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Activated protein C inhibits tissue plasminogen activator–induced brain hemorrhage

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Brain hemorrhage is a serious complication of tissue plasminogen activator (tPA) therapy for ischemic stroke. Here we report that activated protein C (APC), a plasma serine protease with systemic anticoagulant, anti-inflammatory and antiapoptotic activities, and direct vasculoprotective and neuroprotective activities, blocks tPA-mediated brain hemorrhage after transient brain ischemia and embolic stroke in rodents. We show that APC inhibits a pro-hemorrhagic tPA-induced, NF-κB–dependent matrix metalloproteinase-9 pathway in ischemic brain endothelium *in vivo* and *in vitro* by acting through protease-activated receptor 1. The present findings suggest that APC may improve thrombolytic therapy for stroke, in part, by reducing tPA-mediated hemorrhage.

Symptomatic brain hemorrhage[1] is a major complication of thrombolytic therapy for ischemic stroke with tissue plasminogen activator (tPA). tPA activates matrix metalloproteinase-9 (MMP-9) in brain endothelium, which degrades the vascular basement membrane[2] and proteins associated with the blood-brain barrier (BBB)[3]. A number of experimental[2,4–6] and clinical studies[7] have indicated that the tPA–MMP-9 pathway mediates postischemic disruption of the BBB preceding brain hemorrhage. MMP-9 also degrades neuronal laminin, which contributes to neuronal apoptosis[8,9] acutely after stroke[10].

Activated protein C (APC) is a plasma serine protease with systemic anticoagulant, anti-inflammatory and antiapoptotic activities[11–13], and direct neuronal protective activity[14]. APC stabilizes vascular endothelial barriers[15,16] and has a low risk for brain hemorrhage in patients with severe sepsis[17] and in animal models of stroke[13,18,19]. APC reduces brain damage after transient ischemia[13] and embolic stroke[19] in rodents and inhibits the intrinsic and extrinsic apoptotic pathways in injured neurons[14] and ischemic brain endothelium[13,18]. APC can protect neurons and neurovascular cells from tPA toxicity[18], but its effects on tPA-mediated hemorrhage and the tPA–MMP-9 pathway are not known.

RESULTS

APC blocks tPA-induced hemorrhage in stroke models

Infusion of recombinant human tPA (rh-tPA) in mice subjected to focal transient ischemia resulted in brain hemorrhage (**Fig. 1a**) in all studied mice (6 of 6) and increased, by 3.6-fold, hemoglobin levels in the ischemic hemisphere (**Fig. 1b**). Sham-operated mice treated with rh-tPA and mice that had transient middle cerebral artery occlusion (MCAO) did not have hemorrhage (**Fig. 1a,b**). APC infused simultaneously with rh-tPA or 3 h after tPA blocked postischemic rh-tPA–mediated bleeding (**Fig. 1a**) and brain hemoglobin influx (**Fig. 1a,b**). The antihemorrhagic effect of APC on tPA-induced bleeding was dose dependent (**Fig. 1b**). rh-tPA substantially increased brain infarction in mice subjected to transient ischemia (**Fig. 1c**), whereas APC dose-dependently reduced brain infarction in rh-tPA–treated mice (**Fig. 1c**). Infusion of APC alone was neuroprotective (**Fig. 1c**), as reported[13]. We did not observe postischemic hemorrhage or increase in brain hemoglobin influx in vehicle-treated mice or after infusion of APC (**Fig. 1b**), as reported[13,19]. Blood gases and pH were not substantially altered by vehicle, rh-tPA, APC and a combination of rh-tPA and APC before, during and after transient MCAO (**Supplementary Table 1** online).

On the basis of our observation that APC blocks tPA-induced brain hemorrhage, we hypothesized that APC interferes with the tPA–MMP-9 pathway, which mediates BBB breakdown[2,4,6]. Gelatin zymography of brain tissue indicated a marked increase in pro-MMP-9 and a modest increase in the levels of activated MMP-9 in mice subjected to transient ischemia, as reported[9], compared to undetectable MMP-9 levels in sham-operated mice and mice treated with rh-tPA or rh-tPA and APC (**Fig. 1d**). We then observed a substantial increase in pro-MMP-9 and the activated MMP-9 in mice subjected to transient MCAO and rh-tPA compared to mice treated with MCAO and vehicle. Immunostaining analysis confirmed that MMP-9 colocalized with ischemic brain endothelium acutely after stroke (**Fig. 1e**), as reported[3]. Mice subjected to MCAO and rh-tPA therapy exhibited a significant ($P < 0.001$) increase in MMP-9 microvascular staining compared to control mice subjected to ischemia and vehicle (**Fig. 1f**). Collagen IV, a major vascular basement membrane protein, and

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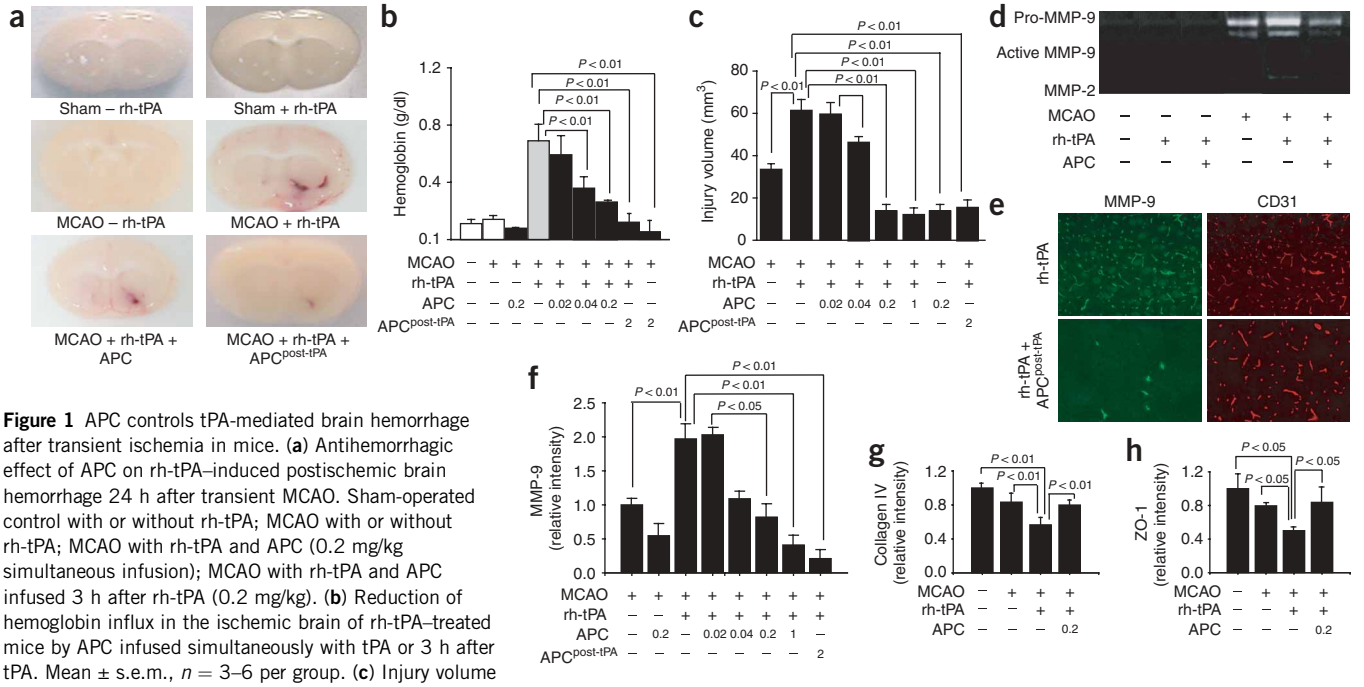

Figure 1 APC controls tPA-mediated brain hemorrhage after transient ischemia in mice. (**a**) Antihemorrhagic effect of APC on rh-tPA–induced postischemic brain hemorrhage 24 h after transient MCAO. Sham-operated control with or without rh-tPA; MCAO with or without rh-tPA; MCAO with rh-tPA and APC (0.2 mg/kg simultaneous infusion); MCAO with rh-tPA and APC infused 3 h after rh-tPA (0.2 mg/kg). (**b**) Reduction of hemoglobin influx in the ischemic brain of rh-tPA–treated mice by APC infused simultaneously with tPA or 3 h after tPA. Mean ± s.e.m., $n = 3$–6 per group. (**c**) Injury volume in mice treated with rh-tPA and APC, as determined at 24 h. Mean ± s.e.m. $n = 3$–6 per group. (**d**) MMP-9 gelatin zymography in the ischemic brain in the presence or absence of rh-tPA and APC (0.2 mg/kg) after MCAO. Typical results from five independent experiments are shown. (**e**) Immunostaining for MMP-9 and brain vascular CD31 after MCAO and rh-tPA or rh-tPA and 3 h after TPA (2 mg/kg). (**f**) Dose-dependent reduction in MMP-9 immunostaining in ischemic brain microvessels in mice treated with rh-tPA and APC. APC was infused either simultaneously with rh-tPA or 3 h after rh-tPA. Mean ± s.e.m. $n = 3$–5. (**g**,**h**) Relative intensity of immunostaining for brain vascular collagen IV and ZO-1 after transient MCAO with and without rh-tPA or rh-tPA and APC. Mean ± s.e.m. $n = 3$–5 per group. APC, mouse recombinant APC (mg/kg). APC$^{post\text{-}tPA}$, APC infused 3 h after TPA. Numbers in *x*-axis labels for panels **b**,**c**,**f**–**h** indicate concentrations of APC in mg/kg.

zonula occludens (ZO-1), a major tight junction BBB protein, were moderately reduced by ischemia, by 14% and 16%, respectively (**Fig. 1g,h**). rh-tPA substantially reduced both collagen IV and ZO-1 abundance in ischemic brain, by 45% and 50%, respectively, relative to sham-operated controls (**Fig. 1g,h**). MMP-2 levels were barely detectable in sham-operated mice and were not substantially altered by ischemia, rh-tPA or APC (**Fig. 1d**).

Next, we tested whether APC at a dose of 0.2 mg per kg body weight, which reduces brain hemoglobin influx and infarction by about 70–75%, can also control tPA-mediated increase in MMP-9 in ischemic brain and microvessels and prevent loss of collagen IV and ZO-1. APC substantially reduced the levels of pro-MMP-9 and the activated MMP-9 in mice treated with rh-tPA and ischemia (**Fig. 1d**). APC infused simultaneously with rh-tPA (data not shown) or 3 h after rh-tPA reduced the intensity of MMP-9 immunostaining in ischemic brain endothelium almost to the background level (**Fig. 1e,f**). The dose-dependent effect of APC on MMP-9 vascular staining (**Fig. 1f**) correlated with APC dose–dependent reduction in hemoglobin influx in mice subjected to rh-tPA and ischemia (**Fig. 1b**). The decrease in collagen IV and ZO-1 caused by rh-tPA and ischemia was reversed by APC (**Fig. 1g,h**; **Supplementary Figs. 1** and **2** online), showing that APC may prevent degradation of proteins crucial for BBB integrity.

We next subjected rats to thromboembolic stroke[20] to determine whether APC can block tPA-induced hemorrhage in a different model. Four hours after stroke, rats received either rh-tPA alone, APC alone or a combination of rh-tPA and APC. rh-tPA induced substantial microscopic hemorrhage in this model after 1 d and 7 d, whereas APC reduced hemorrhage by >90% and by 80% after 1 d and 7 d, respectively (**Fig. 2a,b**). rh-tPA alone did not reduce brain infarction or improve the neurological severity score (NSS) or the foot fault test score after 1 d and 7 d (**Fig. 2c–f**), consistent with earlier reports with tPA in the clot model[20,21]. In contrast, APC alone or in combination with rh-tPA substantially reduced brain injury at 1 d and 7 d, and improved functional recovery compared to that in vehicle-treated or rh-tPA–treated rats. Consistent with the reductions in microscopic hemorrhage, APC reduced the number of MMP-9–positive brain microvessels and increased the number of collagen IV–positive brain microvessels compared to those in vehicle-treated and rh-tPA–treated rats (**Fig. 2g,h**), whereas rh-tPA alone had the opposite effect, as reported[20].

APC blocks a tPA-induced NF-κB-MMP-9 pathway

To investigate the mechanism underlying APC-mediated downregulation of MMP-9 in tPA-treated ischemic brain endothelium, we used a primary human brain endothelial cell (BEC) culture system. Human BEC exposed to oxygen and glucose deprivation (OGD) and rh-tPA showed a substantial 3.5- to 4-fold increase in pro-MMP-9 and activated MMP-9 levels in the medium compared to OGD-treated BEC (**Fig. 3a**). The levels of both MMP-9 forms were barely detectable in normoxic controls (data not shown). The tPA-mediated increase in pro-MMP-9 and activated MMP-9 was dose-dependently inhibited by APC (**Fig. 3a**). hr-tPA rapidly increased *MMP9* mRNA levels in OGD-treated BEC (**Fig. 3b**), indicating transcriptional upregulation of *MMP9* (ref. 2), which was blocked by APC (**Fig. 3b**).

tPA upregulated MMP-9 activity in OGD-treated BEC via the low-density lipoprotein receptor related protein 1 (LRP1)[2], as confirmed by blockade of MMP-9 release into the medium by an

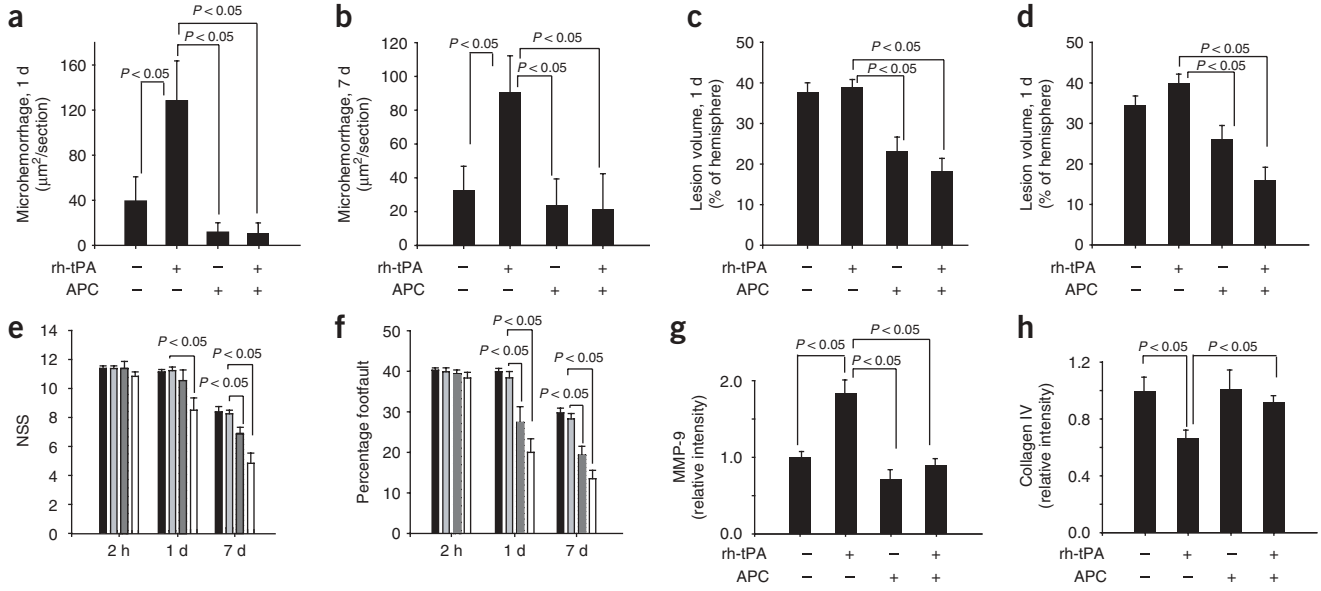

Figure 2 APC blocks tPA-induced brain hemorrhage after embolic stroke in rats. (**a**–**d**) Microscopic hemorrhage 24 h (**a**) and 7 d (**b**) after stroke, and brain injury volume 24 h (**c**) and 7 d (**d**) after stroke, with and without rh-tPA, APC or rh-tPA + APC. (**e**,**f**) Neurological severity score (NSS) (**e**) and foot fault test (**f**) 2 h, 24 h and 7 d after stroke in rats treated with saline (black), rh-tPA (light gray), APC (dark gray) and rh-tPA + APC (white). (**g**,**h**) Relative intensity of immunostaining for MMP-9 in ischemic brain microvessels (**g**) and collagen IV (**h**) 24 h after stroke with and without rh-tPA, APC and rh-tPA + APC. Mean ± s.e.m. $n = 6$–8 per group.

LRP1-specific antibody and by knockdown of LRP1 expression using short hairpin RNA targeting LRP1 (shLRP1) (**Fig. 3c**). In contrast, blockade of LRP2 and the very low density lipoprotein receptor (VLDLR) by receptor-specific antibodies did not inhibit tPA-mediated upregulation of MMP-9 (**Fig. 3c**). α2-macroglobulin, a specific endocytotic ligand for LRP1 (ref. 22), did not affect MMP-9 levels, suggesting that tPA-mediated signaling via LRP1 and its endocytotic function as a cargo receptor are distinct, as shown before[2]. APC's inhibition of MMP-9 required the protease-activated receptor 1 (PAR1) and endothelial protein C receptor (EPCR), as demonstrated by blockade of APC's effect with a PAR1-specific blocking antibody and an EPCR antibody that inhibits the APC binding site (**Fig. 3c**), as reported[12,13].

As the *MMP9* gene promoter has binding sites for NF-kB and AP-1 (ref. 2), both of which can be activated by ischemia[23,24], we next studied whether these transcription factors have a role in tPA-mediated MMP-9 upregulation in our OGD model. First, we studied the effects of pyrollidine dithiocarbamate (PDTC), a relatively selective NF-kB inhibitor, and curcumin, a potent AP-1 inhibitor[2] on pro-MMP-9 and activated MMP-9. PDTC, but not curcumin, blocked MMP-9 release in OGD/hr-tPA–treated BEC (**Fig. 3d**). We confirmed the crucial role of NF-kB in MMP-9 upregulation in the present model by demonstrating a complete inhibition of MMP-9 upregulation in OGD/rh-tPA–treated BEC overexpressing a mutant form of IκB-α (S32A,S36A), which irreversibly and selectively blocks NF-κB activation (ref. 25 and **Fig. 3e**). We did not observe a substantial change in MMP-2 activity in OGD-treated BEC with or without hr-tPA (**Fig. 3e**).

To show which form of MMP-9 is responsible for the breakdown of the neurovascular matrix and disruption of the BBB, we studied *ex vivo* degradation of vascular collagen IV by exogenous pro-MMP-9 and activated MMP-9. Double immunolabeling of collagen IV and brain endothelium with CD31 antibody in mouse brain revealed that only activated MMP-9 degraded collagen IV (**Fig. 3f**). Latent pro-MMP-9 did not degrade collagen IV. Note that the sodium dodecyl sulfate used in this experiment to separate pro-MMP-9 from MMP-9 can chemically activate pro-MMP-9 in the absence of cleavage[26]. We confirmed this by demonstrating that exogenous pro-MMP-9 exhibited gelantinolytic activity with sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (**Supplementary Fig. 3** online), although it cannot degrade neuronal laminin[9] or vascular collagen (**Fig. 3f**) in the mouse brain.

Because APC blocks NF-κB activation after exposure of the endothelial cells to tumor necrosis factor-α (ref. 11), we hypothesized that APC inhibits the tPA–MMP-9 pathway by inhibiting NF-κB. Indeed, APC blocked nuclear translocation of NF-κB in BEC subjected to rh-tPA and OGD, as shown by gel shift and immunostaining assays (**Fig. 4a**,**b**). In contrast, rh-tPA enhanced NF-κB nuclear translocation (**Fig. 4a**,**b**) and induced a transitory increase in *RELA* which encodes the p65 subunit of NF-kB and which was downregulated by APC (**Fig. 4c**). This result is consistent with microarray data demonstrating suppression of *RELA* and *Nfkb2* mRNAs in endothelial cells by APC (refs. 11,27). We also showed that APC blocked NF-κB (p65) expression in the ischemic mouse brain, assessed 24 h after transient ischemia and rh-tPA treatment (**Fig. 4d**,**e**).

PDTC blocks NF-κB nuclear translocation in ischemic neurons *in vivo* and therefore protects against brain ischemia in rats[28]. We tested whether PDTC can inhibit tPA-induced hemorrhage and MMP-9 induction in brain microvessels *in vivo*. PDTC reduced brain hemoglobin influx, the number of MMP-9–positive vessels and the injury volume in mice subjected to hr-tPA and ischemia (**Fig. 4f–h**). These data confirm that rh-tPA–mediated MMP-9 upregulation *in vivo* in this stroke model depends on NF-κB, as it does in OGD- and rh-tPA–treated BEC *in vitro* (**Fig. 3d**,**e**). Autoradiographic analysis of ^{125}I-labeled hr-tPA in the absence and presence of APC indicated no proteolytic degradation of hr-tPA by APC (**Supplementary Fig. 4** online).

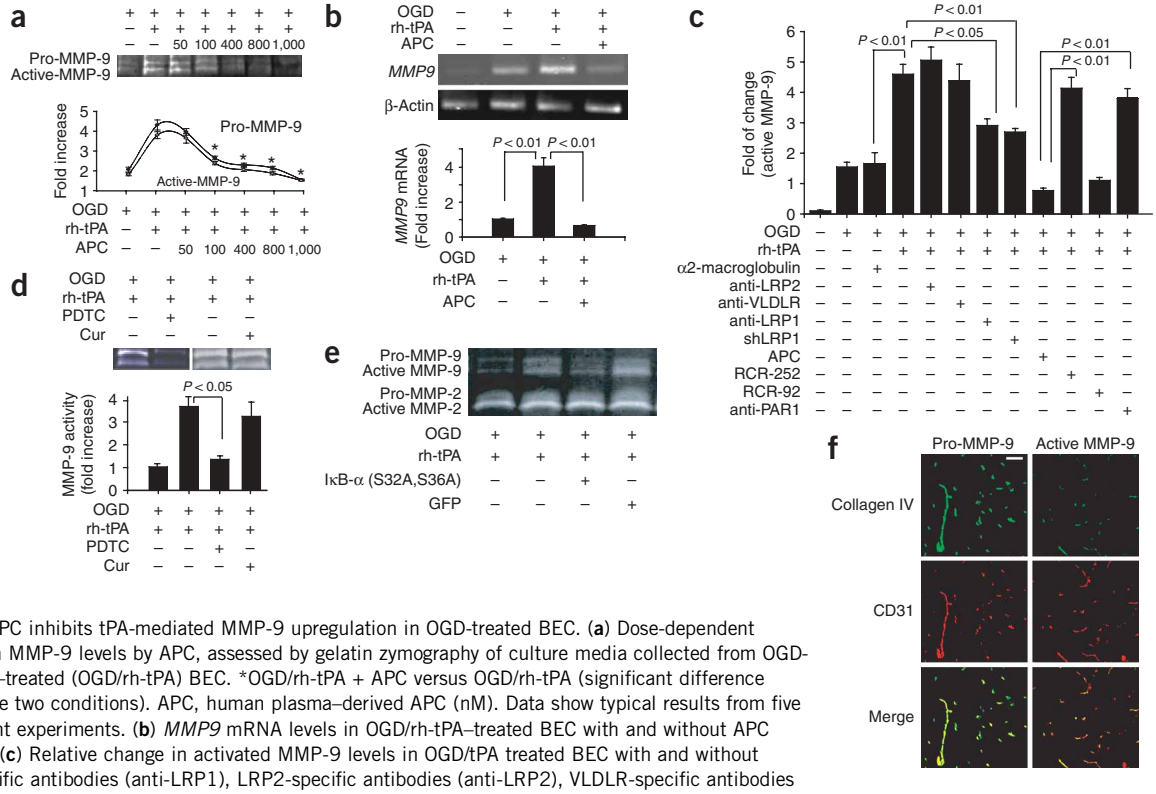

Figure 3 APC inhibits tPA-mediated MMP-9 upregulation in OGD-treated BEC. (**a**) Dose-dependent decrease in MMP-9 levels by APC, assessed by gelatin zymography of culture media collected from OGD- and rh-tPA–treated (OGD/rh-tPA) BEC. *OGD/rh-tPA + APC versus OGD/rh-tPA (significant difference between the two conditions). APC, human plasma–derived APC (nM). Data show typical results from five independent experiments. (**b**) *MMP9* mRNA levels in OGD/rh-tPA–treated BEC with and without APC (400 nM). (**c**) Relative change in activated MMP-9 levels in OGD/tPA treated BEC with and without LRP1-specific antibodies (anti-LRP1), LRP2-specific antibodies (anti-LRP2), VLDLR-specific antibodies (anti-VLDLR), shLRP1, APC, RCR-92 (EPCR control antibody), RCR-252 (antibody to EPCR that blocks the APC binding site), or H111 (antibody that blocks the PAR1 catalytic site, anti-PAR1); OGD treated BEC were also treated with α2-macroglobulin. (**d**) Zymographic analysis of MMP-9 release into culture media from OGD/tPA–treated BEC 2 h after treatment with PDTC, an NF-κB inhibitor or curcumin (cur), an AP-1 inhibitor. (**e**) Lack of rh-tPA–induced MMP-9 upregulation in OGD-treated BEC expressing IκB-α (S32,S36A) compared to BEC expressing green fluorescent protein (GFP). (**f**) Effects of exogenous pro-MMP-9 and MMP-9 on vascular collagen IV in the mouse brain. Mean ± s.e.m. $n = 3$–6 per group.

PAR1 mediates APC's antihemorrhagic pathway

To demonstrate clearly that the observed APC effects *in vivo* and on cells *in vitro* require PAR1, we used *Par1*-null and control mice and performed two types of studies: (i) *in vivo* studies in the MCAO model using recombinant mouse tPA (rm-tPA) with and without mouse APC; and (ii) *in vitro* studies of *Par1*-null and control mouse BEC treated with OGD and rm-tPA with and without APC. Because the response of *Par1*-null mice to brain ischemia *in vivo* depended on the duration of the occlusion (ischemia) phase (that is, the lack of PAR1 was protective for a 30-min transient MCAO, but did not have any effect on ischemic brain injury after 1-h transient MCAO (ref. 29)), we used the 1-h transient MCAO model with 24 h reperfusion to eliminate possible neuroprotective effects of *Par1* gene deletion at shorter ischemia times. We confirmed that deletion of the *Par1* gene did not have any substantial effect on ischemic brain injury and neurological scores after 1-h transient MCAO (**Fig. 5a**,**b**), as reported[29]. This finding is consistent with the finding that the lack of PAR1 does not affect excitotoxic brain lesions *in vivo*[14]. Treatment with rm-tPA alone caused comparable (that is, 49% and 47%) increases in brain injury in both control and *Par1*-null mice, respectively (**Fig. 5a**), and also caused substantial increases in neurological scores (**Fig. 5b**) compared to nontreated controls. Mouse APC exerted a substantial neuroprotective effect in control $Par1^{+/+}$ mice, but was not neuroprotective in $Par1^{-/-}$ mice (**Fig. 5a**,**b**), suggesting PAR1 is required for *in vivo* APC neuroprotection, as reported[13,14]. rm-tPA produced similar >3.5-fold increases in bleeding in control and *Par1*-null mice, and APC substantially prevented hemorrhage in control mice, but not in PAR1-deficient mice (**Fig. 5c**). In addition, when comparing $Par1^{-/-}$ mice and $Par1^{+/+}$ mice, we found that APC did not reduce tPA-induced MMP-9 expression in ischemic brain microvessels or tPA-induced NF-κB (p65) expression in the ischemic brain (**Fig. 5d**,**e**). These findings demonstrate that PAR1 is required for the antihemorrhagic effect of APC *in vivo* and the suppression by APC of the tPA-induced NF-κB–dependent upregulation of MMP-9.

Ischemic brain endothelium is a major cell type in the brain responsible for tPA-induced ischemic hemorrhage and BBB breakdown[10]. But neurons and astrocytes do not express MMP-9 within 24 h of ischemic brain injury[30]; therefore, we extended our studies of the OGD-treated mouse BEC model[31]. rm-tPA, similar to rh-tPA (**Fig. 4b** and **Fig. 3a**), induced NF-κB nuclear translocation in OGD-treated $Par1^{+/+}$ and $Par1^{-/-}$ mouse BEC at 1 h (**Fig. 6a**,**b**); this was followed by an increase in MMP-9 expression at 3 h (**Fig. 6c**). Mouse APC (5 nM) substantially reduced both NF-κB nuclear translocation (**Fig. 6a**,**b**) and MMP-9 expression (**Fig. 6c**) in OGD- and rm-tPA–treated $Par1^{+/+}$ mouse BEC. This effect of APC was abolished by *Par1* gene deletion as shown by its inability to inhibit rm-tPA–induced NF-κB nuclear translocation and MMP-9 expression in $Par1^{-/-}$ BEC (**Fig. 6a–c**). These *in vitro* findings extend our *in vivo* findings by showing that PAR1 is required for APC-mediated downregulation of tPA-induced, NF-κB–dependent upregulation of MMP-9 in BEC.

DISCUSSION

Here we show that APC blocks tPA-mediated brain hemorrhage after transient brain ischemia and embolic stroke in rodents. Our

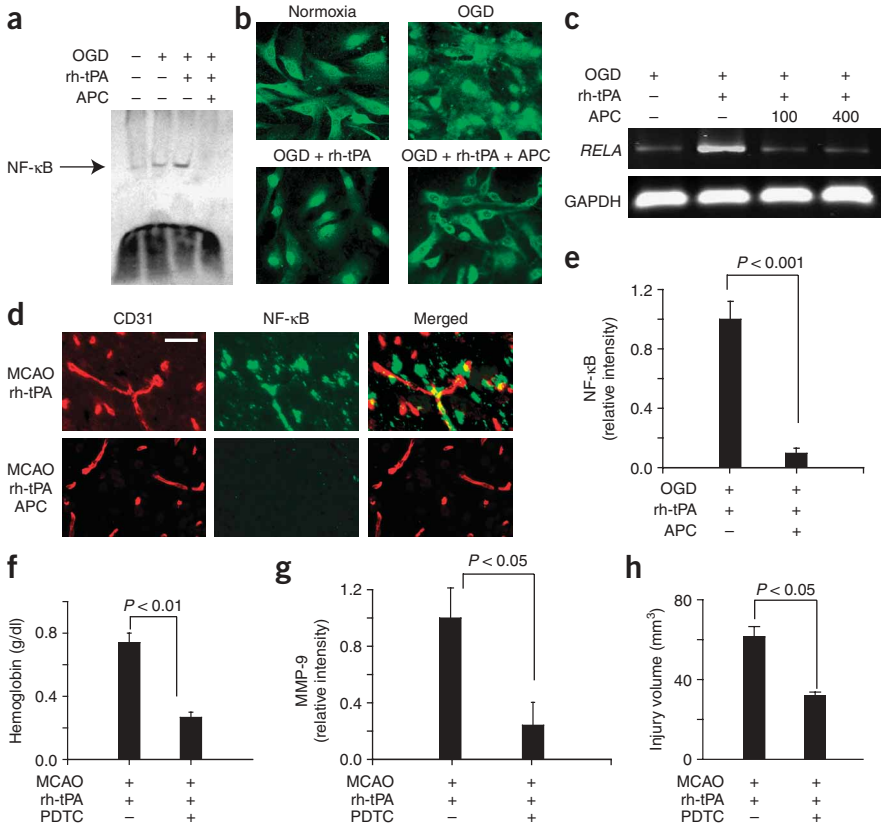

Figure 4 APC inhibits tPA-induced NF-kB activation. (**a**,**b**) NF-κB nuclear translocation in OGD/rh-tPA–treated BEC with and without human APC, assessed by gel shift assay (**a**) and immunostaining (**b**). (**c**) *RELA* mRNA levels in BEC treated with OGD and rh-tPA for 1 h with and without human APC. (**d**) Immunostaining for NF-κB (p65) and CD31 after transient brain ischemia in mice treated with rh-tPA without and with mouse APC (0.2 mg/kg, simultaneous infusion). (**e**) Relative intensity of NF-κB immunostaining in ischemic mouse brain after rh-tPA without and with mouse APC (0.2 mg/kg). (**f**,**h**) Effects of PDTC on hemoglobin level (**f**), MMP-9 vascular immunostaining (**g**) and brain injury (**h**) after transient ischemia in mice. Mean ± s.e.m. $n = 3–6$ per group.

findings demonstrate that APC controls tPA-mediated brain hemorrhage by inhibiting a tPA–NF-κB–MMP-9 pathway in ischemic endothelium, which in turn blocks tPA's proteolytic attack on the BBB. We also show that these neuroprotective effects of APC *in vivo* and *in vitro* require PAR1.

We want to emphasize that extensive controls in our previous papers[13,14] showed absolute specificity for APC effects on BEC and neurons in culture. For example, among other controls, a well-recognized monoclonal antibody to protein C (known as "C3" Mab, which was used by Eli Lilly and company in their clinical development and trials for human APC) blocks the cytoprotective actions of human APC (ref. 32). Second, human and mouse APC have essentially no detectable thrombin activity based on thrombin clotting time assays (using purified fibrinogen). Third, all experiments involving *in vitro* studies of cells treated with APC included hirudin, which blocks thrombin signaling as we and others have reported[12,13,27,33].

In considering APC pharmacological doses, we note that mouse APC was effective at 5 nM on cultured mouse BEC (**Fig. 6**), which compares very well with *in vivo* neuroprotective levels of mouse APC, 6.5 nM, that are achieved in mouse plasma as a 45-min 'plateau' after administration of mouse APC (0.2 mg per kg body weight intravenous (i.v.), 50% as a bolus and 50% as a 30-min infusion after the bolus; **Supplementary Fig. 5** online). At this dose, mouse APC is highly neuroprotective in mouse stroke models[13,18,19,33].

Evaluation of therapeutically effective concentrations of APC depends very much on how APC is studied and from which cells and which species APC is obtained. For example, some studies of human umbilical vein cells (HUVEC) in culture were unable to demonstrate human APC efficacy at low nanomolar concentrations

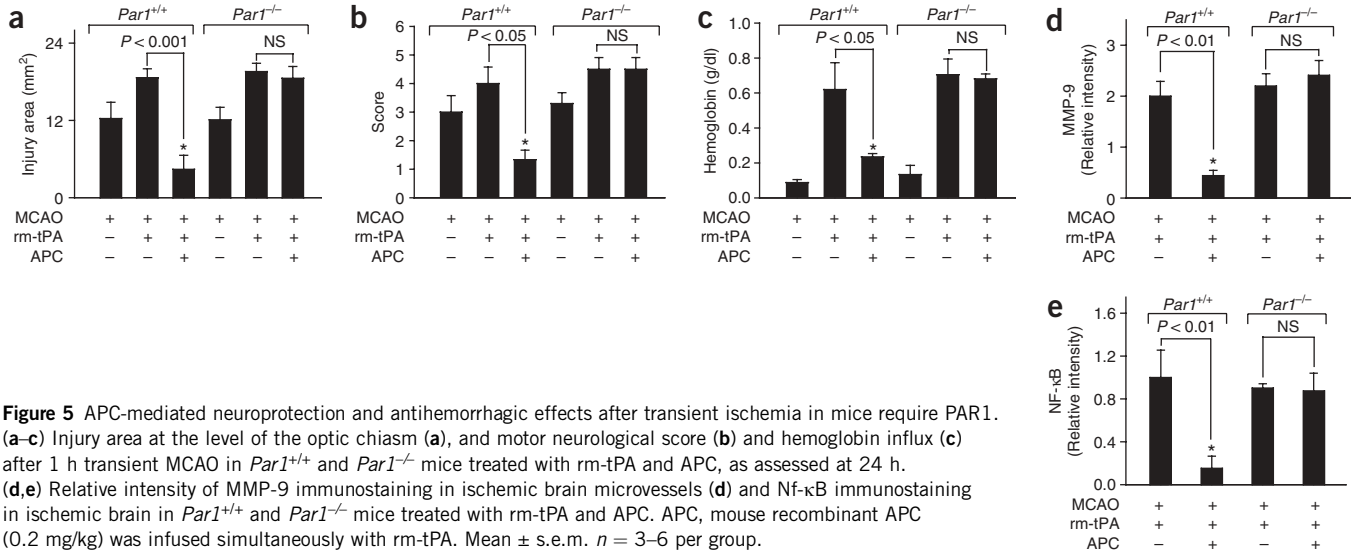

Figure 5 APC-mediated neuroprotection and antihemorrhagic effects after transient ischemia in mice require PAR1. (**a**–**c**) Injury area at the level of the optic chiasm (**a**), and motor neurological score (**b**) and hemoglobin influx (**c**) after 1 h transient MCAO in $Par1^{+/+}$ and $Par1^{-/-}$ mice treated with rm-tPA and APC, as assessed at 24 h. (**d**,**e**) Relative intensity of MMP-9 immunostaining in ischemic brain microvessels (**d**) and Nf-κB immunostaining in ischemic brain in $Par1^{+/+}$ and $Par1^{-/-}$ mice treated with rm-tPA and APC. APC, mouse recombinant APC (0.2 mg/kg) was infused simultaneously with rm-tPA. Mean ± s.e.m. $n = 3–6$ per group.

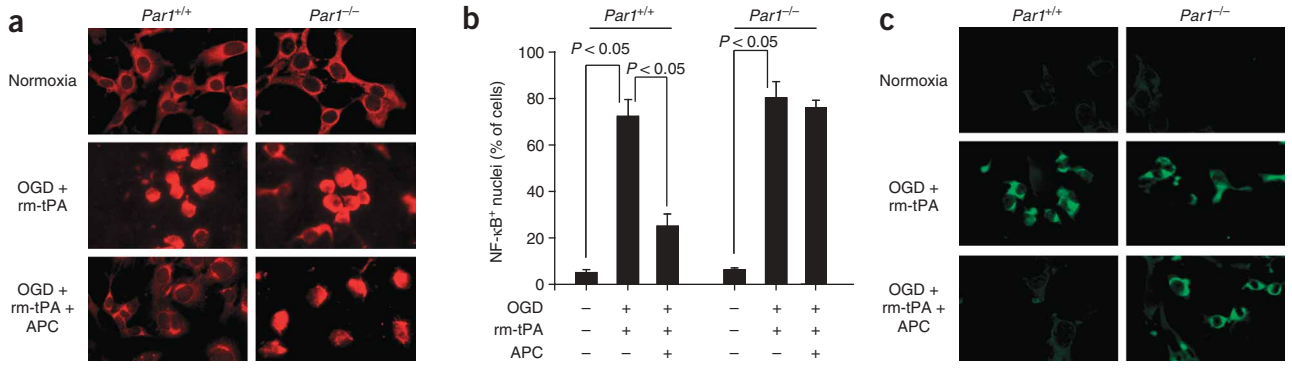

Figure 6 APC-mediated inhibition of the NF-κB-MMP-9 pathway in OGD/tPA-treated mouse BEC requires PAR1. (**a**,**b**) NF-κB nuclear translocation in OGD/rm-tPA–treated BEC from *Par1*$^{+/+}$ and *Par1*$^{-/-}$ mice with and without APC. (**c**) MMP-9 immunostaining in cytoplasm of OGD/mr-tPA–treated BEC from *Par1*$^{+/+}$ and *Par1*$^{-/-}$ mice with and without APC. APC, mouse recombinant APC (5 nM). Mean ± s.e.m. $n = 3$ per group.

and suggested that APC activates PAR1 only at a concentration of 100 nM (ref. 34). However, several studies have shown that APC is effective at concentrations well below 100 nM. For example, human recombinant APC at 8 nM counteracts staurosporine-induced apoptosis in HUVEC (refs. 11,35). Human plasma–derived APC at 10 nM influences gene expression in endothelial cells via PAR1, and PAR1 signaling by APC is distinct from thrombin signaling[27]. The half-maximal inhibitory concentration (IC_{50}) for human plasma–derived APC antiapoptotic effects in human BEC exposed to OGD is 15 nM (ref. 13). Mouse recombinant APC protects mouse neurons against NMDA-induced apoptosis with an IC_{50} of 5 nM (ref. 14), and mouse APC is generally an order of magnitude more potent in mouse *in vivo* and *in vitro* models of ischemic and neuronal injury than human APC (refs. 13,14,33). Whether this is due to higher activity of mouse APC or species homology is not clear at present. In all but one of these studies, PAR1 was required for APC's actions. Human APC at 20 nM influences antiapoptotic gene expression in PAR1-transduced mouse fibroblasts[12]. It is also of note that our pharmacokinetic *in vivo* measurements of mouse APC in plasma after administration of 0.02 mg APC per kg body weight in a mouse model of stroke indicated that neurological deficits can be substantially improved by APC plasma levels at about 0.5 nM (data not shown). In addition, human plasma–derived APC stimulates Ca^{2+} currents in human BEC and HUVEC under physiological conditions with half-maximum effect at 0.23 nM and can effect Ca^{2+} release from the endoplasmic reticulum in BEC at concentrations of around 0.1 nM, and this requires PAR1 (ref. 36).

In conclusion, although tPA thrombolysis is certainly beneficial for stroke[37], its neurotoxicity[18,38–40] and hemorrhagic transformation[1] need to be controlled. Here we demonstrate that in addition to direct neuronal[14] and vascular protective activities of APC in ischemic brain[13,18], APC may limit tPA's extravascular undesirable effects by blocking BBB breakdown and hemorrhage. By acting through PAR1, APC inhibits the prohemorrhagic tPA-induced, NF-κB–dependent-MMP-9 pathway in ischemic brain endothelium *in vitro* and *in vivo*. For that reason, APC could be an ideal neuroprotectant candidate for tPA adjunctive therapy for ischemic stroke.

METHODS

Detailed protocols used in this study are described in the **Supplementary Methods** online.

Reagents. We used rh-tPA (Alteplase, Genentech, San Francisco). In some *in vitro* studies, we used rh-tPA from Sigma (St. Louis). mr-tPA was obtained from Innovative Research (Southfield, Michigan). We prepared human plasma–derived APC and mouse recombinant APC as described[13,33]. We purchased exogenous activated MMP-9 and pro-MMP-9 from R & D Systems (Minneapolis).

Transient MCAO model. Procedures were approved by the Animal Care Committee at the University of Rochester using US National Institutes of Health (NIH) guidelines. We subjected C57Bl6 mice (28–30 g; Jackson Laboratory, Bar Harbor, Maine) anesthetized with 100 mg per kg body weight intraperitoneal ketamine and 10 mg per kg body weight xylazine to 45 min MCAO and 24 h reperfusion. Vehicle, rh-tPA (10 mg/kg, 10% bolus and 90% infusion), rh-tPA and mouse recombinant APC (0.02–0.2 mg/kg, 50% bolus and 50% infusion) or both were administered via the femoral vein during MCAO 10 min before reperfusion and for 20 min into reperfusion. APC was also administered 3 h after hr-tPA infusion. Cerebral blood flow (CBF) was monitored by laser Doppler flowmetry (Transonic Systems). Physiological parameters and arterial blood gases were measured. Neuropathological analysis was performed at 24 h. Hemoglobin levels in the brain were determined by a spectrophotometric assay using Drabkin's reagent (Sigma). Unfixed 1-mm coronal brain slices were incubated in 2% triphenyltetrazolium chloride in phosphate buffer (pH 7.4) to determine volume of injury.

We subjected control and *Par1*-null mice to 1 h MCAO and 24 h reperfusion. *Par1*-null mice and controls received rm-tPA, 1 mg/kg via the femoral vein, 10% as a bolus 10 min before reperfusion and 90% as a 30-min infusion after the bolus injection. Mouse APC or vehicle were infused simultaneously with rm-tPA.

Embolic stroke model. Procedures were approved by the Animal Care Committee at the Henry Ford Health Sciences Center Detroit using NIH guidelines. Embolic stroke was induced by placing a single intact homologous clot at the origin of the MCA in male Wistar rats (350–450 g; Charles Rivers Laboratories, Wilmington, Massachusetts) anesthetized with 3.5% halothane delivered through a face mask. rh-tPA (10 mg/kg) was infused intravenously 4 h after stroke, 10% as a bolus and 90% as a 30-min infusion in combination with APC (0.4 mg/kg as a single bolus) or vehicle. We determined the NSS and the foot fault scores at 2 h, 1 d and 7 d. Rats were killed at 1 d or 7 d. The injury volumes were measured as described[33]. Microscopic hemorrhage was calculated as reported[41].

Brain tissue immunostaining. Mice were transcardially perfused at 24 h after ischemia. Frozen sections were cut in the coronal plane at 10 μm. Immunohistochemistry was performed on acetone fixed sections. For details regarding MMP-9, NF-κB (p65), collagen IV, ZO-1 and CD31 primary antibodies, secondary antibodies and quantification, see **Supplementary Methods**.

OGD/tPA model in brain endothelial cells. Primary human BEC were isolated from cortical tissue after brain surgery for epilepsy. BEC were cultured and characterized as described[13]. BEC were exposed for 1–4 h to rh-tPA (20 μg/ml), and to either normoxia (20% oxygen, 5 mM glucose), OGD (<1% oxygen, no

glucose) or both rh-tPA and OGD. Human APC was added at the time of tPA/hypoxia treatment. Short hairpin RNA for LRP1 (shLRP1) was used as described[42]. We used the following antibodies to endothelial protein C receptor (EPCR): RCR-252, which blocks the APC binding site, and RCR-92, which does not block the APC binding site[43].

Mouse BEC from *Par1*-null and control mice were isolated as described[31] and incubated with rm-tPA (20 μg/ml) and either normoxia or OGD (the latter with and without mouse APC (5 nM)). NF-κB nuclear translocation and MMP-9 expression were studied at 1 h and 3 h after OGD and rm-tPA treatment with and without APC.

In all studies with APC, we used hirudin to block any possible thrombin signaling, as reported[12,13,27,36]. For details regarding experimental conditions and blocking antibodies, see **Supplementary Methods**.

Gelatin zymography. Zymography was performed on ischemic mouse brain samples and in culture media harvested from different OGD conditions with and without tPA and APC, as described[2]. For details see **Supplementary Methods**.

Gel mobility assay. NF-κB and AP-1 consensus sequences were labeled using DIG-11-ddUTP (Roche, Indianapolis). For details see **Supplementary Methods**.

RT-PCR. Semiquantitative RT-PCR was performed with human *MMP-9* and *RELA* primer sets (Maxim Biotech, San Francisco). For details see **Supplementary Methods**.

Adenoviral BEC transduction. We used adenoviruses encoding GFP or a mutant form of IκB-α (S32,36A). For details see **Supplementary Methods**.

***Ex vivo* collagen IV degradation.** Ten-micron frozen mouse brain sections were incubated with 1 μg/ml pro-MMP-9 or activated MMP-9. For details see **Supplementary Methods**.

Statistical analysis. Data were presented as mean ± s.e.m. An analysis of variance (ANOVA) followed by a Tukey-Kramer test was used to compare the treatment effects of ordinal data between groups. Nonparametric data were compared by Kruskal-Wallis test. $P < 0.05$ was considered statistically significant.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

T.C. conducted and performed *in vitro* studies of human BEC. A.L.P. conducted and performed *in vivo* stroke studies in the mouse MCA model. Z.L. conducted and performed *in vivo* stroke studies in the rat embolic model. M.T. performed *in vivo* studies in *Par1*-null mice. Z.Z. performed *in vitro* studies in mouse BEC. Z.W. performed immunostaining studies. D.L. performed *in vivo* mouse MCA studies. S.B.M. performed *in vitro* studies and developed IkkB reagent for the study. R.D. conducted and performed studies of physiological parameters. J.A.F. prepared different preparations of mouse and human APC. B.L. performed physiological analysis in mouse models and *in vitro* assays. J.H.G. contributed to experimental design and data discussion and analysis. M.C. designed studies in rat embolic model, and contributed to data analysis and interpretation. B.V.Z. designed the entire study, supervised all segments of the study, and wrote the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare that competing financial interests (see the *Nature Medicine* website for details).

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