ACTIVATED PROTEIN C β-GLYCOFORM PROMOTES ENHANCED NON-CANONICAL PAR1 PROTEOLYSIS AND SUPERIOR RESISTANCE TO ISCHEMIC INJURY

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Key points
• Novel mechanistic role for a specific N-linked glycan on APC in regulating PAR1 proteolysis
• First description of an APC variant with enhanced therapeutic cytoprotective activity in vivo

Abstract
Activated protein C (APC) is an anticoagulant protease that initiates cell signaling via protease-activated receptor 1 (PAR1) to regulate vascular integrity and inflammatory response. In this study, a recombinant APC variant (APC\textsuperscript{N329Q}) mimicking the naturally occurring APC-β plasma glycoform was found to exhibit superior PAR1 proteolysis at a cleavage site that selectively mediates cytoprotective signaling. APC\textsuperscript{N329Q} also enhanced integrin $\alpha_M\beta_2$-dependent PAR1 proteolysis to exert significantly improved anti-inflammatory activity on macrophages compared to wild type APC. Recent therapeutic applications of recombinant APC in ischemic stroke models have utilized 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 (mAPC\textsuperscript{PS}) was compared to an identical APC variant except for the absence of glycosylation at the APC-β sequon (mAPC\textsuperscript{PS/N329Q}). Remarkably, mAPC\textsuperscript{PS/N329Q} limited cerebral ischemic injury and reduced brain lesion volume significantly more effectively than mAPC\textsuperscript{PS}. Collectively, this study reveals the importance of APC glycosylation in controlling the efficacy of PAR1 proteolysis by APC and demonstrates the potential of novel APC variants with superior
cytoprotective signaling function as enhanced therapeutic agents for the treatment of ischemic stroke.

**Introduction**

Activated protein C (APC) plays multiple regulatory roles that cumulatively serve to limit thrombus development\(^1\) and attenuate inflammation\(^2\). APC engagement with the endothelial cell protein C receptor (EPCR) facilitates proteolysis-dependent activation of protease-activated receptor 1 (PAR1)\(^3\), which protects the endothelial cell barrier from disruptive agents\(^4\), inhibits apoptosis\(^5,6\) and limits cytokine expression from cells exposed to pro-inflammatory stimuli\(^6,7\).

Recombinant APC signaling attenuates neurovascular sequelae associated with experimental murine ischemic stroke\(^8-10\) and limits neurotoxicity due to 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 non-anticoagulant 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 (APC\(^\text{N329Q}\)) that mimics the glycosylation pattern of the endogenous plasma APC-β glycoform\(^15\) exhibits significantly enhanced PAR1-dependent cytoprotective activity on endothelial cells compared to wild type APC\(^16\). In this study, we sought to determine the molecular basis for superior APC-β cytoprotective signaling and establish whether ablation of the APC-β glycosylation
sequon to accelerate cytoprotective signaling represents a novel means by which to increase the therapeutic efficacy of APC in an ischemic stroke model.

Methods

Materials

A complete description of the reagents used in this study is included in ‘Supplementary Material’.

Preparation of recombinant APC

Recombinant human and murine wild type protein C and variants were generated, expressed and purified largely as previously described.\textsuperscript{16,17} A detailed description is provided in ‘Supplementary Material’.

Detection of PAR1 proteolysis on HEK 293T cells

PAR1 reporter-transfected 293T cells were incubated with APC in serum-free DMEM supplemented with 3mM CaCl\textsubscript{2} and 0.6mM MgCl\textsubscript{2} for 3 hours, after which the supernatant was removed and AP activity measured using an AP substrate (QUANTI-Blue, Invivogen) at 650nm.

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\textsuperscript{18}. TNF\textalpha and IL-6 were measured by ELISA (R&D Systems).
Mouse model of ischemic stroke

Ischemic stroke in mice was induced as previously described\textsuperscript{19} and further detailed in ‘Supplementary Materials’. Briefly, 200\,\mu l of 10mg/kg t-PA (10\% bolus, 90\% perfusion during 20 minutes) with or without 0.2mg/kg mAPC\textsuperscript{PS} or mAPC\textsuperscript{PS/N329Q} (50\% bolus, 50\% perfusion during 20 minutes) were given intravenously 3 hours after vessel occlusion. After 24 hours, mice were euthanized and brains removed and frozen in isopentane. 20\,\mu m cryostat-cut coronal brain sections were stained with thionine and analyzed (Image J 1.48V). For volume analysis, one section out of every 10 was stained covering the entire lesion, and lesion (unstained) areas were measured.

Results & Discussion

We hypothesized that enhanced cytoprotective PAR1 signaling by APC-\beta was a consequence of accelerated EPCR-dependent PAR1 proteolysis. To test this, 293T cells co-transfected with EPCR and PAR1 linked to an N-terminal alkaline phosphatase (AP) cleavage reporter\textsuperscript{20} were treated with recombinant APC-\beta (APC\textsuperscript{N329Q}) or N-glycosidase PNGase F-treated APC (APC\textsuperscript{PNG}), in which all N-linked glycans were excised\textsuperscript{16}. Interestingly, both APC\textsuperscript{PNG} and APC\textsuperscript{N329Q} demonstrated up to 4-fold increased PAR1 proteolysis when compared to wild type APC (Figure 1a) or other APC glycoform variants (data not shown).

Generation of unique tethered ligands by APC or thrombin, due to 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\textsuperscript{21,22}. To determine whether the enhanced cytoprotective activity exhibited by
APC^{N329Q} was a consequence of enhanced PAR1 proteolysis at the non-canonical Arg-46 cleavage site, 293T cells were co-transfected with EPCR and either PAR1^{R41Q}-AP or PAR1^{R46Q}-AP reporter variants (Figure 1b and 1c). Notably, cleavage at the Arg-46 site on PAR1 was especially sensitive to loss of N-linked glycosylation following 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 APC^{PNG} and APC^{N329Q} provides a novel molecular explanation for the enhanced cytoprotective activity of APC^{N329Q} on endothelial cells and supports the emerging concept of biased PAR1 agonism to achieve downstream cytoprotective signaling.

α_{Mβ2} can replace EPCR as a co-receptor for PAR1-dependent APC anti-inflammatory signaling on macrophages. To determine whether APC glycosylation also negatively regulates α_{Mβ2}-dependent PAR1 signaling, we tested the ability of APC^{PNG} and APC^{N329Q} to attenuate pro-inflammatory cytokine release from LPS-stimulated macrophages. APC^{PNG} and APC^{N329Q} reduced TNFα and IL-6 release from LPS-treated macrophages significantly more effectively than wild type APC (Figure 1d-g). 293T cells co-expressing recombinant human α_{Mβ2} and PAR1-AP were used to determine whether enhanced anti-inflammatory activity was mediated by increased α_{Mβ2}-dependent PAR1 proteolysis by APC^{N329Q}. Interestingly, the α_{M} subunit mediated all co-receptor activity for α_{Mβ2}-PAR1 proteolysis by APC, and enabled APC cleavage of PAR1 at both canonical (Arg-41) and non-canonical (Arg-46) sites (Supplementary Figure 1). Both APC^{PNG} and APC^{N329Q} significantly enhanced α_{Mβ2}-dependent PAR1 proteolysis (Figure
1h), indicating N-linked glycans at Asn-329 regulate cytoprotective signaling by APC by impairing PAR1 proteolysis, irrespective of APC co-receptor used.

Thrombolysis with t-PA is the only FDA-approved thrombolytic treatment for ischemic stroke, but can only be safely administered within 3 hours of symptom onset due to 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 non-anticoagulant APC that consists solely in the β-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 (mAPC<sup>N329Q</sup>) enhanced PAR1 proteolytic and anti-inflammatory activity (Figure 2a-c). To determine whether the therapeutic activity of an APC variant with reduced anticoagulant activity could be enhanced by selective de-glycosylation, a signaling-competent mAPC variant (mAPC-D36A/L38D/A39V; mAPC<sub>PS</sub>) with severely impaired anticoagulant activity in murine plasma was prepared. The therapeutic efficacy of this variant was compared to an identical mAPC variant prepared on an APC-β background (mAPC<sub>PS/N329Q</sub>) (Figure 2d & 2e). As anticipated, sham-operated mice did not develop cerebral injury (Supplementary Figure 2). Like other mAPC variants with reduced anticoagulant activity, mAPC<sub>PS</sub> 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 & 2g). mAPC<sub>PS/N329Q</sub>, however, significantly reduced brain lesion size.
compared to when t-PA was administered alone following the ischemic event (p=0.006; Figure 2f & 2g). Remarkably, mAPC$_{PS/N329Q}$ was also significantly superior in preventing brain lesion compared to mAPC$_{PS}$ (p=0.004; figure 2f & 2g), 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-β sequon disruption boosts recombinant non-anticoagulant APC amelioration of cerebral ischemic injury following 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 \textit{in vivo}$^{13}$ are particularly enhanced by APC$_{PS/N329Q}$ 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 contributions

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

Conflict of interest disclosure

R.J.S.P. has received honoraria from Octapharma and research funding from Bayer Healthcare Pharmaceuticals.

References


FIGURE LEGENDS:

Figure 1: APC glycosylation at Asn-329 specifically restricts PAR1 proteolysis and signaling activity. 293T cells transfected with EPCR and either (a) PAR1-AP (b) PAR1R41Q-AP (c) PAR1R46Q-AP were treated with APC (black circles), APCPNG (red squares) or APCN329Q (green triangles) (all 6.25-50nM) for 3 hours, before AP activity was measured using an AP substrate. An extended period of incubation with the AP substrate was required to observe any AP activity in cell supernatants from APC-treated EPCR/PAR1R46Q-AP transfected 293T cells. Murine macrophages (RAW264.7) were incubated with APC (black bars) or APCPNG (red bars) (5-20nM) for 3 hours prior to stimulation with LPS (50ng/ml) for 18 hours. (d) TNFα and (e) IL-6 secretion was measured by ELISA. Similarly, APC (black bars) and APCN329Q (yellow bars; 10-20nM) were incubated with RAW264.7 macrophages for 3 hours prior to LPS (50ng/ml) incubation for 18 hours. The resultant supernatants were assessed for the presence of (f) TNFα and (g) IL-6 as before. 293T transfected cells with (h) PAR1-AP alone (black), EPCR/PAR1-AP (red) or αMβ2/PAR1-AP (green) were treated with thrombin (5nM), wild type APC or APCN329Q (both 50nM) for 3 hours before AP activity was assessed. All
experiments were performed at least in triplicate and results are presented as the mean±/ S.E.M.; *p<0.05, **p<0.01 by Students t-test.

**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} (6.25-50nM) 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) (12.5-50nM) for 3 hours prior to stimulation with LPS (50ng/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) SDS-PAGE 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 20μm coronal brain sections with thionine, which is unable to stain lesion areas. For volume analysis, one section of every 10 covering the entire lesion was analyzed. Individual values and the median of each group are represented. n=9-15; *p=0.006, **p=0.004. (g) Images corresponding to two thionine-stained sections of representative mice from each group are shown. Lesion areas are unstained, whereas remaining healthy tissue is stained purple.
Figure 1

(a) EPCR/PAR1WT-AP
(b) EPCR/PAR1R41Q-AP
(c) EPCR/PAR1R458Q-AP

(d) TNFa (pg/ml)
(e) IL-6 (pg/ml)
(f) TNFa (pg/ml)
(g) IL-6 (pg/ml)
(h) PAR1AP activity (OD650)

Figure 1
Figure 2

(a) PAR1 activity (OD650) for mAPC and mAPC<sub>N329Q</sub>.

(b) TNF-α levels (ng/ml) for mAPC and mAPC<sub>N329Q</sub>.

(c) IL-6 levels (pg/ml) for mAPC and mAPC<sub>N329Q</sub>.

(d) Diagram showing the process of thrombin formation, stable clot formation, and brain lesion size assessment.

1. Tissue plasminogen activator (t-PA)
2. t-PA + APC<sup>PS</sup>
3. t-PA + APC<sup>PS/N329Q</sup>

(e) Gel electrophoresis showing proteins α HC, β HC, and LC.

(f) Graph showing lesion size (mm<sup>2</sup>) for different treatments.

(g) Images of brain lesion size for t-PA, mAPC<sup>PS</sup>, and mAPC<sup>PS/N329Q</sup> conditions.
Activated protein C β-glycoform promotes enhanced non-canonical PAR1 proteolysis and superior resistance to ischemic injury

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