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Research Article

Neuroscience

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Signaling via the prostaglandin E₂ 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 PGE₂ 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 PGE₂, PGD₂, PGF_{2α}, TXA₂, and PGI₂, 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 neurological 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.

PGE₂ is a major product downstream of COX-2 enzymatic activity. PGE₂ 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 PGE₂ are markedly upregulated (11), and COX-2 can exert neurotoxicity via the Gα_q-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 PGE₂ receptor EP4, which is positively coupled to Gα_s, in a mouse model of cerebral ischemia. Using pharmacologic and conditional knockout (cKO) 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_i, 0.4 nM) for EP4 has been previously established (17,

Conflict of interest: The authors have declared that no conflict of interest exists.

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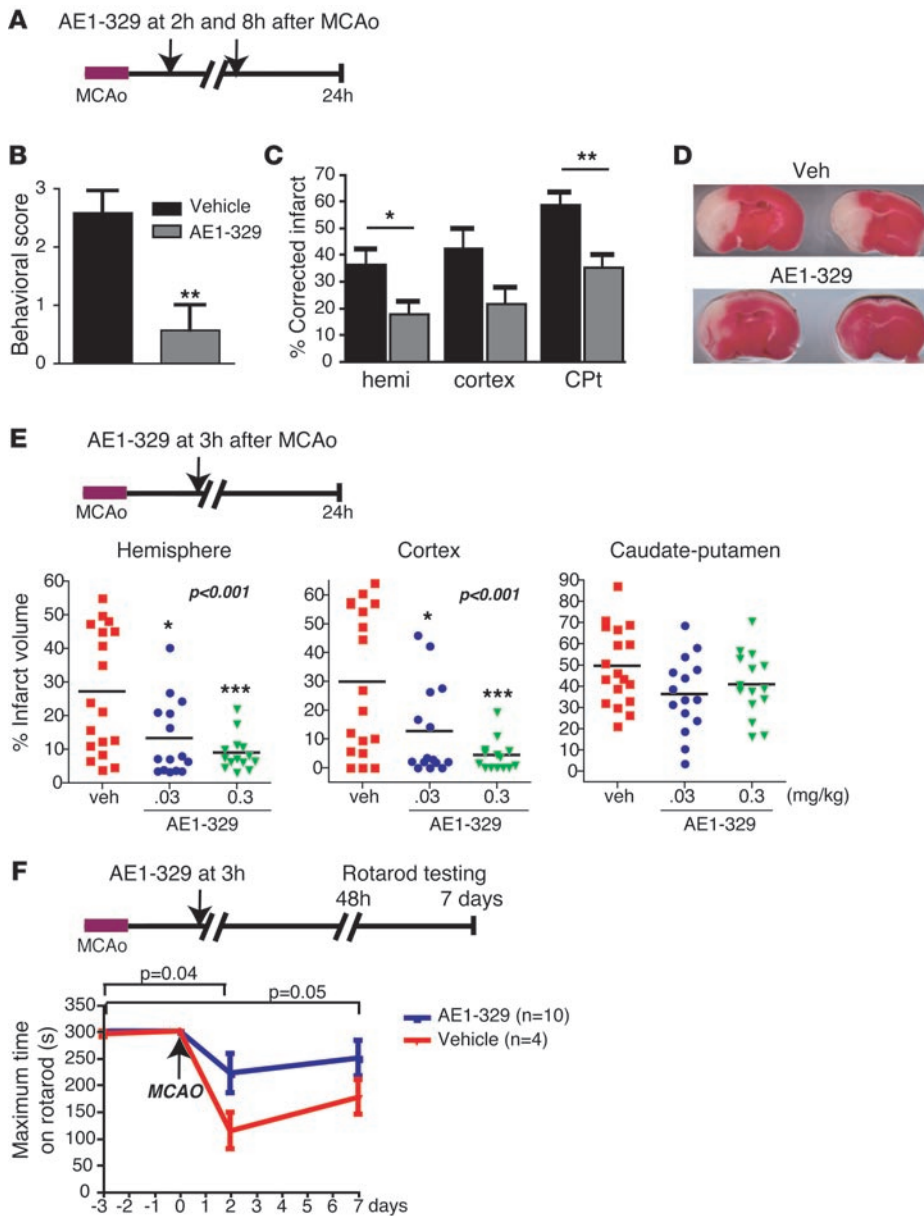


Figure 1

EP4 agonist administration after cerebral ischemia is protective. (A) 60 minutes MCAo and 23 hours reperfusion were carried out in 3-month-old male C57B6/J mice. EP4 agonist AE1-329 (0.3 mg/kg s.c.) or vehicle was administered 2 and 8 hours after MCAo. ****P** < 0.01 vs. vehicle. (B) Behavioral scores at 24 hours in mice treated as in A. (C) Infarct volume, corrected for cerebral edema, in hemisphere, cerebral cortex, and caudate-putamen (CPT) of mice treated as in A. *n* = 7–12 per group. **P* ≤ 0.05; ***P* < 0.01. (D) Representative TTC staining of coronal brain sections from mice treated as in A. (E) Dose response of a single AE1-329 injection 3 hours after 60 minutes MCAo resulted in a dose-dependent reduction in stroke infarct volume at 24 hours (1-way ANOVA, *P* < 0.001; post-hoc, **P* < 0.05 for 0.03 mg/kg, ****P* < 0.001 for 0.3 mg/kg). *n* = 15–18 per group. (F) A single 0.03-mg/kg dose of AE1-329 administered 3 hours after 60 minutes MCAo improved rotarod performance 48 hours and 7 days after infarction in a second background in 3-month-old male F1 hybrid B6D2F1/J mice. *P* values were determined by 2-way ANOVA.

18). Administration of AE1-329 (0.3 mg/kg) at 2 and then 8 hours after 1 hour of middle cerebral artery occlusion (MCAo; Figure 1A) significantly improved behavioral scores (*P* = 0.05) and reduced hemispheric infarct size by 50.8% at 24 hours (Figure 1, B–D). 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. This paradigm, a stringent outcome measure quantifying functional recovery, was carried out in a second genetic background: 3-month-old F1 hybrid B6D2F1/J male mice. 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,

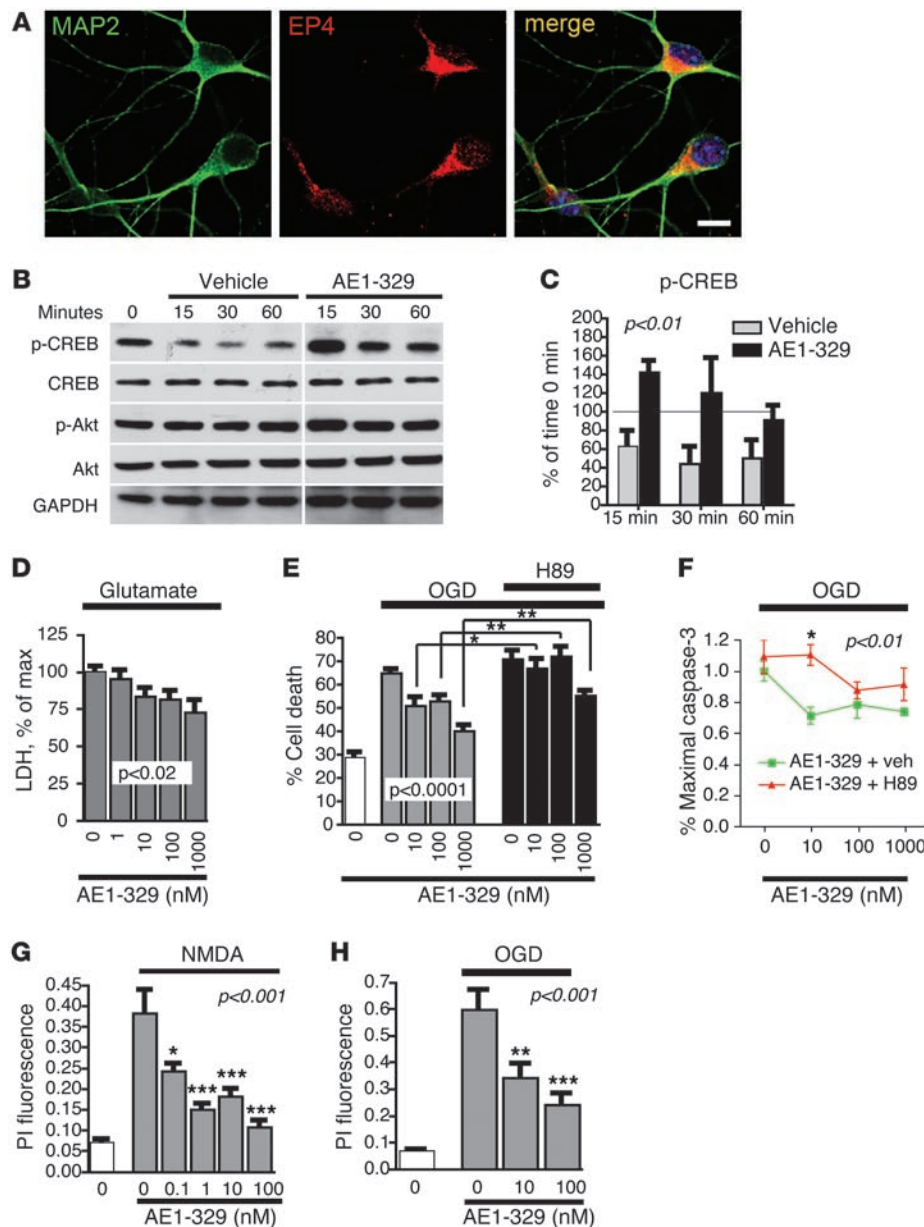


Figure 2

EP4 signaling protects neurons in ischemic and excitotoxic paradigms in vitro. See Results for detailed statistical reporting. **(A)** EP4 expression in mouse cortical neurons stained for MAP2 (see Supplemental Figure 1). Scale bar: 10 μm . **(B)** Rapid induction of phospho-CREB after AE1-329 (100 nM) stimulation of cortical neurons from day 10 in vitro C57B6 mice. A representative Western blot is shown; lanes were run on the same gel but were noncontiguous (white lines). **(C)** Effect of AE1-329 on CREB phosphorylation. Densitometry values were calculated relative to 0 minutes, assigned as 100% (gray line). **(D)** Dose response demonstrated a protective effect of AE1-329 (0–1,000 nM) in cortical neurons stimulated with 250 μM glutamate for 24 hours, assayed by LDH release. $n = 6$ –8 per group. **(E)** AE1-329 rescued cortical neurons subjected to 3 hours of OGD and assayed for PI fluorescence relative to total Hoechst nuclei. Cotreatment with 1 μM H89 reversed EP4 neuroprotection at all doses. $n = 6$ per group. $*P < 0.05$; $**P < 0.01$. **(F)** In cortical neurons subjected to OGD for 3 hours, AE1-329 reduced caspase-3 activation; this was reversed with 1 μM H89 cotreatment. $*P < 0.01$ vs. AE1-329 plus vehicle. **(G and H)** Dose response of AE1-329 rescue of CA1 neurons in organotypic hippocampal 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.

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 rever-

sal 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 2, G and H, and Supplemental Figure 3, the protective effect of EP4 signaling was confirmed in hippocampal organotypic slices subjected to NMDA excitotoxicity (ANOVA, $P < 0.001$; post-test, $P < 0.05$ for 0.1 nM AE1-329, $P < 0.001$ for 1–100 nM AE1-329) or OGD (ANOVA, $P < 0.001$; post-test, $P < 0.01$ for 10 nM AE1-329, $P < 0.001$ for 100 nM AE1-329). This organotypic culture system allows for the examination of neuronal, astrocytic and microglial effects, but does not address vascular function, as endothelial cells are rapidly lost in this preparation.

Given that neuronal EP4 signaling elicited 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

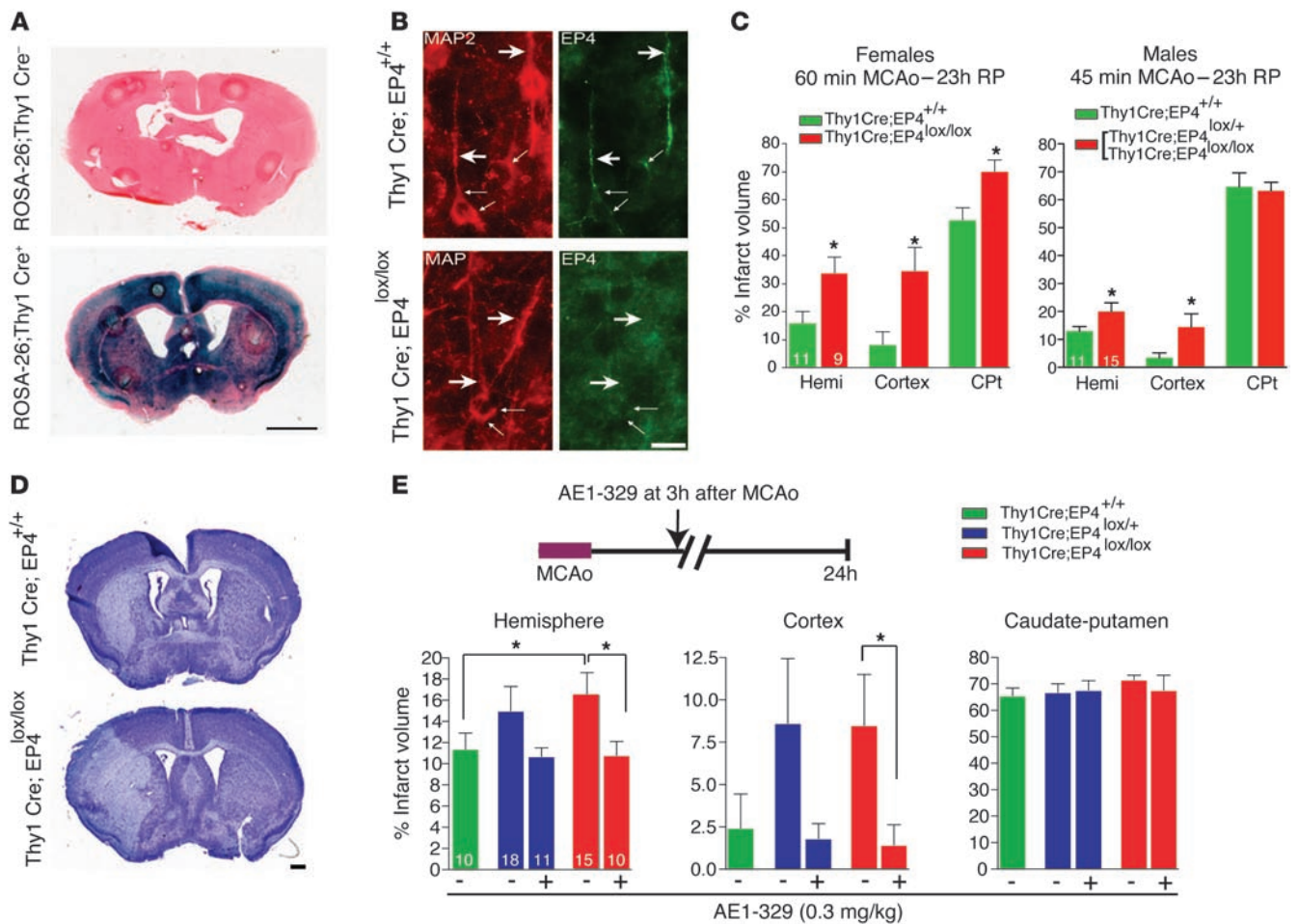


Figure 3

Deletion of neuronal EP4 increases cerebral injury in the MCAo-RP model. **(A)** X-gal staining in ROSA-26;Thy-1 Cre transgenic mouse brain revealed high Cre recombinase activity in cerebral cortex and striatum, the principal structures affected in the MCAo-RP model (Supplemental Figure 5). Scale bar: 2 mm. **(B)** Immunofluorescent staining of cerebral cortical EP4 with anti-mouse EP4 IgY antibody (Supplemental Figures 1 and 2) showed expression of EP4 basally in neurons (arrows) of Thy-1 Cre;EP4^{+/+} mice that was reduced in Thy-1 Cre;EP4^{lox/lox} mice. Scale bar: 15 μ m. **(C)** Thy-1 Cre;EP4^{lox/lox} and Thy-1 Cre;EP4^{+/+} 3-month-old female mice were subjected to 60 minutes MCAo and 23 hours reperfusion (left); male Thy-1 Cre;EP4^{lox/+} and Thy-1 Cre;EP4^{lox/lox} 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;EP4^{lox/lox} brains showed larger infarct size in cKO brain, with loss of blue stain. Scale bar, 500 μ m. **(E)** AE1-329 administration (0.3 mg/kg 3 hours after MCAo) reversed the increase in infarct size in Thy-1 Cre;EP4^{lox/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.

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-1Cre;EP4^{lox/lox} neuronal cKO mice and control Thy-1Cre;EP4^{+/+} mice were subjected to transient focal ischemia using the MCAo-RP model. We first confirmed activity of Thy-1 Cre recombinase in ROSA-26;Thy-1 Cre mice (see Methods) in cerebral cortex and striatum, as these are the principal structures affected in this isch-

emic model, and found robust Cre recombinase activity in these structures by lacZ staining and immunostaining (Figure 3A and Supplemental Figure 5). Excision of floxed EP4 sequences by Thy-1 Cre was confirmed with immunofluorescence staining for EP4 that showed loss of EP4 signal in Thy-1 Cre;EP4^{lox/lox}, but not Thy-1 Cre;EP4^{+/+}, cerebral cortex in neurons (Figure 3B). In 3-month-old females that underwent 60 minutes MCAo and 23 hours reperfusion, neuronal deletion of EP4 produced a 115% increase in hemispheric infarct size (*P* = 0.02), a 331% increase in cortical infarct size (*P* = 0.01), and a 33% increase in caudate-putamen infarct size (*P* = 0.01), compared with wild-type littermates (Figure 3, C and D, Supplemental Figure 6, and Supplemental Figure 7A), which supported the *in vitro* findings in Figure 2. In male cohorts, infarct size

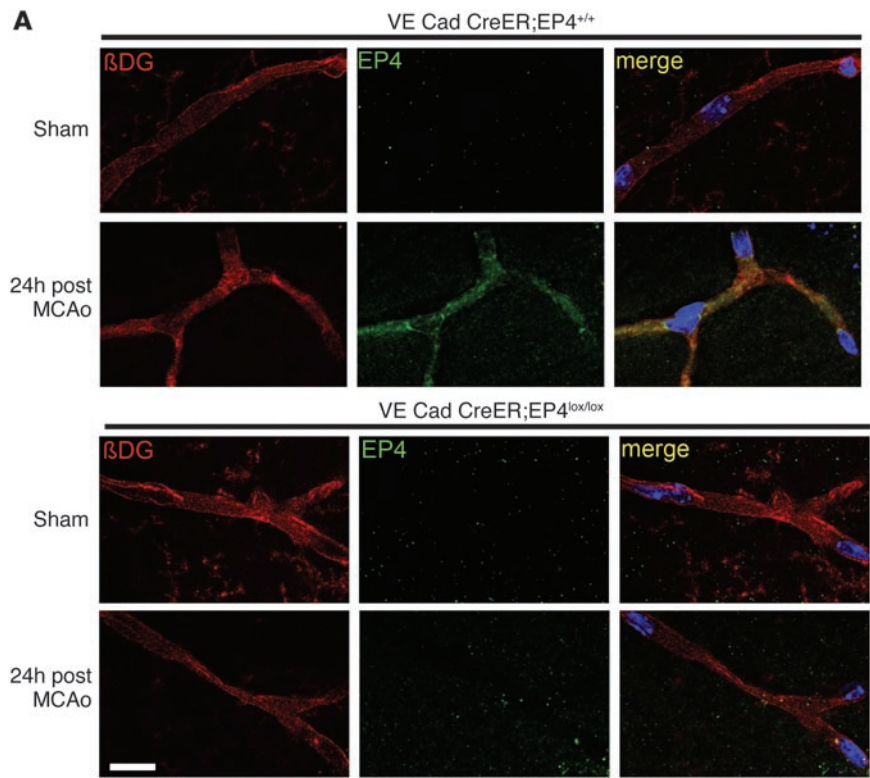
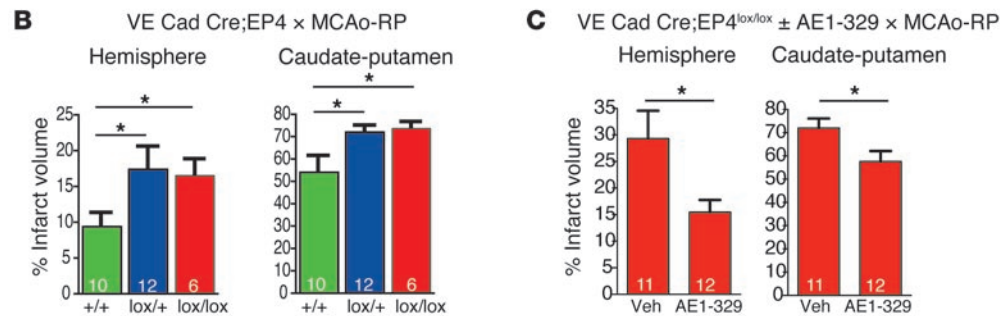


Figure 4

Endothelial EP4 is cerebroprotective. **(A)** Confocal microscopy demonstrated low to undetectable levels of EP4 in control VECad-Cre-ER^{T2};EP4^{+/+} and VECad-Cre-ER^{T2};EP4^{lox/lox} cerebral cortical vasculature at 24 hours after sham surgery. Shown is induction of EP4 expression in VECad-Cre-ER^{T2};EP4^{+/+}, but not VECad-Cre-ER^{T2};EP4^{lox/lox}, cerebral cortical vasculature 24 hours after MCAo. 4 hours after MCAo, EP4 expression was upregulated in endothelium and colocalized with Factor VIII and with β -dystroglycan (Supplemental Figure 8, A and B). Scale bar: 20 μ m. **(B)** Male mice were subjected to 30 minutes of MCAo, and infarct volumes were quantified at 24 hours in cresyl violet-stained brain sections. Infarct size was increased in both VECad-Cre-ER^{T2};EP4^{lox/lox} and VECad-Cre-ER^{T2};EP4^{lox/+} mice compared with control VECad-Cre-ER^{T2};EP4^{+/+} mice. Body temperature and physiological measurements did not differ between genotypes (Supplemental Figure 9B and Supplemental Table 1). * $P \leq 0.05$. **(C)** AE1-329 administration (0.3 mg/kg 3 hours after MCAo) reduced infarct size in VECad-Cre-ER^{T2};EP4^{lox/lox} male mice subjected to 45 minutes MCAo followed by 23 hours of reperfusion. * $P < 0.05$. In **B** and **C**, numerals within bars denote n in each group.

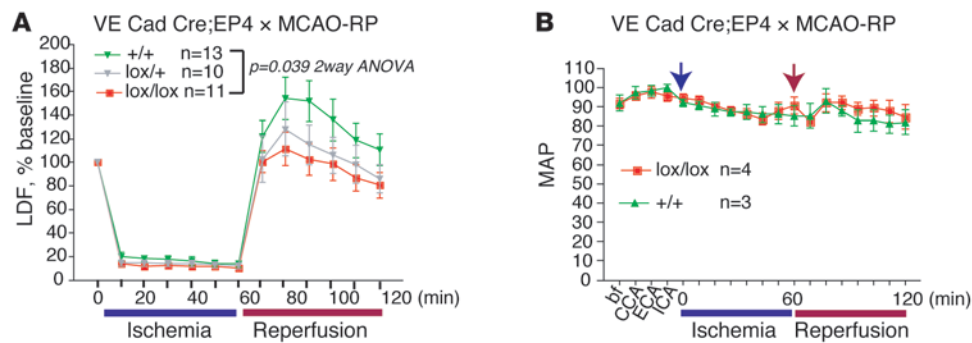


was also increased in a combined cohort of Thy-1 Cre;EP4^{lox/+} and Thy-1 Cre;EP4^{lox/lox} mice compared with control Thy-1 Cre;EP4^{+/+} littermates ($P < 0.05$ for cortex and hemisphere; Figure 3C). Taken together, these data are consistent with an endogenous protective function of neuronal EP4 signaling 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 $n = 6$ 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 ($n = 6$) to the C57B6 background resulted in increased mortality in Thy-1 Cre;EP4^{lox/lox} 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% ($P = 0.05$) in Thy-1 Cre;EP4^{lox/lox} mice compared with controls. Interestingly, AE1-329 administered 3 hours after MCAo significantly reduced infarct

size in Thy-1 Cre;EP4^{lox/lox} mice (hemispheric infarct decreased by 34.9%, $P = 0.03$; cortical infarct decreased by 83.7%, $P = 0.04$). 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^{+/+} plus vehicle, 80%; Thy-1 Cre;EP4^{lox/+} plus vehicle, 61%; Thy-1 Cre;EP4^{lox/lox} plus vehicle, 40%; Thy-1 Cre;EP4^{lox/+} plus AE1-329, 63%; Thy-1 Cre;EP4^{lox/lox} 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 pathway 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 PGE₂ (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

**Figure 5**

Endothelial EP4 regulates CBF during reperfusion. **(A)** Measurement of relative CBF by LDF revealed a significant decrease in cerebral perfusion in male VECad-Cre-ER^{T2};EP4^{lox/lox} mice compared with VECad-Cre-ER^{T2};EP4^{+/+} mice after 60 minutes of MCAo (effect of genotype, $F_{1,22} = 4.769$, $P = 0.039$; effect of time, $F_{12,22} = 74.73$, $P < 0.001$; effect of interaction, $F_{12,22} = 2.168$, $P < 0.05$). Heterozygous VECad-Cre-ER^{T2};EP4^{lox/+} values were intermediate between homozygous and control wild type values, but did not differ significantly from either EP4^{lox/lox} or EP4^{+/+} genotypes. **(B)** MAP before, during, and after ischemia (60 minutes MCAo) did not show differences between genotypes. bf, before any surgery; CCA, common carotid artery ligation; ECA, external carotid artery ligation; ICA, internal carotid ligation. MAP units are mm Hg.

inducible endothelial VECad-Cre-ER^{T2} line (28). Previous studies have demonstrated that tamoxifen effectively triggers excision of floxed sequences in cerebral vasculature of ROSA-26;VECad-Cre-ER^{T2} transgenic mice (28). Using immunostaining, we confirmed using 2 antibodies and 2 different time points after MCAo (4 and 24 hours) that endothelial EP4 was induced in microvasculature in VECad-Cre-ER^{T2};EP4^{+/+} mice, but was absent in VECad-Cre-ER^{T2};EP4^{lox/lox} mice, at 4 and 24 hours after MCAo compared with sham surgery control mice (Figure 4A and Supplemental Figure 8, B and C). After MCAo-RP, cKO of endothelial EP4 significantly increased hemispheric and striatal infarct sizes compared with control littermates at 24 hours in VECad-Cre-ER^{T2};EP4^{lox/lox} mice ($P = 0.05$ and $P = 0.03$, respectively) as well as in VECad-Cre-ER^{T2};EP4^{lox/+} mice ($P = 0.05$ and $P = 0.02$, respectively; Figure 4B; edema shown in Supplemental Figure 7C), indicative of an endogenous cerebroprotective effect of endothelial EP4 signaling in this model. Administration of AE1-329 significantly reduced infarct volume at 24 hours in VECad-Cre-ER^{T2};EP4^{lox/lox} mice, similar to the effect of AE1-329 demonstrated in neuronal cKO mice (Figure 3E). Astrocytes did not express EP4 either basally or after MCAo, and microglial EP4 expression remained low after MCAo (Supplemental Figure 10), which suggests that neuronal EP4 might also be a target of AE1-329 effects.

PGE₂ elicits vasodilation in systemic vasculature through EP2 and EP4 signaling (26, 27, 29). We tested whether deletion of endothelial EP4 would alter cerebral perfusion during and after ischemia. We measured relative cerebral blood flow (CBF) using laser Doppler flowmetry (LDF) in mice subjected to 60 minutes of ischemia followed by 60 minutes of reperfusion. Conditional deletion of endothelial EP4 in VECad-Cre-ER^{T2};EP4^{lox/lox} mice resulted in a significant reduction in reperfusion blood flow compared with VECad-Cre-ER^{T2};EP4^{+/+} mice ($P = 0.039$; Figure 5A). Importantly, this decrease in cerebral perfusion was not associated with changes in systemic mean arterial pressure (MAP) during or after MCAo-RP (Figure 5B). These findings indicate that cerebral endothelial EP4 signaling is protective and enhances cerebral blood perfusion after ischemia.

An important mechanism underlying increased vascular perfusion is eNOS-mediated vasodilation, in which activation of eNOS through phosphorylation at Ser¹¹⁷⁷ leads to increased NO production, cGMP-dependent smooth muscle cell relaxation,

and increased blood flow (30, 31). We therefore investigated whether activation of endothelial EP4 signaling in vivo regulated levels and phosphorylation state of eNOS in cerebral microvasculature (Figure 6). Levels of total Akt and phospho-Akt, which phosphorylates eNOS at Ser¹¹⁷⁷ (32, 33), as well as total eNOS and Ser¹¹⁷⁷ phospho-eNOS, were measured in microvessels isolated from cerebral cortices of mice 1 and 5 hours after systemic administration of AE1-329 (0.3 mg/kg). AE1-329 caused a modest increase in total Akt and a marked increase in phospho-Akt 5 hours after systemic administration ($P < 0.05$). Importantly, levels of total eNOS and phospho-eNOS were significantly elevated at 5 hours after AE1-329 administration ($P < 0.01$ and $P < 0.001$, respectively; Figure 6, A and B). To further validate this finding, we carried out a complementary set of genetic experiments – systemic administration of AE1-329 to VECad-Cre-ER^{T2};EP4^{+/+} and VECad-Cre-ER^{T2};EP4^{lox/lox} mice – and tested whether absence of endothelial EP4 abrogates phosphorylation and activation of eNOS 5 hours after administration of AE1-329. Microvessels from both genotypes were examined for levels of total Akt, eNOS, phospho-Akt, and phospho-eNOS (Figure 6, C and D). Quantitative Western analysis demonstrated that levels of phospho-Akt and phospho-eNOS in cerebral microvessels robustly increased in VECad-Cre-ER^{T2};EP4^{+/+} mice, as expected, but remained unchanged in VECad-Cre-ER^{T2};EP4^{lox/lox} mice. Total levels of Akt and eNOS were also significantly increased in VECad-Cre-ER^{T2};EP4^{+/+} mice stimulated with AE1-329, but this effect was blunted in VECad-Cre-ER^{T2};EP4^{lox/lox} mice. Taken together, these pharmacologic and genetic data suggest that endothelial EP4 signaling in vivo may enhance cerebral perfusion through increased eNOS expression and activation.

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. Pharmacologic stimulation in vivo of EP4 3

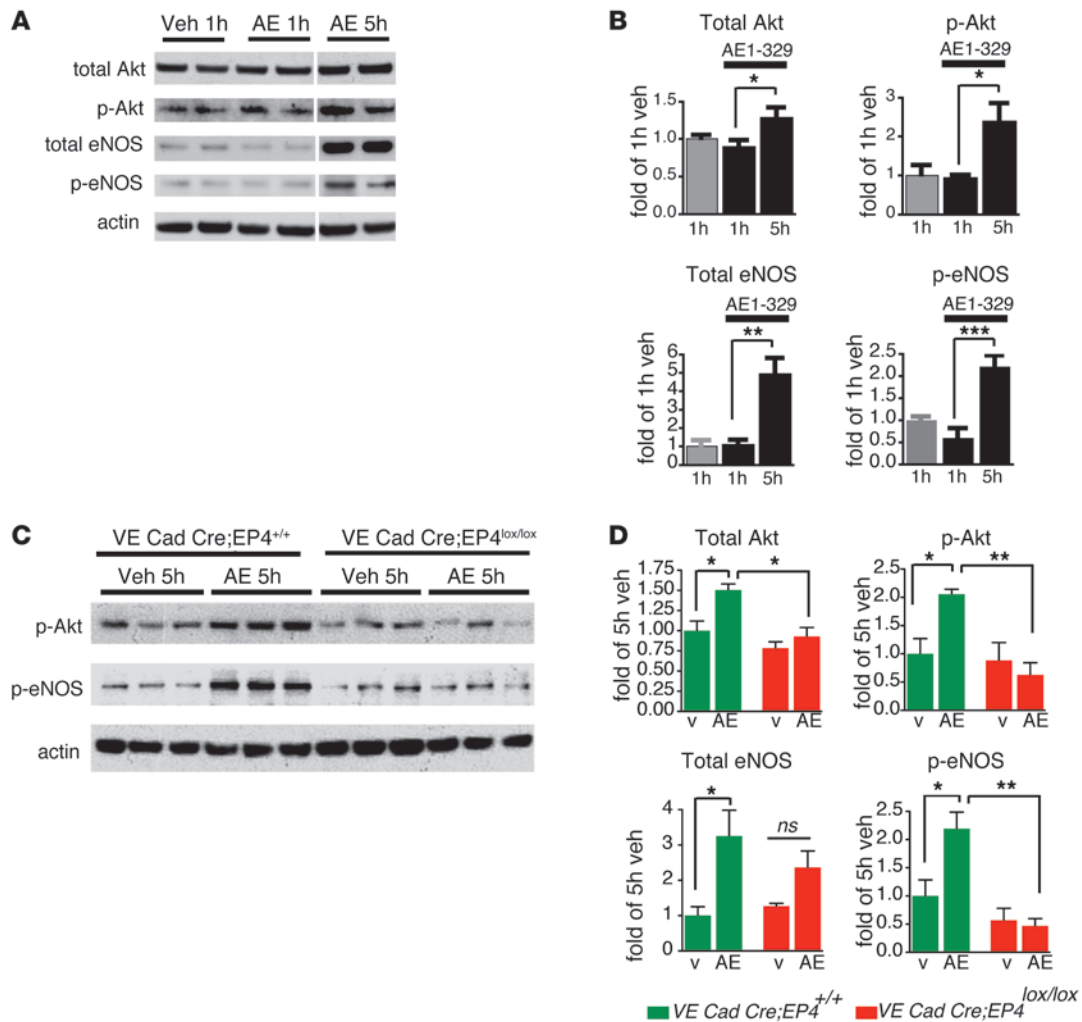


Figure 6

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^{T2};EP4^{+/+} and VECad-Cre-ER^{T2};EP4^{lox/lox} 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^{T2};EP4^{+/+} mice that was absent in VECad-Cre-ER^{T2};EP4^{lox/lox} 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^{T2};EP4^{+/+} mice; such increases were not seen in VECad-Cre-ER^{T2};EP4^{lox/lox} mice. *n* = 3 per group. **P* < 0.05; ***P* < 0.01.

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



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 stroked conditions and was induced to high levels in endothelium after ischemia (21). Examination of EP4 expression in other cell types in sham and stroked 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-ERT² 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-ERT²;EP4^{lox/lox} and VECad-Cre-ERT²;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-ERT²;EP4^{lox/lox} brains, we cannot exclude an excision efficiency of less than 100% by the VECad-Cre-ERT² recombinase; success-

ful excision of floxed sequences by this Cre recombinase requires both tamoxifen-mediated nuclear translocation of Cre-ERT² 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; stock no. 100006) were used to test pharmacologic effects of AE1-329. C57B6 EP4^{lox/lox} mice were provided by R. Breyer and M. Breyer (Vanderbilt University School of Medicine, Nashville, Tennessee, USA). C57B6 VECad-Cre-ER^{T2} mice were provided by L.I. Arispe (UCLA, Los Angeles, California, USA) (28). Tamoxifen (Sigma-Aldrich) was administered to all VECad-Cre-ER^{T2} 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/ethanol), 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(Thy1-cre)1Vln/J] 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-Gt(ROSA)26Soy^{tm1Sor}/J (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-ER^{T2} experiments, Cre:EP4^{lox/lox}, Cre:EP4^{lox/+}, and Cre:EP4^{+/+} littermate mice were generated by serial crosses of Cre and EP4^{lox/lox} or EP4^{lox/+} 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-RP model. All MCAo-RP 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 cresyl 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 physiological measurements. The femoral artery was cannulated for measurement of arterial blood gases and MAP as previously described (14). Artery blood samples were analyzed for blood gas and electrolytes using an I-STAT analyzer (I-STAT). MAP was measured with a microcannula connected to a pressure transducer (Harvard Apparatus).

Cultured primary neurons and organotypic hippocampal slices and assessment of neuronal injury. Primary neuronal cell cultures and organotypic hippocampal slices were established and analyzed as previously described (14).

Immunohistochemistry. Free-floating 40- μ m coronal brain sections were generated and processed for immunostaining as previously described (20). The cerebral cortical peri-infarct areas examined are adjacent to the infarct border (defined as a loss of NeuN and/or MAP2 neuronal staining). The peri-infarct area examined lies within 150–200 μ m of the infarct border. See Supplemental Methods for details of antibodies used. Images were acquired

by sequential scanning using the Leica TCS SPE confocal system and DM 5500 Q microscope (Leica Microsystems) with laser lines 405, 488, and 532 nm. Sections corresponding to 2 μ m (tissue slices) or 4 μ m (cultured cells) were selected and equally processed in Leica LAS AF (Leica Microsystems); collapsed stacks were obtained with ImageJ software (NIH).

Western blot analysis. Quantitative Western blot analysis was performed as previously described (20).

Cerebral microvessel preparation. Cerebral microvessels were isolated from fresh cerebral cortex and hippocampus devoid of meninges, as described previously (53), with modifications. Homogenized samples were mixed with an equal volume of 30% dextran and centrifuged at 3,345 g for 10 minutes at 4°C. The top fatty layer was removed, and the supernatant was mixed with an equal volume of Earle balanced salt solution (EBBS) and recentrifuged. Pellets were resuspended in EBSS and passed first through a 70- μ m and then a 40- μ m nylon filter. Microvessels retained on this filter were washed with EBSS, collected by centrifugation at 209 g for 10 minutes at 4°C, and resuspended in lysis buffer for protein extraction. Microvessel purity was confirmed using immunocytochemistry and Western quantification, which demonstrated marked enrichment of endothelial cell-cell adhesion molecules (e.g., claudin-5) and total depletion of neuronal markers (MAP-2) compared with cerebral cortical lysates (data not shown).

Statistics. Statistical analysis was performed by Student's *t* test or 1-way ANOVA, followed by Tukey post-hoc analysis. For behavioral analyses and LDF measurements, 2-way ANOVA with repeated measures (time and genotype or time and treatment) was used, with genotype or treatment as the main effect. All data are reported as mean \pm SEM. *P* values of 0.05 or less were considered significant.

Study approval. This study was conducted in accordance with NIH guidelines for the use of experimental animals. Protocols were approved by the Institutional Animal Care and Use Committee at Stanford University.

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1. Lloyd-Jones D, et al. Heart disease and stroke statistics--2010 update: a report from the American Heart Association. *Circulation*. 2010;121(7):e46–e215.
2. Fang MC, Cutler DM, Rosen AB. Trends in thrombolytic use for ischemic stroke in the United States. *J Hosp Med*. 2010;5(7):406–409.
3. Chavez JC, Hurko O, Barone FC, Feuerstein GZ. Pharmacologic interventions for stroke: looking beyond the thrombolysis time window into

- the penumbra with biomarkers, not a stopwatch. *Stroke*. 2009;40(10):e558–563.
4. Breyer RM, Bagdassarian CK, Myers SA, Breyer MD. Prostanoid receptors: subtypes and signaling. *Annu Rev Pharmacol Toxicol*. 2001;41:661–690.
5. Hewett SJ, Bell SC, Hewett JA. Contributions of cyclooxygenase-2 to neuroplasticity and neuropathology of the central nervous system. *Pharmacol Ther*. 2006;112(2):335–357.

6. Funk CD, FitzGerald GA. COX-2 inhibitors and cardiovascular risk. *J Cardiovasc Pharmacol*. 2007;50(5):470–479.
7. Cheng Y, et al. Role of prostacyclin in the cardiovascular response to thromboxane A2. *Science*. 2002;296(5567):539–541.
8. Narumiya S, FitzGerald GA. Genetic and pharmacological analysis of prostanoid receptor function. *J Clin Invest*. 2001;108(1):25–30.



9. Nataraj C, et al. Receptors for prostaglandin E(2) that regulate cellular immune responses in the mouse. *J Clin Invest*. 2001;108(8):1229–1235.
10. Andreasson K. Emerging roles of PGE2 receptors in models of neurological disease. *Prostaglandins Other Lipid Mediat*. 2010;91(3–4):104–112.
11. Nogawa S, Zhang F, Ross ME, Iadecola C. Cyclooxygenase-2 gene expression in neurons contributes to ischemic brain damage. *J Neurosci*. 1997;17(8):2746–2755.
12. Kawano T, et al. Prostaglandin E2 EP1 receptors: downstream effectors of COX-2 neurotoxicity. *Nat Med*. 2006;12(2):225–229.
13. Zhou P, Qian L, Chou T, Iadecola C. Neuroprotection by PGE2 receptor EP1 inhibition involves the PTEN/AKT pathway. *Neurobiol Dis*. 2008;29(3):543–551.
14. McCullough L, et al. Neuroprotective function of the PGE2 EP2 receptor in cerebral ischemia. *J Neurosci*. 2004;24(1):257–268.
15. Bilak M, et al. PGE2 receptors rescue motor neurons in a model of amyotrophic lateral sclerosis. *Ann Neurol*. 2004;56(2):240–248.
16. Liang X, Wu L, Hand T, Andreasson K. Prostaglandin D2 mediates neuronal protection via the DP1 receptor. *J Neurochem*. 2005;92(3):477–486.
17. Maruyama T, et al. Design and synthesis of a highly selective EP4-receptor agonist. Part 1: 3,7-dithiaPG derivatives with high selectivity. *Bioorg Med Chem Lett*. 2001;11(15):2029–2031.
18. Suzawa T, et al. The role of prostaglandin E receptor subtypes (EP1, EP2, EP3, and EP4) in bone resorption: an analysis using specific agonists for the respective EPs. *Endocrinology*. 2000;141(4):1554–1559.
19. Fujino H, Regan JW. Prostanoid receptors and phosphatidylinositol 3-kinase: a pathway to cancer? *Trends Pharmacol Sci*. 2003;24(7):335–340.
20. Shi J, Johansson J, Woodling NS, Wang Q, Montine TJ, Andreasson K. The prostaglandin E2 E-prostanoid 4 receptor exerts anti-inflammatory effects in brain innate immunity. *J Immunol*. 2010;184(12):7207–7218.
21. Li J, Liang X, Wang Q, Breyer RM, McCullough L, Andreasson K. Misoprostol, an anti-ulcer agent and PGE(2) receptor agonist, protects against cerebral ischemia. *Neurosci Lett*. 2008;438(2):210–215.
22. Barone FC, Knudsen DJ, Nelson AH, Feuerstein GZ, Willette RN. Mouse strain differences in susceptibility to cerebral ischemia are related to cerebral vascular anatomy. *J Cereb Blood Flow Metab*. 1993;13(4):683–692.
23. Connolly ES Jr, Winfree CJ, Stern DM, Solomon RA, Pinsky DJ. Procedural and strain-related variables significantly affect outcome in a murine model of focal cerebral ischemia. *Neurosurgery*. 1996;38(3):523–531.
24. Maeda K, Hata R, Hossmann KA. Regional metabolic disturbances and cerebrovascular anatomy after permanent middle cerebral artery occlusion in C57black/6 and SV129 mice. *Neurobiol Dis*. 1999;6(2):101–108.
25. Majid A, et al. Differences in vulnerability to permanent focal cerebral ischemia among 3 common mouse strains. *Stroke*. 2000;31(11):2707–2714.
26. Audoly LP, et al. Identification of specific EP receptors responsible for the hemodynamic effects of PGE2. *Am J Physiol*. 1999;277(3 pt 2):H924–930.
27. Zhang Y, Guan Y, Schneider A, Brandon S, Breyer RM, Breyer MD. Characterization of murine vasopressor and vasodepressor prostaglandin E(2) receptors. *Hypertension*. 2000;35(5):1129–1134.
28. Monvoisin A, Alva JA, Hofmann JJ, Zovein AC, Lane TF, Iruela-Arispe ML. VE-cadherin-CreERT2 transgenic mouse: a model for inducible recombination in the endothelium. *Dev Dyn*. 2006;235(12):3413–3422.
29. Narumiya S, Sugimoto Y, Ushikubi F. Prostanoid receptors: structures, properties, and functions. *Physiol Rev*. 1999;79(4):1193–1226.
30. Gallis B, et al. Identification of flow-dependent endothelial nitric-oxide synthase phosphorylation sites by mass spectrometry and regulation of phosphorylation and nitric oxide production by the phosphatidylinositol 3-kinase inhibitor LY294002. *J Biol Chem*. 1999;274(42):30101–30108.
31. Endres M, Laufs U, Liao JK, Moskowitz MA. Targeting eNOS for stroke protection. *Trends Neurosci*. 2004;27(5):283–289.
32. Yano S, et al. Activation of Akt/protein kinase B contributes to induction of ischemic tolerance in the CA1 subfield of gerbil hippocampus. *J Cereb Blood Flow Metab*. 2001;21(4):351–360.
33. Bozulic L, Hemmings BA. PIKING on PKB: regulation of PKB activity by phosphorylation. *Curr Opin Cell Biol*. 2009;21(2):256–261.
34. Dumont I, et al. Developmental regulation of endothelial nitric oxide synthase in cerebral vessels of newborn pig by prostaglandin E(2). *J Pharmacol Exp Ther*. 1999;291(2):627–633.
35. Zhang Y, Jin Y, Behr MJ, Feustel PJ, Morrison JP, Kimelberg HK. Behavioral and histological neuroprotection by tamoxifen after reversible focal cerebral ischemia. *Exp Neurol*. 2005;196(1):41–46.
36. Kimelberg HK, et al. Acute treatment with tamoxifen reduces ischemic damage following middle cerebral artery occlusion. *Neuroreport*. 2000;11(12):2675–2679.
37. Feng Y, Fratkins JD, LeBlanc MH. Treatment with tamoxifen reduces hypoxic-ischemic brain injury in neonatal rats. *Eur J Pharmacol*. 2004;484(1):65–74.
38. Kennedy CR, et al. Salt-sensitive hypertension and reduced fertility in mice lacking the prostaglandin EP2 receptor. *Nat Med*. 1999;5(2):217–220.
39. Tilley SL, et al. Reproductive failure and reduced blood pressure in mice lacking the EP2 prostaglandin E2 receptor. *J Clin Invest*. 1999;103(11):1539–1545.
40. Zhang F, White JG, Iadecola C. Nitric oxide donors increase blood flow and reduce brain damage in focal ischemia: evidence that nitric oxide is beneficial in the early stages of cerebral ischemia. *J Cereb Blood Flow Metab*. 1994;14(2):217–226.
41. Morikawa E, Moskowitz MA, Huang Z, Yoshida T, Irikura K, Dalkara T. L-arginine infusion promotes nitric oxide-dependent vasodilation, increases regional cerebral blood flow, and reduces infarction volume in the rat. *Stroke*. 1994;25(2):429–435.
42. Huang Z, et al. Enlarged infarcts in endothelial nitric oxide synthase knockout mice are attenuated by nitro-L-arginine. *J Cereb Blood Flow Metab*. 1996;16(5):981–987.
43. Dalkara T, Morikawa E, Panahian N, Moskowitz MA. Blood flow-dependent functional recovery in a rat model of focal cerebral ischemia. *Am J Physiol*. 1994;267(2 pt 2):H678–H683.
44. Sun CW, Falck JR, Okamoto H, Harder DR, Roman RJ. Role of cGMP versus 20-HETE in the vasodilator response to nitric oxide in rat cerebral arteries. *Am J Physiol Heart Circ Physiol*. 2000;279(1):H339–H350.
45. Endres M, et al. Stroke protection by 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitors mediated by endothelial nitric oxide synthase. *Proc Natl Acad Sci U S A*. 1998;95(15):8880–8885.
46. Wolfrum S, et al. Simvastatin acutely reduces myocardial reperfusion injury in vivo by activating the phosphatidylinositol 3-kinase/Akt pathway. *J Cardiovasc Pharmacol*. 2004;44(3):348–355.
47. McNeill AM, Kim N, Duckles SP, Krause DN, Kontos HA. Chronic estrogen treatment increases levels of endothelial nitric oxide synthase protein in rat cerebral microvessels. *Stroke*. 1999;30(10):2186–2190.
48. Stirone C, Boroujerdi A, Duckles SP, Krause DN. Estrogen receptor activation of phosphoinositide-3 kinase, akt, and nitric oxide signaling in cerebral blood vessels: rapid and long-term effects. *Mol Pharmacol*. 2005;67(1):105–113.
49. Darby DG, et al. Pathophysiological topography of acute ischemia by combined diffusion-weighted and perfusion MRI. *Stroke*. 1999;30(10):2043–2052.
50. Davis RJ, et al. EP4 prostanoid receptor-mediated vasodilatation of human middle cerebral arteries. *Br J Pharmacol*. 2004;141(4):580–585.
51. Hayashi S, McMahon AP. Efficient recombination in diverse tissues by a tamoxifen-inducible form of Cre: a tool for temporally regulated gene activation/inactivation in the mouse. *Dev Biol*. 2002;244(2):305–318.
52. Dewachter I, et al. Neuronal deficiency of presenilin 1 inhibits amyloid plaque formation and corrects hippocampal long-term potentiation but not a cognitive defect of amyloid precursor protein [V717I] transgenic mice. *J Neurosci*. 2002;22(9):3445–3453.
53. Tontsch U, Bauer HC. Isolation, characterization, and long-term cultivation of porcine and murine cerebral capillary endothelial cells. *Microvasc Res*. 1989;37(2):148–161.