patients and rodent epilepsy models, there is agreement that efflux transporters are overexpressed in epileptic brain tissue (Löscher and Potschka, 2005; Kwan and Brodie, 2006; Hughes, 2008). However, it is a matter of an ongoing debate whether transporter overexpression contributes to pharmacoresistance (Löscher and Sills, 2007). Experimental key findings, including studies that describe a correlation among increased P-glycoprotein expression, reduced antiepileptic drug brain penetration, and limited drug efficacy indicate that P-glycoprotein plays a critical role in antiepileptic pharmacotherapy (Rizzi et al., 2002; Potschka et al., 2004; Volk and Löscher, 2005; van Vliet et al., 2007; Wen et al., 2008). Proof-of-principle for the transporter hypothesis in rodent models came from studies in which efficacy of the antiepileptic drugs phenobarbital or phenytoin was significantly improved by add-on treatment with a selective P-glycoprotein inhibitor (Brandt et al., 2006; van Vliet et al., 2006). Regarding its clinical relevance it is discussed whether putative species differences might hamper conclusions for human patients. Therefore, it is of specific interest that a correlation between blood-brain barrier penetration and P-glycoprotein expression levels has been demonstrated using specimen from human epileptic patients. In patients with oxcarbazepine-resistant epilepsy, the brain-tissue expression of ABCB1 mRNA encoding P-glycoprotein was inversely correlated with brain levels of the active oxcarbazepine metabolite 10,11-dihydro-10-hydroxy-5*H*-dibenzo(*b*,*f*)azepine-5-carboxamide (Marchi et al., 2005). Using an in vitro blood-brain barrier model with human capillary endothelial cells from either normal or epileptic brain, Cucullo et al. (2007) demonstrated a significantly reduced permeability of phenytoin across the in vitro blood-brain barrier from cells of patients with pharmacoresistant epilepsy. In these experiments, the reduced permeability was partly counteracted by the selective P-glycoprotein inhibitor tariquidar. Recent data indicated that levetiracetam, lamotrigine, and phenobarbital are also transported by the human P-glycoprotein isoform (Luna-Tortós et al., 2008) and therefore are probably affected by P-glycoprotein expression rates in epileptic patients. Further scepticism regarding the clinical role of efflux transporters in pharmacoresistance is based on pharmacogenetic studies that resulted in controversial data regarding an association between the P-glycoprotein encoding gene and pharmacosensitivity (Leschziner et al., 2007; Löscher et al., 2009). However, the lack of a clear and reproducible association in these genetic studies is not surprising because experimental studies suggest that P-glycoprotein overexpression in

pharmacoresistant individuals is rather acquired and not intrinsic. Thus, differences are expected in events regulating P-glycoprotein expression in response to seizure activity. The identification of complex signaling events now raises new challenges to pharmacogenetic studies because genetic differences may exist not only in the promoter region of the P-glycoprotein encoding gene but also regarding several factors directly or indirectly involved in the described signaling pathway.

Overall, final conclusions regarding the clinical relevance of transporter overexpression have to await progress with positron emission tomography methods aiming to image P-glycoprotein transport function in individual patients. Moreover, other mechanisms of pharmacoresistance, such as alterations in target sites, need to be considered.

The success of P-glycoprotein inhibition in chronic epilepsy models indicates that prevention of seizure-associated induction of P-glycoprotein should render comparable results. In view of the efficacious control of P-glycoprotein expression by the EP1-receptor antagonist SC-51089, we studied the consequences of subchronic treatment with the antagonist in a massive kindling paradigm with frequent induction of seizures. The subsequent testing of the anticonvulsant efficacy of phenobarbital revealed a significant potentiation by SC-51089 pretreatment. These data provide preliminary evidence that prevention of P-glycoprotein induction by EP1 receptor antagonism might improve pharmacosensitivity toward P-glycoprotein substrates. However, further studies are necessary to substantiate these data, including thorough dose-response studies or testing in chronic models of pharmacoresistant epilepsy with spontaneous seizures.

Based on our findings in the present study, we suggest EP1 as a target for preventing P-glycoprotein overexpression and improving antiepileptic drug efficacy. As demonstrated, this strategy retains P-glycoprotein at basal levels, which is a major advantage. This is of particular relevance considering the protective function P-glycoprotein has in blood-tissue barriers, excretory organs, and hematopoietic cells (Fromm, 2004). P-glycoprotein-mediated active efflux transport protects sensitive tissues from xenobiotics and accelerates xenobiotic elimination, thereby reducing exposure times. Thus, interfering with basal P-glycoprotein transport function is a major limitation of direct P-glycoprotein inhibition. Therefore, it would be advantageous to control P-glycoprotein expression levels by targeting its regulatory pathways in the epileptic brain. Because enhanced glutamate release is also a hallmark in ischemic brain damage, P-glycoprotein induction in the ischemic brain (Spudich et al., 2006) may also be caused by glutamate and activation of inflammatory events. Targeting P-glycoprotein regulation might therefore also be successful in this condition.

When suggesting the present data as a departure point to design subsequent translational studies, one needs to carefully analyze tolerability issues. Because targeting the upstream effector of EP1 receptors, COX-2, might differently affect seizure thresholds, seizure activity, and its consequences (Baik et al., 1999; Kim and Jang, 2006), we carefully analyzed respective seizure data in animals treated with the EP1 receptor antagonist. This analysis gave no evidence for an impact of EP1 receptor antagonism on seizure thresholds or seizure severity. Thus, EP1 receptors seem to represent a more promising target in the P-glycoprotein regulatory cascade compared with COX-2. This is further underlined by the enhanced risk for cardiovascular and cerebrovascular complications that has been attributed to the use of selective COX-2 antagonists. However, in view of the controversial data existing for COX-2 inhibitors in rodent epilepsy models with seizure aggravation in some studies and lack of an effect in others (Baik et al., 1999; Kim and Jang, 2006), this aspect requires further investigational efforts to allow definite conclusions regarding safety concerns.

COX-2 is implicated in the neurotoxicity resulting from glutamate release in brain ischemia, traumatic brain injury, and epilepsy. Accordingly, COX-2 inhibition was associated with neuroprotective effects in a variety of experimental studies involving rodent epilepsy models (Takemiya et al., 2006; Bauer et al., 2008). Kawano et al. (2006) identified EP1 as a downstream effector in COX-2 neurotoxicity. In this study, EP1 activation caused NMDA receptor-induced Ca^{2+} dysregulation and affected the Ca^{2+}/Na^{2+} exchange. Inhibiting EP1 pharmacologically improved brain injury in a mouse focal cerebral ischemia model. Because glutamate excitotoxicity also triggers neuronal cell loss in the epileptic brain (Meldrum, 1993; Mody and MacDonald, 1995), we analyzed the impact of blocking EP1 in the pilocarpine status epilepticus model. In our experiments, we did not find a neuroprotective effect of SC-51089, which is in agreement with data that were obtained in the kainate model (Kawano et al., 2006). Thus, the different outcomes in blocking EP1 might be due to differences in the molecular events leading to neurotoxicity in brain ischemia and epilepsies. However, overall the result is surprising as both, NMDA receptor blockade or COX inhibition, can exert neuroprotective effects in the pilocarpine model (Fujikawa et al., 1994; Zibell et al., 2009). Thus, the lack of neuroprotection in the hippocampal hilus might also be related to kinetic effects, in that neuronal concentrations necessary for cell protection were not reached.

The activation of inflammatory pathways in the epileptic brain has been suggested as a central event in the pathophysiology of epilepsies, which might contribute or predispose to the occurrence of seizures and cell death (Vezzani and Granata, 2005). Antiinflammatory drugs are therefore discussed for future therapeutic approaches raising hope for disease modulation and inhibition of disease progression or development. The identification of a signaling pathway that involves the inflammatory enzyme COX-2 as well as the EP1 receptor and that drives endothelial P-glycoprotein expression now reveals a novel relevance of brain inflammation. The induction of this inflammatory pathway in brain capillaries may critically affect pharmacosensitivity. Our findings suggest blockade of the pathway by EP1 receptor antagonism as an innovative approach to control P-glycoprotein expression and to enhance antiepileptic drug efficacy.

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