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# Protein S modulates the anticoagulant action of recombinant human activated protein C: a comparison between neonates and adults

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**1** Recombinant human-activated protein C (rhAPC, Drotrecogin alpha (activated), Xigris™) has been shown to reduce organ damage and decrease mortality in severe sepsis. Since protein S (PS) serves as a potentiating cofactor of activated protein C and since PS levels are low in neonatal plasma, we hypothesized that the anticoagulant effect of rhAPC would be decreased in cord plasma compared to adult plasma.

**2** We demonstrate that the anticoagulant action of 0.3 $\mu g\,ml^{-1}$ rhAPC (5 $nmol\,l^{-1}$) was decreased in cord plasma compared to adult plasma, and dose dependently increased in cord plasma in the presence of increasing activities of PS.

**3** Correspondingly, the anticoagulant action of rhAPC decreased in adult plasma in the presence of decreasing activities of PS.

**4** The low anticoagulant action of rhAPC in cord compared to adult plasma is attributable to low neonatal levels of PS, and as previously shown, to low neonatal levels of TFPI and AT.

**5** Our laboratory experiments do not allow definite conclusions for clinical situations. However, we speculate that the anticoagulant efficacy of rhAPC is impaired in neonates and in clinical situations associated with consumption and/or inhibition of PS, AT, and TFPI, such as severe sepsis.
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**Abbreviations:** APC, activated protein C; APTT, activated partial thromboplastin time; AT, antithrombin; CT, clotting time; F 1 + 2, prothrombin fragment 1 + 2; PS, protein S; rhAPC, recombinant activated protein C; PT, prothrombin time; TFPI, tissue factor pathway inhibitor; TP, thrombin potential

## Introduction

Recombinant human-activated protein C (rhAPC, Drotrecogin alpha (activated), Xigris™) is approved for adult patients with severe sepsis and a high risk of death (Bernard *et al*., 2001; Dhainaut *et al*., 2004; Fourrier, 2004). Since severe sepsis, defined as sepsis associated with acute organ dysfunction, remains a major cause of morbidity and mortality among neonates and children, treatment with rhAPC might also be beneficial in this age group (Martinot *et al*., 1997; Rawicz *et al*., 2002; Barton *et al*., 2004).

However, one would expect that doses of rhAPC required in neonates are different from that in adults due to different physiological levels of natural inhibitors present in neonates compared to adults known to modulate the anticoagulant efficacy of rhAPC (Andrew *et al*., 1987). For example, tissue factor pathway inhibitor (TFPI) and antithrombin (AT) amplify the anticoagulant action of APC in a synergistic manner in purified systems and also in TF-activated plasma samples (Van't Veer *et al*., 1997). As a result, the anticoagulant action of rhAPC is more pronounced in adult than in cord plasma due to physiologically higher levels of TFPI and AT present in adults (Cvirn *et al*., 2004).

In addition to TFPI and AT, protein S (PS) is known to modulate the anticoagulant action of APC (Dahlbäck, 1991; Dahlbäck & Villoutreix, 2003; Heeb *et al*., 2004). The capability of APC to prolong clotting times (CTs) and suppress thrombin formation was shown to dose dependently increase in the presence of increasing amounts of PS in plasma samples activated *via* the intrinsic pathway (Cvirn *et al*., 2002). Since total PS antigen levels are physiologically low in neonates (approximately 35% of adult value, Andrew *et al*., 1987), a different anticoagulant effect of rhAPC would be expected in neonates compared to adults.

Therefore, the aim of our present study was to evaluate the anticoagulant action of rhAPC in the presence of various levels of PS in cord plasma and adult plasma. The anticoagulant action of rhAPC was investigated with respect to prolongation of CTs, suppression of thrombin potential (TP), and suppression of prothrombin fragment 1 + 2 (F1 + 2) generation in (a) cord plasma spiked to contain increasing activities of PS,

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(b) in adult plasma adjusted to contain decreasing activities of PS, and (c) in native cord and adult plasma. Activation of plasma samples was performed by addition of low amounts of lipidated tissue factor (TF), a condition that apparently better reflects the *in vivo* situation than the strong activation applied in routine determination of clotting parameters (prothrombin time (PT); activated partial thromboplastin time (APTT), Davie *et al*., 1991; Butenas *et al*., 1999; Mann *et al*., 2003). This assay system allows sensitive detection of the effects of different levels of pro- and anticoagulants on thrombin generation.

## Methods

### *Reagents*

Buffer A contained 0.05 mol l$^{-1}$ Tris-HCl at pH 7.4, 0.1 mol l$^{-1}$ NaCl and 0.5 mol l$^{-1}$ bovine serum albumin. Buffer B was analogous to buffer A but contained, in addition, 20 mmol l$^{-1}$ EDTA. RhAPC (rhAPC, Drotrecogin alpha (activated), Xigris™) was purchased from Eli Lilly GmbH, Vienna, Austria. A stock solution was prepared by dissolving 5 mg of the lyophilized glycoprotein in 2.5 ml of distilled water. After subsequent 1 : 10 dilution in 0.9% NaCl, 1 ml aliquots were stored at −70°C. Innovin® (recombinant human TF thromboplastin) from Dade Behring, Marburg GmbH, Marburg, Germany, was used as a source of TF. The lyophilized product was dissolved in 10 ml of distilled water and subsequently diluted at a ratio of 1 : 1000 in NaCl solution (TF-stock solution). Soybean trypsin inhibitor was purchased from CoaChrom Diagnostics, Vienna, Austria. PS from human plasma was obtained from ICN Biomedicals. In all, 100 μg of PS was dissolved in 500 μl 0.9% NaCl, 100 μl aliquots of this PS-stock solution were stored at −70°C. Affinity-purified rabbit antibody to human PS was purchased from HYPHEN BioMed, Neuville, France. The fibrin polymerization-inhibitor H-Gly-Pro-Arg-Pro-OH (GPRP, Pefabloc™ FG) was purchased from Pentapharm LDT (Basel, Switzerland). PS activity kit was purchased from Behring Diagnostics GmbH, Marburg, Germany. Total PS antigen-test was from Haemochrom, Essen, Germany. The chromogenic substrate used for thrombin determination was H-D-Phe-Pip-Arg-pNA · 2HCl (pNAPEP™ 0238) from CoaChrom Diagnostics (Vienna, Austria). Of the lyophilized substrate, 25 mg was dissolved in 7 ml of distilled water to a concentration of 5.7 mmol l$^{-1}$. Testkit F 1 + 2 micro was purchased from Behring Diagnostics GmbH, Marburg, Germany. The stopping solution for F 1 + 2 determination consisted of Na-citrate (3.8%)/EDTA (0.2 mol l$^{-1}$)/Trasylol™ = 8/1/1 and 110 μmol l$^{-1}$ PPACK (D-Phe-Pro-Arg chloromethyl ketone) from Sigma, Vienna, Austria. Trasylol™ from Bayer, Vienna, Austria, contains the protease inhibitor aprotinin.

### *Collection and preparation of plasma*

Cord blood was obtained immediately following the delivery of 17 full-term infants (38–42 weeks gestational age). Informed consent was obtained from their parents. Newborns with Apgar scores of 9 or less 5 min after delivery were excluded from the study. Blood (2.7 ml) was collected in precitrated S-Monovette™ premarked tubes from Sarstedt (Nümbrecht, Germany), containing 300 μl 0.106 mol l$^{-1}$ citrate, centrifuged at room temperature for 15 min at 2800 × *g* and stored at −70°C in propylene tubes. Subsequently, plasma samples were thawed, pooled, and stored at −70°C until assayed. Pro- and anticoagulant factors of the pooled plasma was in the normal range for neonates (Andrew *et al*., 1987). In the same way, plasma from 12 healthy adults was collected from the antecubital vein, prepared, and checked. Total PS antigen level of cord plasma was 36%, and PS activity of cord plasma was 45% of the respective adult value.

### *Preparation of plasma containing various concentrations of PS*

The PS content of pooled adult plasma was decreased by using affinity-purified rabbit antibody to human PS coupled to Sepharose 4B. Other vitamin K-dependent proteins were not affected by the procedure due to the high selectivity of the immobilized antibody. The PS activity of pooled cord plasma was increased by spiking with different amounts of purified PS concentrate.

### *Determination of the PS concentration*

Total PS antigen level was determined by means of monoclonal total PS antigen-test from Haemochrom, Essen, Germany, applying monoclonal antibody directed against PS and peroxidase-conjugated polyclonal-detecting antibody. Determination of the PS activity of plasma samples was performed by means of standard coagulation method using PS-deficient plasma on a Behring Coagulation Timer from Behring Diagnostics, Marburg, Germany. In all, 85 μl of PS-immunodepleted plasma was preincubated at 37°C for 4 min. Then 100 μl of APTT reagent (Behring Diagnostics, Marburg, Germany) was added simultaneously with 100 μl APC (10 μg ml$^{-1}$) and incubated for 4 min. Subsequently, 15 μl of test plasma or a normal plasma sample was added, and after incubation for 30 s, 100 μl of 25 mmol l$^{-1}$ $CaCl_2$ was added and the CT was measured. Standard curves were constructed with normal human plasma dilutions. Subsequently, for the preparation of cord plasma containing increasing levels of PS and adult plasma containing decreasing levels of PS, the PS activity was checked in each plasma sample as described.

### *Activation of plasma*

Plasma (250 μl) containing various amounts of PS was incubated with 50 μl of NaCl solution containing rhAPC (0.3 μg ml$^{-1}$ or 5 nmol l$^{-1}$ final concentration), soybean trypin inhibitor (18 μg ml$^{-1}$ final concentration), and H-Gly-Pro-Arg-Pro-OH (GPRP, Pefabloc™ FG, 1.0 mg ml$^{-1}$ final concentration) to inhibit fibrin polymerization for 1 min at 37°C (Rijkers *et al*., 1998). Subsequently, 20 μl of TF-stock solution (approximately 0.35 pmol l$^{-1}$ final TF concentration, Sorensen *et al*., 2003) was added. Finally, citrated plasma samples were recalcified by addition of 20 μl 0.2 mol l$^{-1}$ $CaCl_2$.

### *Determination of CT*

Plasma was activated as described above except that Pefabloc$^{TM}$ FG was replaced by NaCl solution. CTs were determined by means of optomechanical coagulation analyzer Behring Fibrintimer II from Behring Diagnostics GmbH (Marburg, Germany), which applies the turbodensitometric measuring principle.

### *Determination of thrombin generation*

We used a subsampling method derived from a recently described technique (Hemker *et al*., 1986; Wielders *et al*., 1997). Plasmas were prepared and activated as described above. At timed intervals (30 s), 10 $\mu$l aliquots were withdrawn from the activated plasma and subsampled into 490 $\mu$l buffer B containing 255 $\mu$mol l$^{-1}$ pNAPEP$^{TM}$ 0238. Amidolysis of pNAPEP$^{TM}$ 0238 was stopped after 6 min by addition of 250 $\mu$l 50% acetic acid. The amount of thrombin generated was quantitated by measuring the absorbance by double wavelength (405–690 nm) in the Anthos microplate-reader 2001, from Anthos Labtec Instruments GmbH (Salzburg, Austria). The total amidolytic activity measured is caused by the simultaneous activity of free thrombin and the alpha 2-macroglobulin–thrombin complex. Free thrombin generation curves were calculated by mathematical treatment of total amidolytic activity curves using a method developed by Hemker *et al*. (1993). The area under the free thrombin generation curve has been called 'TP'. TP has been shown to be a suitable parameter to reflect the thrombogenic potency in a given plasma sample.

### *Determination of prothrombin fragment 1 + 2*

Plasmas were prepared and activated as described above. At timed intervals 10 $\mu$l aliquots were withdrawn from the plasma and subsampled into 490 $\mu$l stopping solution. After subsequent 1 : 10 dilution in the stopping solution, the amount of F1 + 2 generated was quantitated by using standard immunoenzymatic test kit from Behring Diagnostics GmbH, Marburg, Germany.

### *Statistical analysis*

Results obtained in cord and adult plasmas were compared by means of Mann–Whitney *U*-test. Results are expressed as mean $\pm$ s.d. ($n = 5$). The effects of different activities of PS in the presence of rhAPC on CT, TP, and F1 + 2 generation were analyzed using Wilcoxon's test. The significance level of *P*-values was set at 5%.

## Results

CTs of native cord and adult plasmas were 285 $\pm$ 16 and 365 $\pm$ 18 s ($P < 0.01$), TPs were 253 $\pm$ 15 and 298 $\pm$ 19 nmol l$^{-1}$ min ($P < 0.01$), and F1 + 2 generation was 0.487 $\pm$ 0.02 and 0.634 $\pm$ 0.03 $\mu$mol l$^{-1}$ ($P < 0.01$), respectively, in the presence of 0.35 pmol l$^{-1}$ TF to induce clot formation. All experiments were performed in the presence of 18 $\mu$g ml$^{-1}$ of soybean trypsin inhibitor in order to abolish contact factor activation.

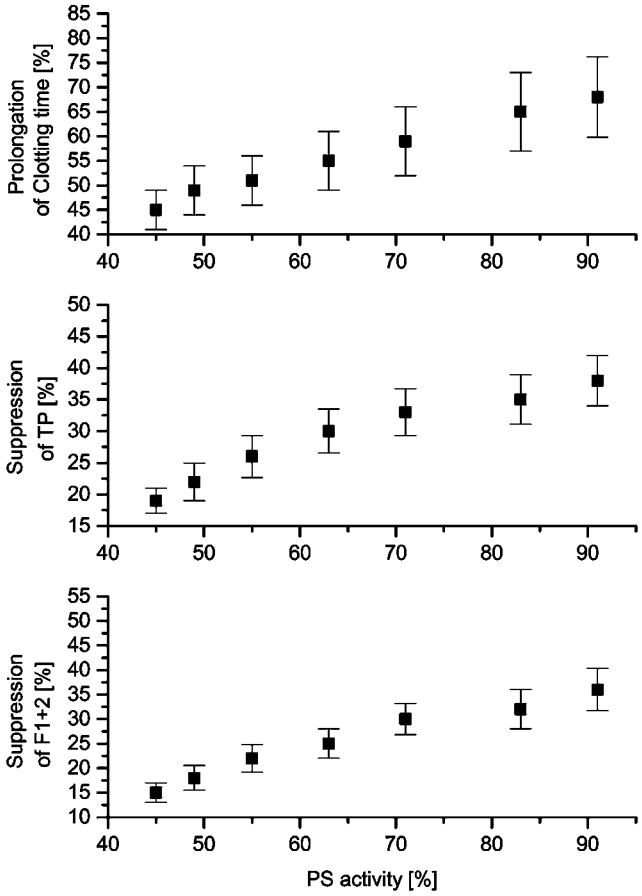


**Figure 1** Effect of PS on the anticoagulant action of rhAPC in cord plasma. Effect of increasing activities of PS on the anticoagulant action of 0.3 $\mu$g ml$^{-1}$ rhAPC in cord plasma. The PS activity of native cord plasma was 45%.

Pooled cord plasma contained 36% total PS antigen and 45% PS activity level compared with adults.

### *Effect of increasing levels of PS on the anticoagulant action of rhAPC in cord plasma*

The PS activity of cord plasma was successively raised by the addition of increasing amounts of purified PS concentrate. Prolongation of CT (Figure 1a), suppression of TP (Figure 1b), and suppression of F1 + 2 generation (Figure 1c) in cord plasma due to addition of 0.3 $\mu$g ml$^{-1}$ (5 nmol l$^{-1}$) rhAPC dose dependently increased in the presence of increasing amounts of PS.

### *Effect of decreasing levels of PS on the anticoagulant action of rhAPC in adult plasma*

The PS activity of adult plasma was successively decreased by means of PS-antibody coupled to sepharose. Prolongation of CT (Figure 2a), suppression of TP (Figure 2b), and suppression of F1 + 2 generation (Figure 2c) due to addition of 0.3 $\mu$g ml$^{-1}$ (5 nmol l$^{-1}$) rhAPC dose-dependently decreased in the presence of decreasing amounts of PS.

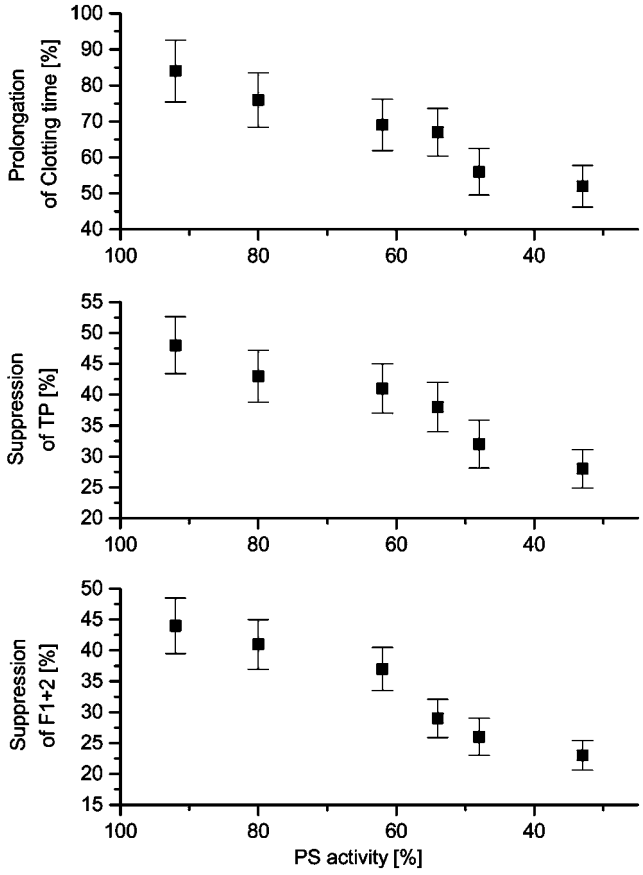


**Figure 2** Effect of PS on the anticoagulant action of rhAPC in adult plasma. Effect of decreasing activities of PS on the anticoagulant action of 0.3 $\mu$g ml$^{-1}$ hAPC in adult plasma. The PS activity of native adult plasma was 92%.

### *The anticoagulant action of rhAPC in cord plasma compared with that in adult plasma adjusted to contain PS at neonatal level*

Prolongation of CT (45±4 vs 56±6.5%), suppression of TP (19±2 vs 32±3.9%), and suppression of F1+2 generation (15±2 vs 26±3%) due to addition of 0.3 $\mu$g ml$^{-1}$ (5 nmol l$^{-1}$) rhAPC, was significantly less pronounced in cord plasma than in adult plasma adjusted to contain PS at neonatal level by means of PS-antibody coupled to sepharose ($P$ of all differences $<0.01$).

### *The anticoagulant action of rhAPC in adult plasma compared with that in cord plasma spiked with purified PS concentrate to contain PS at adult level*

Prolongation of CT (84±8.6 vs 68±8.2%), suppression of TP (48±4.6 vs 38±4), and suppression of F1+2 generation (44±4.5 vs 36±4.3%) due to addition of 0.3 $\mu$g ml$^{-1}$ (5 nmol l$^{-1}$) rhAPC was significantly more pronounced in adult plasma than in cord plasma spiked with PS concentrate to contain PS at adult level ($P$ of all differences $<0.01$).

## Discussion

In the previous work, we have shown that the anticoagulant efficacy of rhAPC is significantly lower in cord than in adult plasma using low amounts of TF to induce clot formation (Cvirn *et al*., 2004). We demonstrated that this low anticoagulant action of rhAPC is partly attributable to the physiologically low neonatal levels of TFPI and AT (Cvirn *et al*., 2003a, b) two natural inhibitors known to influence the PC pathway (Van't Veer *et al*., 1997). In the present study, we investigated the effects of PS on the anticoagulant action of rhAPC since PS serves as a cofactor for APC (Fulcher *et al*., 1984; Petäjä & Manco-Johnson, 2003) and its levels are also low in neonates (Kuhle *et al*., 2003). We measured 36% total PS antigen but 45% PS activity level compared with adults. This difference is attributable to the low physiological levels of C4B-binding protein in neonates (Schwarz *et al*., 1988). We demonstrate that the anticoagulant action of 0.3 $\mu$g ml$^{-1}$ (5 nmol l$^{-1}$) rhAPC is significantly decreased in (native) cord plasma compared to adult plasma. Furthermore, we demonstrate that the anticoagulant action of rhAPC dose dependently increases in cord plasma in the presence of increasing activities of PS, and, correspondingly, decreases in adult plasma in the presence of decreasing activities of PS. However, the anticoagulant action of 0.3 $\mu$g ml$^{-1}$ (5 nmol l$^{-1}$) rhAPC was significantly greater in adult plasma than in cord plasma spiked to contain PS at adult level. Similarly, the anticoagulant action of rhAPC was less pronounced in cord plasma than in adult plasma adjusted to contain PS at neonatal levels by means of PS-antibody coupled to sepharose. This finding can be assumed to be mainly attributable to the lower levels of TFPI and AT present in cord plasma samples, as previously shown (Cvirn *et al*., 2004). In conclusion, the anticoagulant effect of rhAPC depends, at least, on the levels of PS, TFPI, and AT present in the plasma. Consequently, rhAPC exerts lower anticoagulant action in neonates compared to adults due to the low physiological levels of PS, TFPI, and AT present in neonates. Factor V is also a cofactor of APC in proteolysis of factor VIII. Since factor V levels are similar in cord and adult plasmas (Andrew *et al*., 1987), we suggest that factor V does not significantly contribute to the different anticoagulant action of rhAPC in cord vs adult plasma.

Our laboratory experiments do not allow definite conclusions for clinical situations. However, we speculate that the anticoagulant effect of rhAPC is decreased in neonates resulting in higher dose requirement. Moreover, the anticoagulant effect of rhAPC would be expected to be impaired in all clinical situations (pediatric or adult patients) associated with consumption or inhibition of PS, AT, and TFPI (Lorente *et al*., 1993; Martinez *et al*., 1999).

Bleeding events experienced during rhAPC infusion might be attributable to the synergistically enhanced anticoagulant action of rhAPC during progressive normalization of natural anticoagulants. Therefore, we suggest to take actual levels of the natural inhibitors PC, PS, AT, and TFPI into account in future clinical studies, in order to evaluate the optimal rhAPC dosage. Furthermore, administration of recombinant human PS (not available to date) might be beneficial in improving the anticoagulant action of rhAPC, particularly during initial sepsis therapy (where levels of natural PS are markedly decreased).

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