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REGULAR ARTICLE

Antithrombotic and anticoagulant effects of wild type and Gla-domain mutated human activated protein C in rats

Karl Malm [a,*], **Björn Arnljots** [a], **Ing-Marie Persson** [b], **Björn Dahlbäck** [b]

[a] *Department of Clinical Sciences, Division of Reconstructive Surgery, University Hospital, Malmö, Sweden*
[b] *Department of Laboratory Medicine, Division of Clinical Chemistry, University Hospital, Malmö, Sweden*

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Abstract The antithrombotic and anticoagulant effects of recombinant wild type (WT) and mutated human activated protein C (hAPC) were investigated using a rat model of arterial thrombosis. Recent *in vitro* studies using human plasma have shown enhanced anticoagulant effects of hAPC by mutagenesis of either loop 148 in the serine protease domain or of the Gla domain. The Gla-domain mutant QGNSEDY-hAPC (=H10Q/S11G/S12N/D23S/Q32E/N33D/H44Y) was found to be particularly active as an anticoagulant. We now combined the two mutations to create the variant QGNSEDY-hAPC:B148 and investigated the *in vivo* effects of this variant as well as of QGNSEDY-hAPC and WT hAPC using a rat model of arterial thrombosis. *In vitro* clotting experiments using rat plasma demonstrated WT hAPC to be inefficient, whereas both mutant hAPC variants yielded distinct dose dependent anticoagulant effects. In the arterial injury model, a segment of the left common carotid artery was opened longitudinally. An endarterectomy was performed and the arteriotomy was closed, whereafter the vessel was reperfused and the patency rate determined after 31 min. Three treatment groups each containing 10 rats and a control group of 20 animals were in a blind random fashion given intravenous bolus injections of 0.8 mg/kg WT or mutant hAPC or vehicle only. The *ex vivo* clotting times of plasma drawn 3 min after the injections, as compared to baseline clotting times, were approximately doubled by QGNSEDY-hAPC and tripled by QGNSEDY-hAPC:B148 infusions, while WT APC had little effect. Compared to the control group, none of the hAPC preparations had significant antithrombotic effect or increased arteriotomy bleeding.

Abbreviations: WT, wild type; APC, activated protein C; PS, protein S; EPCR, endothelial protein C receptor; PAR, protease activated receptor; APTT, activated partial thromboplastin time; PT, prothrombin time; TBS, tris-buffered saline; BSA, bovine serum albumin; SDS-PAGE, sodium dodecyl sulfate polyacrylamid gel electrophoresis.

* Corresponding author. Department of Plastic and Reconstructive Surgery, University Hospital Malmö, SE-205 02 Malmö, Sweden. Tel.: +46 40 33 10 00; fax: +46 40 33 62 71.
E-mail address: karl.malm@med.lu.se (K. Malm).

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Protein C is a vitamin K-dependent protein that circulates in plasma as a zymogen to a serine protease with anticoagulant properties [1–4]. In response to thrombin generation, protein C is activated on the endothelial surface by the thrombin-thrombomodulin complex [4]. Activated protein C (APC) together with its cofactor protein S (PS) effectively down-regulates blood coagulation by cleaving and inhibiting the two cofactors activated factor V (FVa) and activated factor VIII (FVIIIa) [5,6]. Several cleavage sites have been identified in both FVa and FVIIIa, of which the cleavages at Arg306 and Arg506 in FVa have been studied in detail [7–12].

The physiological importance of the anticoagulant properties of APC is best illustrated by the increased risk of venous thrombosis in individuals with genetic defects affecting the protein C system [1,13,14]. In addition to its anticoagulant properties, APC has been shown to possess anti-inflammatory functions, which appear to depend on the presence of endothelial protein C receptor (EPCR) [15]. EPCR is suggested to direct the proteolytic activity of APC towards the protease activated receptor 1 (PAR1) rather than to FVa and FVIIIa [16]. APC has been shown to be efficient in the treatment of septic shock, the beneficial effects possibly being due to both the anticoagulant and anti-inflammatory properties of APC [15,17].

The anticoagulant properties of APC make it an interesting therapeutic candidate for prevention and treatment of venous and arterial thrombosis. There are several reports on record investigating the effect of APC in different animal models [18–31]. We have previously described powerful antithrombotic effects of bovine APC (bAPC) in combination with bovine PS (bPS) in both rabbit and rat models of deep arterial injury [18–20,32].

We have recently created several recombinant human protein C variants with enhanced anticoagulant activity and the aim of the present investigation was to elucidate their antithrombotic potential in the arterial thrombosis model in the rat. In one of the variants, QGNSEDY-hAPC (H10Q/S11G/S12N/D23S/Q32E/N33D/H44Y), the membrane-binding properties of APC were altered by mutagenesis of the γ-carboxyglutamic acid (Gla)-rich domain. This variant proved to be highly effective as an anticoagulant in human plasma and QGNSEDY-hAPC alone was found to be more potent than WT hAPC together with hPS in a FVa degradation assay [33]. In another recombinant hAPC variant (hAPC:B148), loop 148 in the serine protease domain was exchanged for the corresponding bovine loop, which resulted in an hAPC variant with modestly enhanced catalytic and anticoagulant activity [34]. By combining the mutations in QGNSEDY-hAPC and hAPC:B148 we have now created a new hAPC variant (QGNSEDY-hAPC:B148), which is more effective as an anticoagulant than either of the two original variants.

In this study, we have investigated the antithrombotic, antihaemostatic, and anticoagulant effects of recombinant WT hAPC as well as the two recombinant hAPC variants QGNSEDY-hAPC and QGNSEDY-hAPC:B148. Despite demonstrating strong anticoagulant effects *in vitro* using rat plasma, the two hAPC variants failed to demonstrate antithrombotic effects in the arterial thrombosis model in the rat.

Materials and methods

Recombinant protein C variants

Full length cDNAs for the QGNSEDY-hPC and hPC:B148 introduced in the HindIII and XbaI sites in the pRC/CMV vector were created with recombinant DNA-techniques, as previously described [33–36]. The mutations in the QGNSEDY variant was H10Q/S11G/S12N/D23S/Q32E/N33D/H44Y, where the first letter preceding the amino-acid number is the one-letter abbreviation for the wild-type amino acid, and the letter after the number refers to the amino acid residue introduced by the mutagenesis [33].

The amino-acid numbers refer to the positions in the mature protein, i.e. after removal of the signal sequence and propeptide. In the hPC:B148 mutant, the 148 loop in human protein C, GWGYHSSREKEAKRN (the underlined amino acids correspond to positions 303–310 in the linear sequence, and to 144–149b according to the chymotrypsinogen numbering), was replaced by the four residues shorter corresponding loop in bAPC (GWGYRDETKRN) [34]. The cDNA for the new mutant used in this study QGNSEDY-hPC:B148 was constructed by combining the appropriate fragments of the cDNAs for QGNSEDY and hPC:B148. Thus, the 360 bp HindII-SalI fragment of QGNSEDY cDNA was ligated together with the 1140 bp SalI-Xba I fragment of hPC:B148 cDNA into the pRC/CMV vector.

The cDNAs corresponding to WT hPC and the two variants QGNSEDY-hPC and QGNSEDY-hPC:B148, inserted in the eukaryotic expression vector pRc/CMV, were used to transfect the human kidney cell line 293. High-expressing colonies were selected for large-scale culture and subsequent purification, as previously described [35]. The recombinant protein C variants were activated by thrombin, and then passed through a chromatography column of SP-Sephadex to remove thrombin essentially, as earlier described [36].

In vitro coagulation analyses

Citrated rat plasma was acquired from male Sprague-Dawley albino rats and used for *in vitro* characterization of the anticoagulant activities of the various