Activated protein C β-glycoform promotes enhanced noncanonical PAR1 proteolysis and superior resistance to ischemic injury

Eimear M. Gleeson,1,2 María G. Dichiara,3 Agustina Salicio,3 Louise M. Quinn,1,2 Clive Drakeford,1,2 Shane E. Russell,1,2 Patrick T. Walsh,1,2 Josune Orbe,3 José Hermida,3 Owen P. Smith,2,4 James S. O’Donnell,5 Ramón Montes,3 and Roger J. S. Preston1,2

1Department of Clinical Medicine, School of Medicine, Trinity College Dublin, Dublin, United Kingdom; 2National Children’s Research Centre, Our Lady’s Children’s Hospital Crumlin, Crumlin, Dublin, United Kingdom; 3Atherothrombosis Laboratory, Center for Applied Medical Research, University of Navarra, Pamplona, Spain; 4Department of Haematology, Our Lady’s Children’s Hospital Crumlin, Crumlin, Dublin, United Kingdom; and 5Haemostasis Research Group, School of Medicine, Trinity College Dublin, Dublin, United Kingdom

Introduction

Activated protein C (APC) plays multiple regulatory roles that cumulatively serve to limit thrombus development5 and attenuate inflammation.2 APC engagement with the endothelial cell protein C receptor (EPCR) facilitates proteolysis-dependent activation of protease-activated receptor 1 (PAR1),1 which protects the endothelial cell barrier from disruptive agents,4 inhibits apoptosis,5,6 and limits cytokine expression from cells exposed to proinflammatory stimuli.6,7 Recombinant APC signaling attenuates neurovascular sequelae associated with experimental murine ischemic stroke8-10 and limits neurotoxicity caused by subsequent thrombolytic tissue plasminogen activator (t-PA) administration.11,12 Recombinant APC therefore represents a promising neuroprotective drug candidate to treat ischemic stroke, and a recombinant nonanticoagulant APC variant is currently being evaluated as an adjunctive therapy to minimize the adverse effects of t-PA administration.13,14

A recombinant APC variant (APCN329Q) that mimics the naturally occurring APC-β plasma glycoform was found to exhibit superior PAR1 proteolysis at a cleavage site that selectively mediates cytoprotective signaling. APCN329Q also enhanced integrin αMβ2-dependent PAR1 proteolysis to exert significantly improved antiinflammatory activity on macrophages compared with wild-type APC. Recent therapeutic applications of recombinant APC in ischemic stroke models have used APC variants with limited anticoagulant activity to negate potential bleeding side effects. Using a mouse model of ischemic stroke and late t-PA intervention, the neuroprotective activity of a murine APC variant with limited anticoagulant activity (mAPCPS)15 was compared with an identical APC variant except for the absence of glycosylation at the APC-β sequon (mAPCPS/N329Q), demonstrated the potential of novel APC variants with superior cytoprotective signaling function as enhanced therapeutic agents for the treatment of ischemic stroke. (Blood. 2015;126(7):915-919)

Study design

Materials

A complete description of the reagents used in this study is included in the supplemental Material, found on the Blood Web site.

Preparation of recombinant APC

Recombinant human and murine wild-type protein C and variants were generated, expressed, and purified largely as previously described.16,17 A detailed description is provided in the supplemental Material.

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The online version of this article contains a data supplement.
Detection of PAR1 proteolysis on HEK 293T cells

PAR1 reporter–transfected 293T cells were incubated with APC in serum-free Dulbecco’s modified Eagle medium supplemented with 3 mM CaCl₂ and 0.6 mM MgCl₂ for 3 hours, after which the supernatant was removed and alkaline phosphatase (AP) activity measured using an AP substrate (QUANTIBlue, Invivogen) at 650 nm.

APC inhibition of cytokine secretion from LPS-stimulated macrophages

Analysis of APC inhibition of cytokine secretion from RAW264.7 cells was performed as previously described. Tumor necrosis factor-alpha (TNFα) and IL-6 were measured by enzyme-linked immunosorbent assay (ELISA) (R&D Systems).

Mouse model of ischemic stroke

Ischemic stroke in mice was induced as previously described and is further detailed in the supplemental Material. Briefly, 200 μL of 10 mg/kg t-PA (10% bolus, 90% perfusion during 20 minutes) with or without 0.2 mg/kg mAPCPS or mAPCPS/N329Q (50% bolus, 50% perfusion during 20 minutes) were given intravenously 3 hours after vessel occlusion. After 24 hours, mice were killed and their brains removed and frozen in isopentane. 20 μm cryostat-cut coronal brain sections were stained with thionine and analyzed (ImageJ 1.48V). For volume analysis, one section of every 10 was stained covering the entire lesion, and lesion (unstained) areas were measured.

Results and discussion

We hypothesized that enhanced cytoprotective PAR1 signaling by APC-β was a consequence of accelerated EPCR-dependent PAR1 proteolysis. To test this, 293T cells cotransfected with EPCR and PAR1 linked to an N-terminal alkaline phosphatase (AP) cleavage reporter were treated with recombinant APC-β (APCN329Q) or N-glycosidase PNGase-F–treated APC (APCNG), in which all N-linked glycans were excised. Interestingly, both APCNG and APCN329Q demonstrated up to a fourfold increased PAR1 proteolysis when compared with wild-type APC (Figure 1A) or other APC glycoform variants (data not shown).
Generation of unique tethered ligands by APC or thrombin, caused by distinct cleavage events at Arg-46 or Arg-41, respectively, on PAR1, has been proposed as a potential mechanism to explain the disparate outcomes of PAR1 signaling initiated by each protease. To determine whether the enhanced cytoprotective activity exhibited by APCN329Q was a consequence of enhanced PAR1 proteolysis at the noncanonical Arg-46 cleavage site, 293T cells were cotransfected with EPCR and either PAR1R41Q-AP or PAR1R46Q-AP reporter variants (Figure 1B-C). Notably, cleavage at the Arg-46 site on PAR1 was especially sensitive to the loss of N-linked glycosylation after PNGase F treatment, and particularly loss of glycosylation at the APC-β sequon (Figure 1B). In contrast, Arg-41 proteolysis was limited in the presence of APC and largely insensitive to glycosylation status (Figure 1C). The increased rate of Arg-46 proteolysis on PAR1 by APCN329Q provides a novel molecular explanation for the enhanced cytoprotective activity of APCN329Q on endothelial cells and murine ischemic stroke.

**Figure 2. APC-β exhibits superior inhibition of cerebral injury in murine ischemic stroke.** (A) 293T cells transfected with EPCR and PAR1-AP were incubated with mAPC or mAPC N329Q (6.25-50 nM) for 3 hours. AP activity was assessed using QUANTI Blue AP substrate, as before. RAW264.7 macrophages were incubated with mAPC (black bars) or mAPC N329Q (red bars) (12.5-50 nM) for 3 hours before stimulation with LPS (50 ng/mL) for 18 hours. Secretion of (B) TNFα and (C) IL-6 was measured by ELISA. (D) Schematic description of the ischemic stroke model and study protocol. (E) Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis under reducing conditions of purified recombinant mAPC PS and mAPC PS/N329Q. Proteins were stained with Coomassie Brilliant Blue. The heavy chain of mAPC PS migrates as a diffuse band composed of the APC-α heavy chain and APC-β glycoform heavy chain fractions. The heavy chain of mAPC PS/N329Q is composed solely of the APC-β fraction. (F) Brain infarct volumes post-stroke and t-PA administration were calculated in each mouse by staining 20-μm coronal brain sections with thionine, which is unable to stain lesion areas. For volume analysis, 1 section of every 10 covering the entire lesion was analyzed. Individual values and the median of each group are represented; n = 9-15; *P = .006, **P = .004. (G) Images corresponding to 2 thionine-stained sections of representative mice from each group are shown. Lesion areas are unstained, whereas remaining healthy tissue is stained purple.
supports the emerging concept of biased PAR1 agonism to achieve downstream cytoprotective signaling.

\( \alpha_{\text{M}} \beta_2 \) can replace EPCR as a coreceptor for PAR1-dependent APC antinflammatory signaling on macrophages. To determine whether APC glycosylation also negatively regulates \( \alpha_{\text{M}} \beta_2 \)-dependent PAR1 signaling, we tested the ability of APC\(^{\text{PNG}} \) and APC\(^{\text{N329Q}} \) to attenuate proinflammatory cytokine release from lipopolysaccharide (LPS)-stimulated macrophages. APC\(^{\text{PNG}} \) and APC\(^{\text{N329Q}} \) reduced TNFα and IL-6 release from LPS-treated macrophages significantly more effectively than wild-type APC (Figure 1D-G). 293T cells coexpressing recombinant human \( \alpha_{\text{M}} \beta_2 \) and PAR1-AP were used to determine whether enhanced antinflammatory activity was mediated by increased \( \alpha_{\text{M}} \beta_2 \)-dependent PAR1 proteolysis by APC\(^{\text{N329Q}} \). Interestingly, the \( \alpha_{\text{M}} \) subunit mediated all coreceptor activity for \( \alpha_{\text{M}} \beta_2 \)-PAR1 proteolysis by APC, and enabled APC cleavage of PAR1 at both canonical (Arg-41) and noncanonical (Arg-46) sites (supplemental Figure 1). Both APC\(^{\text{PNG}} \) and APC\(^{\text{N329Q}} \) significantly enhanced \( \alpha_{\text{M}} \beta_2 \)-dependent PAR1 proteolysis (Figure 1H), indicating that N-linked glycans at Asn-329 regulate cytoprotective signaling by APC by impairing PAR1 proteolysis, irrespective of the APC coreceptor used.

Thrombolysis with t-PA is the only Food and Drug Administration–approved thrombolytic treatment of ischemic stroke, but can only be safely administered within 3 hours of symptom onset because of significant neurotoxicity. Recombinant APC variants with severely attenuated anticoagulant activity reduce t-PA–associated toxicity in murine models of ischemic stroke in a PAR1-dependent manner. We hypothesized that a recombinant nonanticoagulant APC that consists solely in the \( \alpha_{\text{M}} \)-glycoform would be more efficacious in reducing t-PA neurotoxicity than existing APC variants in a murine model of ischemic stroke with late t-PA intervention. Similar to its human equivalent, in vitro analysis indicated that loss of N-linked glycan attachment at Asn-329 in murine APC (mAPCN329Q) enhanced PAR1 proteolytic and antinflammatory activity (Figure 2A-C). To determine whether the therapeutic activity of an APC variant with reduced anticoagulant activity could be enhanced by selective deglycosylation, a signaling-competent mAPC variant (mAPC-D36A/L38D/A39V; mAPC\(^{\text{DPS}} \)) with severely impaired anticoagulant activity in murine plasma was prepared. The therapeutic efficacy of this variant was compared with an identical mAPC variant prepared on an APC-\( \beta \) background (mAPC\(^{\text{PS/N329Q}} \)) (Figure 2D-E). As anticipated, sham-operated mice did not develop cerebral injury (supplemental Figure 2). Like other mAPC variants with reduced anticoagulant activity, mAPC\(^{\text{DPS}} \) retained the ability to reduce t-PA–induced cerebral injury 24 hours after stroke and t-PA administration, albeit modestly in this model (Figure 2F-G). mAPC\(^{\text{PS/N329Q}} \), however, significantly reduced brain lesion size compared with when t-PA was administered alone after the ischemic event (\( P = .006; \) Figure 2F-G).

Remarkably, mAPC\(^{\text{PS/N329Q}} \) was also significantly superior in preventing brain lesions compared with mAPC\(^{\text{PS}} \) (\( P = .004; \) Figure 2F-G), despite their structures only differing by the presence or absence of a specific N-linked glycan.

This study shows that effective cytoprotective PAR1 signaling by APC is determined by specific N-linked glycans absent in certain naturally occurring APC glycoforms, revealing new mechanistic insights as to how PAR1 proteolysis by APC is regulated. Moreover, APC-\( \beta \)-sequon disruption boosts recombinant nonanticoagulant APC amelioration of cerebral ischemic injury after late t-PA treatment in ischemic stroke. Although further studies are required to investigate which of the multiple PAR1-dependent neuroprotective functions mediated by APC in vivo are particularly enhanced by APC\(^{\text{PS/N329Q}} \) in this setting, recombinant APC variants capable of hyperactive cytoprotective signaling represent a novel approach to boost the efficacy of APC-based therapeutic strategies without increasing dosage or compromising safety.

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Authorship

Contribution: E.M.G., M.G.D., A.S., L.M.Q., C.D., and S.E.R., performed experiments and analyzed data; P.T.W., J.O., J.H., O.P.S., and J.S.O’D. analyzed data and planned experiments; R.M. and R.J.S.P. analyzed data, planned experiments, and conceived the study; and all authors contributed to the preparation of the manuscript.

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Correspondence: Roger J. S. Preston, School of Medicine, Trinity College Dublin, United Kingdom; e-mail: prestonr@tcd.ie; and Ramón Montes, Center for Applied Medical Research, University of Navarra, Pamplona, Spain; e-mail: montesdiazpamplona@outlook.com.

References


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