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## Invited review

## Can adjunctive therapies augment the efficacy of endovascular thrombolysis? A potential role for activated protein C

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## ABSTRACT

In the management of acute ischemic stroke, vessel recanalization correlates with functional status, mortality, cost, and other outcome measures. Thrombolysis with intravenous tissue plasminogen activator has many limitations that restrict its applicability, but recent advances in the development of mechanical thrombectomy devices as well as improved systems of stroke care have resulted in greater likelihood of vessel revascularization. Nonetheless, there remains substantial discrepancy between rates of recanalization and rates of favorable outcome. The poor neurological recovery among some stroke patients despite successful recanalization confirms the need for adjuvant pharmacological therapy for neuroprotection and/or neurorestoration. Prior clinical trials of such drugs may have failed due to the inability of the agent to access the ischemic tissue beyond the occluded artery. A protocol that couples revascularization with concurrent delivery of a neuroprotectant drug offers the potential to enhance the benefit of thrombolysis. Analogs of activated protein C (APC) exert pleiotropic anti-inflammatory, anti-apoptotic, antithrombotic, cytoprotective, and neuroregenerative effects in ischemic stroke and thus appear to be promising candidates for this novel approach. A multicenter, prospective, double-blinded, dose-escalation Phase 2 randomized clinical trial has enrolled 110 patients to assess the safety, pharmacokinetics, and efficacy of human recombinant 3K3A-APC following endovascular thrombolysis.

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## Contents

1. Introduction .....	00
2. Overview of activated protein C (APC) pathways .....	00
2.1. PAR1 cleavage-dependent signaling .....	00
2.2. The protective effects of APC .....	00
2.2.1. Endothelial cells .....	00
2.2.2. Neurons .....	00
2.2.3. Neurogenic effects of APC .....	00

**Abbreviations:** (AIS), acute ischemic stroke; (Akt), protein kinase B; (APC), activated protein C; (BBB), blood-brain barrier; (CNS), central nervous system; (EPCR), endothelial protein C receptor; (ERK1/2), extracellular signal-regulated kinase 1/2; (FDA), food and drug administration; (GA), general anesthesia; (IV tPA), intravenous tissue plasminogen activator; (LVO), large vessel occlusion; (MMP9), matrix metalloproteinase-9; (NfκB), nuclear factor kappa-light-chain-enhancer of activated B cells; (NMDA), N-methyl-D-aspartate; (NPCs), neural progenitor cells; (NSC), neural stem cell; (NVU), neurovascular unit; (PC), protein C; (PAR1), protease activated receptor 1; (PAR3), protease activated receptor 3; (Rac1), Ras-related C3 botulinum toxin substrate 1; (RhoA), Ras homolog gene family, member A; (Serpins), serine protease inhibitors; (STAIR), stroke therapy academic industry roundtable; (SVZ), subventricular zone; (IIa), thrombin; (TM), thrombomodulin; (tPA), tissue plasminogen activator; (TRAP), thrombin receptor-activating peptides; (wt), wild-type.

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2.3. Stem cells and APC .....	00
3. Augmenting the benefit of thrombolysis with APC .....	00
4. Conclusion .....	00
Conflict of interest .....	00
Acknowledgements .....	00
References .....	00

## 1. Introduction

Stroke is the third leading cause of death worldwide and the number one cause of disability in the United States (Mortality and Causes of Death, 2015). Among the subset of stroke patients with persistent proximal vessel occlusion, up to 80% die within 90 days or fail to regain functional independence (Amar et al., 2015). It is estimated that for each minute during acute ischemic stroke (AIS), 1.9 million neurons, 14 billion synapses, and 12 km (7.5 miles) of myelinated fibers are destroyed (Saver, 2006).

Extensive research efforts have helped elucidate the pathophysiology underlying AIS and have characterized many processes of the ischemic cascade, including dysfunction of all elements of the neurovascular unit (neurons, astrocytes, microglia, and endothelial cells). Although these multiple injury mechanisms suggest myriad potential therapeutic approaches, the only medication approved by the United States Food and Drug Administration (FDA) for AIS remains intravenous tissue plasminogen activator (IV tPA), which targets the occlusive thrombus within a blood vessel. However, IV tPA has many limitations that restrict its widespread application, including a relatively short time window for delivery, low rates of recanalization in large vessel occlusion (LVO), and risks of intracranial bleeding (Amar et al., 2015). As a result of these and other reasons, only about 5% of AIS patients receive IV tPA (Jauch et al., 2013; Mozaffarian et al., 2015; Schwamm et al., 2013). Adjunctive drugs that counteract these limitations may expand the applicability of this therapy in the future.

In the last few years, the role of mechanical neurothrombectomy in AIS therapy has expanded significantly. The current generation of aspiration and stent retrieval devices achieves recanalization in the majority of patients with LVO (Almekhlafi et al., 2014; Berkhemer et al., 2015; Campbell et al., 2015; Goyal et al., 2015; Nogueira et al., 2012; Saver et al., 2012, 2015) and detailed analysis of safety data confirms that neurothrombectomy procedures can be performed with minimal morbidity and mortality (Akins et al., 2014).

Nonetheless, the likelihood of functional independence following neurothrombectomy (14–58%) remains poor compared with rates of recanalization (60–90%) (Amar et al., 2015). This disparity underscores the need for adjunctive therapies that enhance the benefit of endovascular thrombolysis, such as pharmacological neuroprotection (Amar et al., 2015).

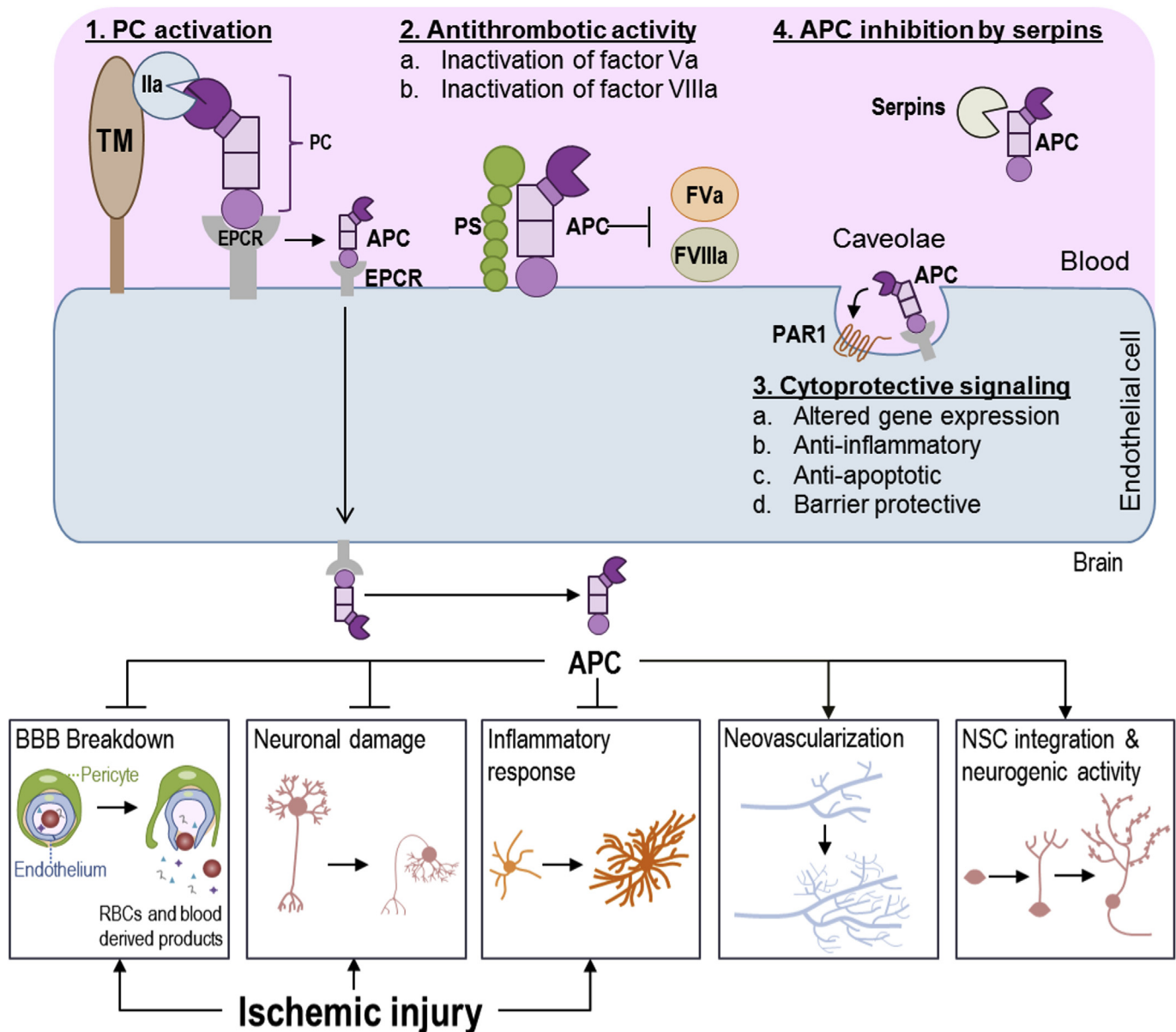
Thousands of preclinical studies and human trials with potential neuroprotective agents in AIS have been reported, but none has proven unequivocally efficacious and none has yet achieved FDA approval (Ginsberg, 2009; Tymianski, 2013). One plausible explanation for this failure is that the agent may not reach the ischemic tissue due to lack of perfusion. When administered systemically, neuroprotective agents might not traverse the occluded artery and must rely instead on collateral flow to ischemic tissue, but such collateral flow may be insufficient for adequate drug delivery. This provides impetus for a strategy coupling revascularization with the ancillary administration of a neuroprotective drug.

We have previously reviewed the foundation of clinical trials

that administer an analogue of activated protein C (APC) after endovascular thrombolysis by IV tPA, mechanical neurothrombectomy, or both (Amar et al., 2015). APC confers pleiotropic benefits, such as stabilizing blood brain barrier (BBB) integrity, preventing propagation of thrombosis, enhancing fibrinolysis, promoting neuroprotection, attenuating inflammation, and facilitating neuroregeneration (Griffin et al., 2002, 2015; Zlokovic and Griffin, 2011). It represents a novel multiple-action multiple-target approach that addresses all components of the pathogenic triad (consisting of vascular damage, neuronal injury, and neuroinflammation) in AIS (Zlokovic and Griffin, 2011). Since the first report of its anti-inflammatory, cytoprotective, and antithrombotic properties in AIS (Shibata et al., 2001), APC has progressively fulfilled Stroke Therapy Academic Industry Roundtable (STAIR) criteria for drug development (Zlokovic and Griffin, 2011). The preclinical safety and pharmacokinetic profile of APC has been well characterized in mice and monkey models (Williams et al., 2012). A phase I safety study in normal human subjects has demonstrated that high dose bolus regimens of modified APC are well-tolerated in normal human subjects (Lyden et al., 2013), and a multicenter phase II dose-escalation clinical trial of intravenous administration for AIS (NCT02222714, NN104) is currently in progress (<https://clinicaltrials.gov/ct2/show/record/NCT02222714>) (Lyden et al., 2016).

## 2. Overview of activated protein C (APC) pathways

Protein C (PC) is a 62 kDa vitamin K-dependent secretory glycoprotein produced mainly by liver (Griffin et al., 1993). PC circulates at 70 nM in the blood as an inactive zymogen of the natural anticoagulant serine protease APC (Gruber and Griffin, 1992; Mosnier et al., 2007). PC binds to the endothelial cell protein C receptor (EPCR) at the endothelial cell surface (Fukudome and Esmon, 1994) and is activated by thrombomodulin (TM) receptor-bound thrombin (IIa) by cleavage at Arg169 and removal of a peptide fragment at the amino-terminal of PC heavy chain (Esmon, 2003; Essalmani et al., 2017) (Fig. 1). EPCR is also required for transport of APC across the BBB (Deane et al., 2009). APC associated with EPCR in caveolae microdomains (Russo et al., 2009) cleaves protease-activated receptor-1 (PAR-1) initiating cytoprotective signaling including altered gene expression, and anti-inflammatory, anti-apoptotic and barrier protective activities (Fig. 1). Normally plasma levels of APC are about 40 pM in healthy humans (Mosnier et al., 2007). APC is inactivated and cleared from plasma by serine protease inhibitors (Serpins) (Fig. 1). APC is a unique protease having potent anticoagulant and anti-inflammatory activities (Griffin et al., 2002). APC along with its cofactor protein S partially degrade and inactivate coagulation factors Va and VIIIa on the platelet membrane (Esmon, 2003; Marlar et al., 1982) (Fig. 1). APC is physiologically very important as heterozygous PC deficiency increases the risk for venous thrombosis in adults and the rare homozygous PC deficiency in neonates results in a fatal syndrome known as purpura fulminans if untreated (Griffin et al., 1981; Marlar et al., 1989). In mice, total



**Fig. 1.** The Protein C (PC) pathway and the protective effects of activated protein C (APC) after ischemic injury. There are four major PC pathways. 1. PC activation. PC, bound to its receptor, endothelial protein C receptor (EPCR), is activated by thrombomodulin (TM)-bound thrombin (IIa) complex on the endothelial cell surface. 2. Antithrombotic activity. APC employs its anticoagulant activities by proteolytic inactivation of factor Va (FVa) and factor VIIIa (FVIIIa) aided by the cofactor Protein S (PS). 3. Cytoprotective signaling. APC associated with EPCR cleaves protease-activated receptor-1 (PAR-1) in caveolae initiating cytoprotective signaling including altered gene expression, anti-inflammatory, anti-apoptotic and barrier protective activities. Other receptors (not shown) may also contribute to cytoprotective signaling. 4. APC is inhibited by serine protease inhibitors (Serpins). APC is transported across the blood-brain barrier (BBB) into brain where it has many protective and regenerative effects. APC treatment after ischemic stroke limits brain injuries by protecting the BBB and neurons and reducing inflammatory responses. Furthermore, APC promotes neovascularization and neurogenesis, and it improves neural stem cell (NSC) proliferation, integration and neurogenic activity, aiding functional recovery.

deletion of PC results in death soon after birth, whereas transgenic mice expressing very low levels of PC develop severe thrombosis and inflammation (Lay et al., 2005).

### 2.1. PAR1 cleavage-dependent signaling

Thrombin and APC differentially cleave PAR-1 to determine and trigger different unique downstream signaling cascades (Griffin et al., 2015; Mosnier et al., 2012). Thrombin cleaves PAR1 at Arg41 generating a new N terminus beginning at Ser42 which corresponds to a PAR1 peptide sequence known as thrombin receptor-activating peptides (TRAP, peptides beginning with

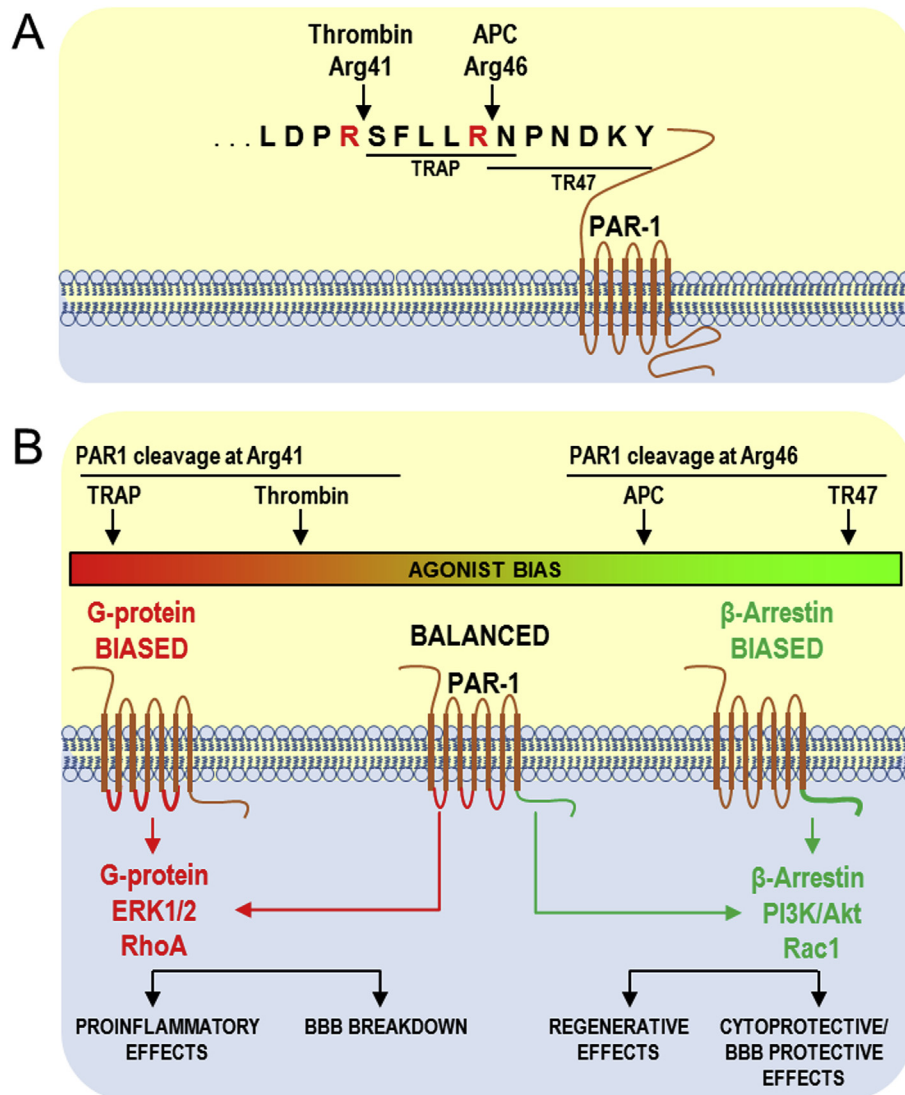
SFLLRN) which mirrors many of thrombin's biological effects (Fig. 2A) (Griffin et al., 2015; Mosnier et al., 2012). APC cleaves PAR1 at Arg46 generating a new N terminus beginning with Asn47 which corresponds to a 20-mer peptide known as TR47 (i.e., a peptide starting with NPNDKY) which acts as a biased agonist of PAR1 mimicking APC's effects (Fig. 2A) (Griffin et al., 2015; Mosnier et al., 2012). In cultured endothelial cells, TRAP or thrombin causes rapid extracellular signal-regulated kinase 1/2 (ERK 1/2) phosphorylation (Griffin et al., 2015; Mosnier et al., 2012). Conversely, TR47 or APC causes delayed protein kinase B (Akt) phosphorylation (Griffin et al., 2016). Therefore, the cleavage of PAR-1 in sites separated by only 5 amino acids has opposite biological effects depending on the

biased agonism of PAR-1 (Fig. 2B) (Griffin et al., 2016; Mosnier et al., 2012). On one hand, PAR-1 can initiate G-protein-coupled receptor-dependent proinflammatory effects via ERK 1/2 activation, and Ras homolog gene family, member A (RhoA) activation, leading to BBB disruption (Fig. 2B) (Griffin et al., 2015, 2016; Mosnier et al., 2012). Alternatively, activated PAR-1 can initiate a  $\beta$ -arrestin-2 pathway involving Ras-related C3 botulinum toxin substrate 1 (Rac1) and promoting BBB stabilization (Soh and Trejo, 2011) and survival signaling via Akt activation (Fig. 2B) (Griffin et al., 2016; Mosnier et al., 2012).

## 2.2. The protective effects of APC

The neuroprotective effect of APC was first discovered in a murine middle cerebral artery occlusion/reperfusion model (Shibata et al., 2001). Treatment of mice with purified human plasma-derived APC reduced brain infarct volume and edema, prevented brain infiltration of neutrophils, and reduced the BBB breakdown (Shibata et al., 2001). This initial study led to the cloning of murine PC for subsequent studies to avoid cross-species

artifacts (Fernandez et al., 2003). Several in vitro and in vivo pre-clinical studies in the past two decades have identified APC's anti-inflammatory, cytoprotective activities which are important for direct endothelial cell protection, BBB stabilization, neuronal protection, neurogenesis and neovascularization in ischemic stroke recovery (Griffin et al., 2006, 2016; Mosnier et al., 2007; Zlokovic and Griffin, 2011) (Fig. 1). Preclinical studies in various injury models showed beneficial effects of human or mouse recombinant wild-type (wt)-APC (Griffin et al., 2015). Recent clinical studies in humans demonstrated effectiveness of local application of APC in the treatment of recalcitrant orthopedic wounds (Wijewardena et al., 2011), chronic skin ulcers (Kapila et al., 2014), chronic diabetic lower leg ulcers (Whitmont et al., 2015), and pressure sores (Wijewardena et al., 2016). Treatment with recombinant wt-APC has been shown to increase the risk for bleeding (Bernard et al., 2003; Christiaans et al., 2013). To understand APC's distinct anticoagulant and cytoprotective actions and minimize risk for intracerebral bleeding, we took advantage of APC's unique structural features and developed several signaling-selective (with significantly reduced, <10% anticoagulant activity) and anticoagulant



**Fig. 2.** PAR-1 signaling depends on the preference of thrombin or APC for different cleavage sites. A) PAR-1 is cleaved at Arg41 by thrombin resulting in an N-terminal tethered peptide. APC cleaves PAR-1 at Arg46, resulting in a different N-terminal tethered agonist. B) Thrombin cleavage of PAR-1 promotes G-protein-dependent signaling, inducing proinflammatory effects and BBB breakdown. APC cleavage of PAR-1 at Arg46 promotes  $\beta$ -arrestin 2-dependent signaling, inducing regenerative and cytoprotective effects. Adapted from (Mosnier et al., 2012).



selective APC mutants (Griffin et al., 2015; Mosnier and Griffin, 2006). Studies with signaling-selective recombinant APC mutants, such as 5A-APC or 3K3A-APC, found significant protection in several brain injury models such as amyotrophic lateral sclerosis (Winkler et al., 2014; Zhong et al., 2009), traumatic brain injury (Petraglia et al., 2010; Walker et al., 2010) and ischemic stroke (Guo et al., 2009a, 2009b). Whereas treatment with anticoagulant-selective E149A-APC variant having >3-fold increased anticoagulant activity but defective cytoprotective activities worsened brain injury in ischemic stroke in mice (Wang et al., 2013a). These studies confirm that primarily, if not exclusively, the cytoprotective activities and not anticoagulant activities are central for neuroprotection after brain injury. Below, we review the literature assessing the protective effects of APC on several cell types of the neurovascular unit (NVU), including endothelial cells, neurons and microglia (Fig. 3).

### 2.2.1. Endothelial cells

Murine APC directly prevented apoptosis via an EPCR/PAR-1-dependent pathway involving the inhibition of tumor suppressor protein p53, normalization of the apoptotic Bax/Bcl-2 ratio and reduction of caspase-3 signaling (Fig. 3A) (Cheng et al., 2003). Furthermore, APC inhibited the pro-hemorrhagic tPA-induced, NFκB-dependent matrix metalloproteinase-9 (MMP-9) pathway in ischemic brain endothelium in vivo and in vitro by acting through PAR-1 (Fig. 3A) (Cheng et al., 2006). Additionally, APC inhibited the tPA-induced caspase-8 activation of caspase-3, shifting the apoptotic signaling from the intrinsic to extrinsic pathway which requires caspase-8 (Liu et al., 2004). Interestingly, APC regulates intracellular Ca<sup>2+</sup> levels in brain endothelial cells by binding to EPCR and signaling via PAR-1 (Domotor et al., 2003).

### 2.2.2. Neurons

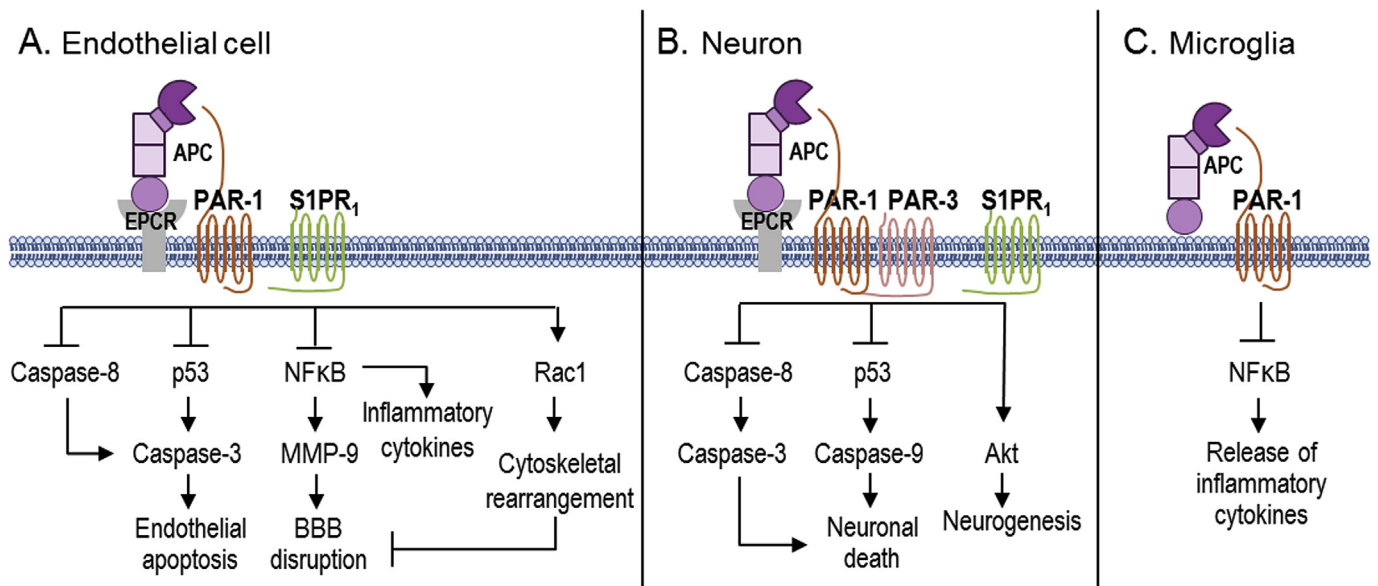
APC inhibits caspase-3 dependent nuclear translocation of apoptosis-inducing factor in *N*-methyl-D-aspartate (NMDA)-treated neurons and reduced tPA-mediated cerebral ischemic injury in mice (Liu et al., 2004; Zlokovic et al., 2005). Also, APC blocked NMDA-dependent apoptosis by inhibiting caspase-8 activation upstream of caspase-3 activation and apoptosis-inducing factor nuclear translocation (Guo et al., 2004). APC also blocked the induction of p53 (Guo et al., 2004). A series of mechanistic studies by others also confirm that APC's neuroprotection is dependent upon PAR-1, PAR-3 and EPCR (Gorbacheva et al., 2007, 2008, 2009, 2010, 2013).

### 2.2.3. Neurogenic effects of APC

The first evidence of APC to promote neurogenesis were in middle cerebral artery occlusion mice which found PAR-1 dependent increased proliferation of neuronal progenitor cells in the subventricular zone (SVZ) by 40–50% and migration of newly formed neuroblasts from the SVZ toward the ischemic border (Thiyagarajan et al., 2008). In vitro studies found that 3K3A-APC stimulated neuronal mitogenesis and differentiation from fetal human neural stem cells (NSCs) and neural progenitor cells (NPCs) that were mediated through PAR-1, PAR-3 and S1PR<sub>1</sub>, and triggering Akt (Guo et al., 2013). Interesting recent studies found that 3K3A-APC promotes the survival and neuronal production of transplanted NPCs into mice, leading to neuronal circuit restoration and improved function (Wang et al., 2016).

### 2.3. Stem cells and APC

Regenerative medicine with human stem cells holds the greatest promise for the treatment of stroke and other neurological disorders and central nervous system (CNS) injuries (George and



**Fig. 3.** Cell specific APC protective signaling pathways. A) In endothelial cells, APC helps to seal the BBB and is vasculoprotective. APC/EPCR activates PAR-1 and inhibits caspase-8 activation of caspase-3, thereby limiting the extrinsic apoptotic pathway in endothelium. APC/EPCR-dependent PAR-1 activation also suppresses the pro-apoptotic p53 transcription factor inhibiting caspase-3 activation blocking the intrinsic apoptotic pathway. Also, APC suppresses the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB)-dependent transcriptional activation of matrix metalloproteinase 9 (MMP-9), thereby blocking the breakdown of the BBB basement membrane. Furthermore, APC blocks the expression of proinflammatory cytokines limiting inflammation by controlling NFκB nuclear translocation. APCs cytoprotective effects on endothelial cells require EPCR and PAR-1 to cross-activate sphingosine 1-phosphate receptor 1 (S1PR<sub>1</sub>). Cross-activation of S1PR<sub>1</sub> triggers Ras-related C3 botulinum toxin substrate 1 (Rac1) leading to stabilization of cytoskeleton, thereby boosting the integrity of the BBB. B) In neurons, APC/EPCR is cytoprotective via PAR-1 and PAR3 which inhibits caspase-8 upstream of caspase-3 and thereby limiting the extrinsic apoptotic pathway. Also, an APC-PAR-1-PAR3 pathway block p53 activation in injured neurons, thereby blocking the caspase-9-dependent intrinsic apoptotic pathway. Furthermore, APC promotes neurogenesis via a PAR-1-PAR3-S1PR<sub>1</sub>-Akt pathway. C) APC's inhibition of NFκB-dependent transcriptional expression of different proinflammatory cytokines suppresses microglial activation.

Steinberg, 2015; Jeong et al., 2014). In the last decade, stem cell therapy has been extensively tested in preclinical experimental stroke models in rodents, large mammals and primates (Lees et al., 2012; Lemmens and Steinberg, 2013; Liu et al., 2014; Popa-Wagner et al., 2014; Shinozuka et al., 2013; *Stem Cell Therapies as an Emerging Paradigm in Stroke*, 2009). These studies, although they are not all consistent due to the differences in the models, cell types and transplantation protocols, have encouragingly suggested that transplanted cells homing to the damaged brain regions can exert multiple beneficial effects, including neuroprotection, anti-inflammation, pro-angiogenesis and pro-neurogenesis, and improve functional outcomes (Bliss et al., 2007; George and Steinberg, 2015; Lees et al., 2012; Liu et al., 2014). Despite recent advances, there is still a lack of mechanistic studies to address the issues regarding poor survival of transplanted cells and indiscriminate differentiation of the progenies in the hostile infarcted environment (Francis and Wei, 2010; Xian and Huang, 2015). In addition, whether transplanted cells can indeed be functional and replace the lost cells in the host neural network due to injuries has been debated. However, a recent report demonstrated that combination therapy with human NSCs and 3K3A-APC improved transplantation tolerance, stimulated cell replacement, accelerated structural recovery and enhanced functional restoration in a pre-clinical animal model of stroke (Wang et al., 2016). Furthermore, this study provided direct evidence showing functional integration of transplanted cells into the host neural circuits which is accompanied by substantial improvement in brain sensory-motor functions (Wang et al., 2016), suggesting that this combination approach may potentially be used for late treatment of stroke in patients.

Given that 3K3A-APC has neuroprotective effects in aged female mice and hypertensive rat models with a larger infarct volume (Wang et al., 2013b), it may be predicted that 3K3A-APC and NSC-repair therapy would successfully translate both to different experimental-stroke models and to humans (Albers et al., 2011). There are ongoing Phase I (NCT01151124) and Phase II (NCT02117635) clinical trials directly injecting manufactured NSCs into the brain of patients that remain moderately to severely disabled following an ischemic stroke. The Phase I trial, known as PISCES, found that a single intracerebral injection of up to 20 million NPCs induced no adverse events and was associated with improved neurological function (Kalladka et al., 2016). The Phase II trial recently completed recruitment and is investigating the benefit of NSC injection in ischemic stroke patients with stable upper-limb paresis. Future studies should determine whether including 3K3A-APC treatment with NPC transplantation in ongoing clinical trials could be a beneficial combination therapy for stroke patients that may help repair stroke-damaged neural circuits.

### 3. Augmenting the benefit of thrombolysis with APC

The premise of endovascular thrombolysis is that timely restoration of blood flow to the ischemic territory improves clinical outcome by salvaging the hypoperfused tissue at risk of converting to infarction. Meta-analysis of more than 50 studies confirms the strong correlation between recanalization and outcome in AIS, and the odds ratio of functional independence or death for those with recanalization compared to those without is 4.43 and 0.24, respectively (Rha and Saver, 2007).

However, recanalization alone—whether achieved by IV tPA, mechanical neurothrombectomy, or both—is often insufficient to achieve good clinical outcome. The current generation of neurothrombectomy devices can achieve recanalization in the overwhelming majority of patients, but even when this procedure is

performed expeditiously among AIS patients with small infarct cores, the rate of good clinical outcomes is comparatively poor (Amar et al., 2015). Analysis of the reasons underlying this disparity reinforces the benefit of a strategy combining endovascular thrombolysis with APC. We have previously reviewed many of the mechanisms by which adjunctive delivery of APC should strengthen the biological relationship between recanalization and outcome (Amar et al., 2015):

- a) Recanalization of upstream large arteries may not restore distal tissue reperfusion. Rethrombosis, migration of emboli, secondary thrombosis of downstream arteries, or microcirculatory occlusion may produce a no-reflow phenomenon despite proximal vessel thrombolysis (Bai and Lyden, 2015). The inherent anticoagulant activity of APC might attenuate the thrombosis that underlies no-reflow. Conversely, excessive anticoagulation could promote intracerebral bleeding. Variants such as 3K3A-APC, which have reduced anticoagulant activity (<8%) compared with wild type APC, might represent a favorable compromise between prothrombotic and anticoagulant forces, but this proposition awaits confirmation in clinical trials.
- b) Restoring flow to ischemic brain tissue following thrombolysis or thrombectomy risks reperfusion injury, hemorrhagic transformation, or cerebral edema, all of which could counteract the benefit of recanalization. By reinforcing the integrity of the damaged BBB within ischemic tissue, adjunctive APC confers vasculoprotective benefits.
- c) It has been proposed that recanalization therapies such as tPA may be neurotoxic, either directly through induction of caspases and other proapoptotic pathways, or through breakdown of the BBB that promotes the toxic accumulation of serum proteins that affect secondary neuronal injury (del Zoppo, 1998; Liu et al., 2004; Zlokovic and Griffin, 2011). The intrinsic neuroprotective actions of adjunctive APC might overcome this damage.
- d) Similarly, APC might protect against the deleterious effects of anesthesia, which is often administered during neurothrombectomy procedures. Unpublished data from the pooled analysis of the HERMES collaboration, reported at the 2017 International Stroke Conference, show that AIS patients given endovascular treatment under general anesthesia (GA) experienced worse neurological outcomes than those treated without GA. Among the potential explanations for this observation is the inherent neurotoxicity of intravenous and inhalational anesthetics. These actions are mediated through neuroapoptosis and inhibition of neurogenesis (Bilotta et al., 2017). The propitious effects of APC on apoptosis and neurogenesis could mitigate these effects of anesthesia on AIS patients receiving GA.
- e) Through neuroprotection of the ischemic penumbra that has not yet converted to infarction, APC might extend the time window for the effectiveness of thrombolytic therapies. Unpublished data from the DAWN (Diffusion weighted imaging or computed tomography perfusion assessment with clinical mismatch in the triage of wake up and late presenting strokes undergoing neurointervention with Trevo) trial, presented at the 2017 European Stroke Organization Conference, indicates that neurothrombectomy is superior to standard medical therapy even when performed beyond 6 h since the last known well time among the subset patients with limited infarct size and clinical-core mismatch. By sustaining the viability of the penumbral tissue through collateral flow, APC might increase the proportion of patients

who can successfully undergo neurothrombectomy beyond the standard 6–8 h time window.

- f) If recanalization occurs too late to benefit the ischemic tissue that has progressed to infarction, the intrinsic neurogenic and angiogenic properties of APC, confirmed in both in vitro and in vivo models, might enhance functional recovery and improve clinical outcome.

These theoretical benefits of APC as an adjunct to endovascular thrombolysis form the underpinnings of the “Safety Evaluation of 3K3A-APC in Ischemic Stroke (RHAPSODY)” trial (NCT02222714, NN104). This multicenter, prospective, double-blinded, dose-escalation Phase 2 randomized clinical trial assesses the safety, pharmacokinetics, and efficacy of four increasing doses of 3K3A-APC following treatment with tPA and/or mechanical neurothrombectomy.

The RHAPSODY protocol utilizes a regimen of intravenous 3K3A-APC bolus doses every 12 h, up to a total of 5 doses following endovascular thrombolysis. Prior studies in sepsis have shown that low-dose continuous infusion of APC is very unlikely to optimize the favorable cell signaling actions leading to cytoprotection and that bolus dosing more effectively promotes the receptor activation leading to altered gene expression profiles, which in turn contribute to the beneficial effects of APC in AIS, such a BBB stabilization and anti-apoptotic and anti-inflammatory activities (Griffin et al., 2015). The previous Phase 1 safety study in normal subjects confirmed that high dose bolus regimens using 3K3A-APC are safe and feasible in adults (Lyden et al., 2013).

The RHAPSODY trial began recruitment in 2014 and recently completed its preplanned enrollment of 110 patients, with approximately half of the study drug patients receiving IV tPA and the other half receiving thrombectomy (Lyden et al., 2016). The study results are anticipated at the end of 2017.

#### 4. Conclusion

Adjunctive delivery of a multiple-action, multiple-target drug may augment the benefit of endovascular thrombolysis with IV tPA and/or mechanical neurothrombectomy. The anti-inflammatory, anti-apoptotic, neuroprotective, and neuroregenerative properties of APC make this agent an ideal candidate for such a strategy.

#### Conflict of interest

Dr. Griffin is a consultant for ZZ Biotech LLC and inventor for some uses of 3K3A-APC.

Dr. Zlokovic is a founder of ZZ Biotech LLC, a biotechnology company with a mission to develop APC and its functional mutants for the treatment of stroke and other neurological disorders.

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