Stroke is the third leading cause of death in the United States. Fewer than 5% of patients benefit from the only intervention approved to treat stroke. Thus, there is an enormous need to identify new therapeutic targets. The role of inducible cyclooxygenase (COX-2) activity in stroke and other neurologic diseases is complex, as both activation and sustained inhibition can engender cerebral injury. Whether COX-2 induces cerebroprotective or injurious effects is probably dependent on which downstream prostaglandin receptors are activated. Here, we investigated the function of the PGE$_2$ receptor EP4 in a mouse model of cerebral ischemia. Systemic administration of a selective EP4 agonist after ischemia reduced infarct volume and ameliorated long-term behavioral deficits. Expression of EP4 was robust in neurons and markedly induced in endothelial cells after ischemia-reperfusion, suggesting that neuronal and/or endothelial EP4 signaling imparts cerebroprotection. Conditional genetic inactivation of neuronal EP4 worsened stroke outcome, consistent with an endogenous protective role of neuronal EP4 signaling in vivo. However, endothelial deletion of EP4 also worsened stroke injury and decreased cerebral reperfusion. Systemic administration of an EP4 agonist increased levels of activated eNOS in cerebral microvessels, an effect that was abolished with conditional deletion of endothelial EP4. Thus, our data support the concept of targeting protective prostaglandin receptors therapeutically after stroke.
Signaling via the prostaglandin E<sub>2</sub> receptor EP4 exerts neuronal and vascular protection in a mouse model of cerebral ischemia

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Stroke is the third leading cause of death in the United States. Fewer than 5% of patients benefit from the only intervention approved to treat stroke. Thus, there is an enormous need to identify new therapeutic targets. The role of inducible cyclooxygenase (COX-2) activity in stroke and other neurologic diseases is complex, as both activation and sustained inhibition can engender cerebral injury. Whether COX-2 induces cerebroprotective or injurious effects is probably dependent on which downstream prostaglandin receptors are activated. Here, we investigated the function of the PGE<sub>2</sub> receptor EP4 in a mouse model of cerebral ischemia. Systemic administration of a selective EP4 agonist after ischemia reduced infarct volume and ameliorated long-term behavioral deficits. Expression of EP4 was robust in neurons and markedly induced in endothelial cells after ischemia-reperfusion, suggesting that neuronal and/or endothelial EP4 signaling imparts cerebroprotection. Conditional genetic inactivation of neuronal EP4 worsened stroke outcome, consistent with an endogenous protective role of neuronal EP4 signaling in vivo. However, endothelial deletion of EP4 also worsened stroke injury and decreased cerebral reperfusion. Systemic administration of an EP4 agonist increased levels of activated eNOS in cerebral microvessels, an effect that was abolished with conditional deletion of endothelial EP4. Thus, our data support the concept of targeting protective prostaglandin receptors therapeutically after stroke.

Introduction
Stroke is the third leading cause of death after cardiovascular disease and cancer, and stroke survivors have a 30%–50% chance of losing functional independence (1). Treatment with recombinant tPA, a thrombolytic agent, is the only approved therapy for acute stroke; however, less than 5% of stroke patients benefit from this intervention (2), in large part because of the limited time window of administration and the risk of hemorrhagic transformation. Translational attempts to validate neuroprotective strategies in the early poststroke setting have been uniformly unsuccessful, even in cases of compelling preclinical animal data. Although many reasons have been raised for this lack of success, there is consensus that single agents targeting early short-lived components of the neurotoxic cascade may not be effective (3). Thus, there is a crucial need to identify new interventions that can be therapeutically implemented after stroke.

The cyclooxygenases COX-1 and COX-2 catalyze the first committed step in the formation of prostaglandins PGE<sub>2</sub>, PGD<sub>2</sub>, PGF<sub>2α</sub>, TXA<sub>2</sub>, and PGI<sub>2</sub>, which activate distinct classes of GPCRs (reviewed in ref. 4). Cyclooxygenase activation and prostaglandin receptor signaling elicits significant injury in models of cerebral ischemia and related models of spinal cord and brain trauma, and also contributes to neurodegeneration in models of Parkinson disease, amyotrophic lateral sclerosis, and Alzheimer disease (reviewed in ref. 5). Thus, pathological induction of cyclooxygenase/prostaglandin signaling is deleterious in a wide range of acute and chronic neurologic diseases. However, recent evidence demonstrates that chronic blockade of cyclooxygenase in patients taking COX-2 inhibitors leads to cerebrovascular and cardiovascular complications (reviewed in ref. 6), which indicates that some prostaglandin signaling pathways may in fact be protective (7). Selective targeting of prostaglandin GPCRs, both toxic and beneficial, therefore represents a promising approach in the treatment of brain disorders.

PGE<sub>2</sub> is a major product downstream of COX-2 enzymatic activity. PGE<sub>2</sub> is a potent lipid messenger and activates 4 distinct GPCRs, receptors EP1–EP4. These receptors have divergent downstream signaling cascades, cellular expression patterns, and functional effects depending on the physiological or pathological context (reviewed in refs. 8–10). In experimental stroke, levels of COX-2 and PGE<sub>2</sub> are markedly upregulated (11), and COX-2 can exert neurotoxicity via the G<sub>q</sub>-coupled receptor EP1 in vivo (12, 13). However, neuronal prostaglandin receptors that are positively coupled to cAMP can elicit paradoxical protective effects in vitro in excitotoxic and hypoxic paradigms (14–16), which indicates that both protective and toxic prostaglandin signaling pathways may be active in cerebral ischemia. In the present study, we examined the function of the PGE<sub>2</sub> receptor EP4, which is positively coupled to G<sub>q</sub>s, in a mouse model of cerebral ischemia. Using pharmacologic and conditional knockout (eKO) genetic strategies to target cell-specific EP4 signaling in brain, we tested the function of EP4 in the middle cerebral artery occlusion–reperfusion (MCAo-RP) model of transient focal cerebral ischemia and identified dual and independent cerebroprotective effects of vascular and neuronal EP4 signaling.

Results
We first examined the effect of pharmacologic activation of EP4 on infarct volume and behavioral function in 3-month-old male C57B6 mice using the EP4 agonist AE1-329. The selectivity of AE1-329 (K<sub>i</sub>, 0.4 nM) for EP4 has been previously established (17,
Administration of AE1-329 (0.3 mg/kg) at 2 and then 8 hours after 1 hour of middle cerebral artery occlusion (MCAo) significantly improved behavioral scores (P = 0.05) and reduced hemispheric infarct size by 50.8% at 24 hours (Figure 1A; n = 7–12 per group). **P < 0.01 vs. vehicle.**}

A second experiment testing a single dose of AE1-329 administered 3 hours after 1 hour MCAo resulted in 51.6% and 67.3% decreases in hemispheric infarct size at 24 hours at doses of 0.03 and 0.3 mg/kg, respectively (Figure 1E; P < 0.001, ANOVA), indicative of a highly significant dose-dependent effect.

A third experiment assayed long-term functional outcome with performance on the rotarod at 48 hours and 7 days after 1 hour MCAo. Here, a single low dose of 0.03 mg/kg AE1-329 was administered 3 hours after 1 hour MCAo. Even with this lower dose, rotarod performance improved at both 48 hours (P = 0.04) and 7 days (P = 0.05; Figure 1F), although hemispheric infarct volume as measured by cresyl violet staining did not show significant differences at 7 days (vehicle, 5.1 ± 1.27; AE1-329, 5.25 ± 2.11). Finally, administration of AE1-329 6 hours after MCAo exerted mild protection in caudate-putamen (0.3 mg/kg; vehicle, 84.7 ± 1.92; AE1-329, 77.34 ± 3.59; P < 0.05; n = 11–23 per group) but was not significantly different for hemispheric infarct volumes, indicating a time window of efficacy up to 6 hours for a single dose of AE1-329 (0.3 mg/kg s.c.).

We then tested the function of neuronal EP4 in vitro in paradigms of excitotoxicity and hypoxia. EP4 was expressed basally in cultured cortical neurons in a perinuclear distribution and in processes (Figure 2A and Supplemental Figures 1 and 2; supplemental material available online with this article; doi:10.1172/JCI46279DS1). Previous studies have demonstrated modulation of both PKA and AKT pathways by EP4 signaling in nonneuronal cells (19, 20). Here,
quantitative Western analysis demonstrated significant induction of phospho-CREB in mouse cortical neurons stimulated with 100 nM AE1-329 (Figure 2, B and C; effect of AE1-329, F_{2,5} = 14.48, P < 0.01). Injury induced by administration of 250 μM glutamate for 24 hours was reduced with AE1-329 treatment in a dose-dependent manner (Figure 2D; ANOVA, P < 0.02). As shown in Figure 2E, administration of AE1-329 also rescued cortical neurons subjected to 3 hours of oxygen-glucose deprivation (OGD; ANOVA, P < 0.0001 for 0–1,000 nM; post-test, P < 0.01 for 10 nM, P < 0.05 for 100 nM, P < 0.001 for 1 μM), and this rescue was reversed with administration of the PKA inhibitor H89 (P < 0.05 to P < 0.01 for all doses of AE1-329). At a dose of 1 μM, H89 does not have any effect on basal cell survival (14, 16), and administration of H89 to cells subjected to OGD also does not alter cell survival. Activation of caspase-3 in OGD-stimulated cultures was significantly decreased with administration of AE1-329; H89 was able to block this reversal at a low dose, but not at higher doses, of AE1-329 (Figure 2F; effect of H89, F_{1,6} = 10.29, P < 0.01; effect of AE1-329, F_{3,6} = 3.14, P < 0.05; post-test, P < 0.01 for 10 nM AE1-329). As shown in Figure 2G and H, and Supplemental Figure 3, the protective effect of EP4 signaling was confirmed in hippocampal organotypic slices subjected to 75 minutes OGD (G) or 1 hour NMDA (H; 10 μM) and assayed for PI fluorescence of the CA1 subregion (Supplemental Figure 3). *P < 0.05, **P < 0.01, ***P < 0.001 vs. 0 nM AE1-329.

Given that neuronal EP4 signaling elicits PKA-dependent neuroprotection in vitro, we tested whether neuronal EP4 was similarly protective in vivo in the MCAo-RP model. We had previously demonstrated increased expression of EP4 in neurons 4 hours...
after MCAo-RP in the peri-infarct area region (21); at 24 hours after MCAo-RP, neuronal EP4 levels persisted in the peri-infarct area of mice subjected to ischemia-reperfusion compared with sham surgery control littermates (Supplemental Figure 4B). This was consistent with separate in vitro measurements of EP4 mRNA in cultured mouse cortical neurons (Supplemental Figure 4A) that showed a transient and significant induction of EP4 mRNA by 4 hours that resolved to baseline levels by 24 hours after OGD. Thy-1 Cre;EP4+/- and Thy-1 Cre;EP4lox/lox 3-month-old female mice were subjected to 60 minutes MCAo and 23 hours reperfusion (left); male Thy-1 Cre;EP4lox/lox and Thy-1 Cre;EP4+/- mice were combined (compared with control Thy-1 Cre;EP4+/- mice) and subjected to 45 minutes MCAo and 23 hours reperfusion (right). Conditional deletion of neuronal EP4 significantly increased infarct volumes in both genders. *P < 0.05. (D) Representative cresyl violet–stained sections from Thy-1 Cre;EP4+/- and Thy-1 Cre;EP4lox/lox brains showed larger infarct size in cKO brain, with loss of blue stain. Scale bar, 500 μm. (E) AE1-329 administration (0.3 mg/kg 3 hours after MCAo) reversed the increase in infarct size in Thy-1 Cre;EP4lox/lox female mice subjected to 30 minutes MCAo and 23 hours reperfusion; an additional cellular EP4 pathway may thus be involved in cerebroprotection. *P < 0.05. Body temperature readings did not differ between genotypes (see Supplemental Figure 9A). In C and E, numerals within bars denote n in each group.
was also increased in a combined cohort of Thy-1 Cre;EP4<sup>lox/lox</sup> and Thy-1 Cre;EP4<sup>lox/lox</sup> mice compared with control Thy-1 Cre;EP4<sup>+/+</sup> littermates (<i>P</i> < 0.05 for cortex and hemisphere; Figure 3C). Taken together, these data are consistent with an endogenous protective function of neuronal EP4 signaling in vivo.

To determine whether the cerebroprotective effect of systemic AE1-329 administration (Figure 1) was mediated exclusively through neuronal EP4 signaling, a second cohort of Thy-1 Cre;EP4 mice (backcrossed <i>n</i> = 6 in the C57B6 background) was generated, subjected to 30 minutes of MCAo followed by 23 hours of reperfusion, and dosed with either vehicle or 0.3 mg/kg AE1-329 3 hours after MCAo (Figure 3E and Supplemental Figure 7B). A shorter occlusion time of 30 minutes was necessary because 60 minutes of occlusion in the higher backcross (<i>n</i> = 6) to the C57B6 background resulted in increased mortality in Thy-1 Cre;EP4<sup>lox/lox</sup> mice (data not shown). Precedent indicates that genetic background can affect the extent of cerebral injury (22–25). With 30 minutes of MCAo, hemispheric infarct size increased by 46.1% (<i>P</i> = 0.05) in Thy-1 Cre;EP4<sup>lox/lox</sup> mice compared with controls. Interestingly, AE1-329 administered 3 hours after MCAo significantly reduced infarct size in Thy-1 Cre;EP4<sup>lox/lox</sup> mice (hemispheric infarct decreased by 34.9%, <i>P</i> = 0.03; cortical infarct decreased by 83.7%, <i>P</i> = 0.04). The percentage of mice in each group with no cortical infarction decreased with deletion of the neuronal EP4 and increased with administration of AE1-329 (Thy-1 Cre;EP4<sup>+/+</sup> plus vehicle, 80%; Thy-1 Cre;EP4<sup>lox/+</sup> plus vehicle, 61%; Thy-1 Cre;EP4<sup>lox/lox</sup> plus vehicle, 40%; Thy-1 Cre;EP4<sup>lox/lox</sup> plus AE1-329, 63%; Thy-1 Cre;EP4<sup>lox/lox</sup> plus AE1-329, 80%). The reversal in ischemic injury in neuronal EP4 cKO mice suggests that although neuronal EP4 exerts endogenous neuroprotection, an additional nonneuronal EP4 signaling path-way may also contribute to AE1-329 cerebroprotection.

Accordingly, we examined the role of the endothelial EP4 in MCAo-RP injury; we had previously determined that endothelial EP4 expression was induced early in core and peri-infarct cortical regions 4 hours after MCAo-RP (21). EP4 colocalized in infarcted brain with the endothelial marker Factor VIII at 4 hours after MCAo (Supplemental Figure 8A). EP4 signaling contributes to the vasodilatory effects of PGE<sub>2</sub> (26, 27) in vascular beds outside of the central nervous system. To test the function of endothelial EP4 signaling in cerebral ischemia, we generated endothelial EP4 cKO mice using the
Endothelial EP4 regulates CBF during reperfusion. (A) Measurement of relative CBF by LDF revealed a significant decrease in cerebral perfusion in male VECad-Cre-ERT²;EP4lox/lox mice compared with VECad-Cre-ERT²;EP4+/+ mice after 60 minutes of MCAo (effect of genotype, $F_{1,22} = 4.769, P = 0.039$; effect of time, $F_{1,22} = 74.73, P < 0.001$; effect of interaction, $F_{1,22} = 2.168, P < 0.05$). Heterozygous VECad-Cre-ERT²;EP4lox/+ values were intermediate between homozygous and control wild type values, but did not differ significantly from either EP4lox/lox or EP4+/+ genotypes. (B) MAP before, during, and after ischemia (60 minutes MCAo) did not show differences between genotypes. If, before any surgery, CCA, common carotid artery ligation; ECA, external carotid artery ligation; ICA, internal carotid ligation. MAP units are mm Hg.

![Figure 5](image)

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Discussion

In this study, we demonstrated a protective function for the PGE₂ receptor EP4 in reducing cerebral injury and improving functional recovery after stroke, a finding that supports the concept of beneficial prostaglandin signaling downstream of COX-2. Moreover, EP4 signaling elicited its protection via dual and independent cell-specific mechanisms of neuroprotection and enhanced vascular perfusion.
hours after onset of ischemia resulted in significant improvements in cerebral stroke injury and in motor performance on the rotarod 1 week after ischemia. In vitro, neuronal EP4 signaling elicited protection through a PKA-dependent pathway; this was confirmed in vivo, in which conditional deletion of EP4 in neurons significantly worsened stroke injury. Administration of AE1-329 3 hours after MCAo to neuronal EP4 cKO mice reversed the increase in cerebral injury seen in these mice, which suggests that systemic AE1-329 elicits its cerebroprotective effect through an additional nonneuronal EP4 signaling pathway. Endothelial EP4, although expressed at nearly undetectable levels in sham brain vasculature, was markedly upregulated in vasculature after ischemia, as assayed by immunolocalization with both β-dystroglycan and Factor VIII. Accordingly, the effect of conditionally deleting EP4 in endothelium was tested, and endothelial cKO mice demonstrated a marked increase in cerebral injury, consistent with an endogenous cerebroprotective effect of endothelial EP4 signaling. Conditional deletion of EP4 in endothelium also resulted in significant reduction in relative CBF during reperfusion, which suggests that loss of endothelial EP4 may increase stroke injury by decreasing reperfusion blood flow. A major mechanism underlying increased CBF is eNOS-mediated vasodilation, in which

Figure 6
Endothelial EP4 regulates eNOS activation in cerebral microvasculature in vivo. (A) AE1-329 or vehicle was administered to 3-month-old male C57B6 mice (0.3 mg/kg s.c.), and levels of total Akt, phospho-Akt, total eNOS, and Ser1177 phospho-eNOS were measured in acutely isolated cerebral microvessels at 1 and 5 hours by quantitative Western analysis. Total Akt, phospho-Akt, total eNOS, and phospho-eNOS were increased 5 hours after AE1-329 administration. A representative Western blot is shown; lanes were run on the same gel but were noncontiguous (white lines). (B) Quantification of densitometric values normalized to actin demonstrated significant increases in total and phospho-Akt and in total and phospho-eNOS 5 hours after AE1-329 administration, n = 6–9 per condition. *P < 0.05; **P < 0.01; ***P < 0.001. (C) AE1-329 or vehicle was administered to 3-month-old male VECad-Cre-ER<sup>T2</sup>;EP4<sup>+/+</sup> and VECad-Cre-ER<sup>T2</sup>;EP4<sup>lox/lox</sup> mice (0.3 mg/kg s.c.), and microvessels were isolated 5 hours after agonist administration. Western analysis demonstrated induction of phospho-Akt and phospho-eNOS in VECad-Cre-ER<sup>T2</sup>;EP4<sup>+/+</sup> mice that was absent in VECad-Cre-ER<sup>T2</sup>;EP4<sup>lox/lox</sup> mice. All samples were on the same blot. (D) Quantification of densitometric values normalized to actin demonstrated significant increases in microvessel total Akt, phospho-Akt, total eNOS, and phospho-eNOS levels 5 hours after AE1-329 administration in VECad-Cre-ER<sup>T2</sup>;EP4<sup>+/+</sup> mice; such increases were not seen in VECad-Cre-ER<sup>T2</sup>;EP4<sup>lox/lox</sup> mice. n = 3 per group. *P < 0.05; **P < 0.01.
activation of eNOS and increased NO production lead to cGMP-dependent smooth muscle cell relaxation and increased blood flow (reviewed in ref. 31). Systemic in vivo administration of AE1-329 robustly increased protein levels of eNOS and activated phospho-Ser1177 eNOS in cerebral microvessels. Conversely, deletion of endothelial EP4 abolished the increase in phospho-eNOS elicited by AE1-329 administration. These experiments suggest that endothelial EP4 may rescue infarct injury by enhancing cerebral reperfusion through increased production of NO. Taken together, our data demonstrate endogenous cerebroprotection mediated by 2 independent cellular EP4 signaling pathways acting through vascular and neuroprotective mechanisms.

Immunostaining for EP4, using a mouse EP4-specific IgY antibody developed for this study, confirmed that EP4 was expressed in neurons under basal and postinfarct conditions and was induced to high levels in endothelium after ischemia (21). Examination of EP4 expression in other cell types in sham and stroke mice revealed undetectable levels in astrocytes, and very low levels in microglia. To test cell-specific mechanisms of EP4 cerebroprotection, we used cKO strategies to selectively delete EP4 in neurons and in endothelial cells. This genetic strategy offered a powerful means to dissect out cell-specific neuronal and endothelial mechanisms of action in vivo of this prostaglandin signaling pathway in cerebral ischemia. Interestingly, the increases in infarct injury in both neuronal and endothelial EP4 cKO mice were reversed with systemic administration of AE1-329, which suggests that both cell types could be targets of AE1-329. We cannot exclude that AE1-329 might act through microglia; however, in separate experiments, we have not detected differences in infarct injury at 48 hours in mice with a conditional deletion of EP4 in microglia (L. Lin, unpublished observations). However, we were able to confirm that endothelial EP4 is activated by systemic administration of AE1-329 in vivo, as demonstrated in acutely isolated microvessel preparations in which AE1-329 stimulation increased levels of eNOS and phospho-eNOS and, conversely, in which conditional deletion of endothelial EP4 abolished this effect.

Conditional deletion of endothelial EP4 increased cerebral infarct volume. Interestingly, the heterozygous genotype had a degree of injury similar to that of the homozygous genotype. It is possible that in the acute setting of ischemia, loss of 1 allele may have a significant effect, whereas in a more chronic context, compensatory mechanisms may have more time to develop and mask the loss of 1 allele. The increase in infarct size with cKO of endothelial EP4 was associated with a significant decrease in relative CBF during reperfusion, as measured by LDF. To mitigate against potential involvement of the endothelial EP4 in early postnatal development (34), we adopted a tamoxifen-inducible VECad-Cre-ER T2 recombinase strategy. Although this precaution was taken, we cannot completely exclude the possibility that endothelial EP4 could function in vascular development or remodeling after 6 weeks of age, the time point at which mice received tamoxifen. Importantly, tamoxifen, which has been shown in several rodent studies to acutely reduce stroke injury (35–37) was administered to both VECad-Cre-ER T2;EP4lox/lox and VECad-Cre-ER T2;EP4;+/+ genotypes; moreover, administration of tamoxifen was completed by 6–7 weeks of age, long before initiation of MCAo-RP studies. Finally, although our immunostaining experiments using 2 independent antibodies to EP4 did not show detectable vascular EP4 expression in VECad-Cre-ER T2;EP4lox/lox brains, we cannot exclude an excision efficiency of less than 100% by the VECad-Cre-ER T2 recombinase; successful excision of floxed sequences by this Cre recombinase requires both tamoxifen-mediated nuclear translocation of Cre-ER T2 and subsequent Cre-mediated excision of floxed sequences. However, precedent indicates that the efficiency of this inducible promoter can reach near-complete levels of excision (28).

Prior studies of systemic vascular responses demonstrate that PGE2 increases blood flow via EP2 and/or EP4 signaling (26, 27, 38, 39), which suggests that EP4 signaling may be protective in MCAo-RP by enhancing arteriolar and/or capillary reperfusion. Supporting this possibility is the observation that mice administered AE1-329 showed significant increases in eNOS protein and phosphorylation of Ser1177 eNOS 5 hours after administration in cerebral microvessels, and the converse genetic experiments demonstrating that deletion of endothelial EP4 blocked this effect. Past studies have demonstrated that eNOS activation is crucial in protecting the brain after cerebral ischemia through increased production of NO, which leads to cGMP-dependent smooth muscle relaxation (40–43) and also to inhibition of synthesis of the vasoconstrictor 20-HETE (44). In increasing levels of total and activated eNOS protein, EP4 acts in a manner similar to that of cerebroprotective agents such as statins (45, 46) and estrogen (47, 48). From a translational perspective, the fact that eNOS levels readily increased in cerebral microvessels after systemic AE1-329 administration indicates that the endothelial EP4 is physically accessible, consistent with the fact that it is a G protein–coupled membrane receptor. Finally, EP4 signaling may enhance outcome after cerebral ischemia by additional eNOS/NO actions, including the antiplatelet, antithrombotic, and antiinflammatory effects of NO.

Our findings broaden the concept of beneficial prostaglandin receptors in cerebrovascular disease. PGI2 has been identified in cardiovascular systems as a beneficial vasodilatory prostaglandin (7), and its synthesis is decreased with sustained COX-2 inhibition, leading to a prothrombotic state from unopposed thromboxane A2 signaling (reviewed in ref. 6). Here, we have identified EP4 as a protective prostaglandin receptor acting through distinct and independent neuronal and endothelial mechanisms to reduce injury in a model of cerebral ischemia. The dual neuronal and vascular mechanisms of cerebroprotection triggered by activation of EP4 support the concept of protective prostaglandin receptors that could be targeted therapeutically after stroke. These data also support the concept of targeting nonneuronal as well as neuronal mechanisms, which could recruit more robust and less time-sensitive pathways than neuroprotective strategies alone (3). Pharmacological effects on cerebral perfusion are particularly attractive, as they may synergistically enhance neuronal survival not only by improving delivery of nutrients and oxygen, but also by extending tissue penetration of the therapeutic agent being administered. In our studies, AE1-329 administration increased levels of activated eNOS in cerebral microvessels, which suggests NO as one mechanism by which endothelial EP4 could improve reperfusion. As human MRI studies now suggest that rescue of cerebral perfusion may be possible at later time points than previously realized (49, 50), reperfusion-based strategies may extend the effective time window for therapeutic interventions in stroke.

**Methods**

Further information can be found in Supplemental Methods.

**Animals.** For in vitro studies, Sprague-Dawley rats were obtained from Charles River for organotypic hippocampal cultures, and C57B6 E17 mouse embryos were used for cortical neuron primary cultures. C57B6 male mice and F1 hybrid B6D2F1/J mice (12–14 weeks of age; Jackson Laboratory) were used for in vivo studies. Mice were genotyped as described above, and littermate wild-type siblings were used as controls. C57B6 male mice and F1 hybrid B6D2F1/J mice (12–14 weeks of age; Jackson Laboratory) were used for in vivo studies. Mice were genotyped as described above, and littermate wild-type siblings were used as controls.
Laboratory; stock no. 100006) were used to test pharmacologic effects of AE1-329, C57B6 EP4+/+ mice were provided by R. Breyer and M. Breyer (Vanderbilt University School of Medicine, Nashville, Tennessee, USA). C57B6 VECad-Cre-ER12 mice were provided by L.I. Arispe (UCLA, Los Angeles, California, USA) (28). Tamoxifen (Sigma-Aldrich) was administered to all VECad-Cre-ER12 genotypes to induce translocation of Cre and excision of floxed EP4 sequences following established protocols (28, 51). Filtered tamoxifen stock solution at 10 mg/ml was prepared in sunflower seed oil (10:1 oil), and 2 mg tamoxifen was administered for 5 consecutive days at 6 weeks of age. Thy-1 Cre mice (52) were purchased from Jackson Laboratories (FVB/N-Tg(Thy-1-cre)1VIm]) and backcrossed to the C57B6 background (n = 4 for the initial series of studies of Thy-1 Cre:EP4 mice; n = 6 for subsequent studies). The Cre reporter mouse B6.129S4-Gr(Rosa)26Sor+/m (abbreviated ROSA-26; stock no. 003474; The Jackson Laboratory) was used to validate Thy-1 Cre activity distribution. For both neuronal Thy-1 Cre and VECad-Cre-ER12 experiments, Cre:EP4+/+, Cre:EP4m, and Cre:EP4m littermate mice were generated by serial crosses of Cre and EP4+/+ or EP4m lines. All mice were housed in an environment controlled for lighting (12-hour light/12-hour dark cycle), temperature, and humidity, with food and water available ad libitum.

Reagents and materials. The EP4-specific agonist AE1-329 [16-[3-methoxy-methyl] phenyl-omega-tetranor-3,7-dithia prostaglandin E1; refs. 17, 18] was a gift from Ono Pharmaceuticals Co. Cell culture media, supplements, and antibiotics were purchased from Invitrogen.

MCao-RO model. All MCao-RO experiments were performed by an experimenter blinded to genotype and/or pharmacological agent, as described previously (14).

Quantification of infarct volume. All infarct quantification was carried out by a second examiner blinded to genotype and/or pharmacological agent. After 23 hours of reperfusion, mice were lethally anesthetized, and brain tissue was harvested for infarct volume quantification using either 1% 2,3,5-triphenyl-tetrazolium chloride (TTC) staining for pharmacological experiments or creosyl violet for genetic experiments, as previously described (14). Infarct volume (corrected for swelling) was represented as a percentage, as previously described (14).

Measurement of relative CBF. LDF was performed in a blinded fashion 2 mm posterior and 3 mm lateral to the bregma over the parietal skull surface, as previously described (14).

MAP and physiologic measurements. The femoral artery was cannulated with an I-STAT analyzer (I-STAT). MAP was measured with a microcan


References


