Thrombosis Research (2008) **122**, 69–76

ELSEVIER

THROMBOSIS RESEARCH

intl.elsevierhealth.com/journals/thre

REGULAR ARTICLE

# On interaction of activated protein C with human aortic smooth muscle cells attenuating the secretory group IIA phospholipase $A_2$ expression

**Mario Menschikowski [a,*], Albert Hagelgans [a], Ute Hempel [b], Peter Lattke [a], Iskander Ismailov [c], Gabriele Siegert [a]**

[a] *Institute of Clinical Chemistry and Laboratory Medicine, Technical University of Dresden, Medical Faculty "Carl Gustav Carus", Dresden, Germany*
[b] *Institute of Physiological Chemistry, Technical University of Dresden, Medical Faculty "Carl Gustav Carus", Dresden, Germany*
[c] *Department of Neuroscience, Baylor College of Medicine, Houston TX, USA*

Received 10 May 2007; received in revised form 14 August 2007; accepted 30 August 2007
Available online 22 October 2007

**KEYWORDS**
Activated protein C;
Secretory phospholipase $A_2$;
Endothelial protein C receptor;
Protease-activated receptors

**Abstract**

*Introduction:* Pharmacological restriction of secretory group IIA phospholipase $A_2$ ($sPLA_2$-IIA) expression is thought to be beneficial in the treatment of inflammatory diseases such as sepsis and septic shock. In this study we investigated the effects of activated protein C (APC) on $sPLA_2$-IIA expression, phosphorylation of extracellular signal-regulated kinase (ERK) 1/2, and on DNA-binding activities of nuclear factor-κB (NF-κB) and CCAAT box enhancer binding protein-β (C/EBP-β) in human aortic smooth muscle cells (HASMC).
*Materials and methods:* To achieve elevated $sPLA_2$-IIA production as occurring during inflammation, HASMC were stimulated with interferon-γ (IFN-γ) alone and in combination with other inductors, thus modeling the strong $sPLA_2$-IIA elevation by inflammation.
*Results and conclusions:* APC inhibited the stimulated expression of $sPLA_2$-IIA in HASMC dose-dependently (1–300 nM). At the same time, APC increased the phosphorylation of ERK 1/2 and decreased NF-κB and C/EBP-β DNA-binding activities in these cells, as

*Abbreviations:* APC, activated protein C; BSA, bovine serum albumin; C/EBP, CCAAT box enhancer binding protein; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunoassay; EPCR, endothelial protein C receptor; ERK 1/2, extracellular signal-regulated kinase; FCS, fetal calf serum; HASMC, human aortic smooth muscle cells; HCAEC, human coronary artery endothelial cells; HRP, horse-radish peroxidase; IFN-γ, interferon-γ; MEK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; PAR-1, protease-activated receptor-1; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-polymerase chain reaction; $sPLA_2$-IIA, secretory phospholipase $A_2$ of group IIA.

* Corresponding author. Fetscherstrasse 74, D-01307 Dresden, Germany. Tel.: +49 351 458 2634; fax: +49 351 458 4332.
*E-mail address:* menschik@rcs1.urz.tu-dresden.de (M. Menschikowski).

0049-3848/$ - see front matter 
doi:10.1016/j.thromres.2007.08.015

compared with respective stimulated controls. Reverse transcriptase-polymerase chain reaction and cell-based ELISA reveal an endothelial protein C receptor (EPCR) expression in HASMC. Application of antibodies against EPCR and protease-activated receptor-1 (PAR-1) reduced the APC-induced ERK 1/2 activation and the treatment of cells with a PAR-1 antagonist diminished the $sPLA_2$-IIA inhibition. The obtained results show that APC effectively suppresses the up-regulated $sPLA_2$-IIA expression, which might contribute to the reported beneficial effects of APC in the treatment of severe inflammatory disorders.


## Introduction

Secretory phospholipase $A_2$ of group IIA ($sPLA_2$-IIA) is a well-characterized entity, which belongs to the diverse and structurally heterogeneous superfamily of phospholipases [1]. Although specific biological functions of $sPLA_2$-IIA are not completely understood, it may be involved in a variety of processes in the mammalian cells including signal transduction, apoptosis, remodelling of cell membranes, host defense, and coagulation [2–4]. Also, large amounts of this enzyme have been found in serum of patients with severe inflammations such as sepsis, septic shock, and polytrauma, in good correlation with the degree of the disorders suggesting that $sPLA_2$-IIA plays a pivotal role in inflammation [3,5,6]. These findings formed the basis for a search of specific inhibitors of $sPLA_2$-IIA as a novel strategy for the treatment of acute and chronic inflammatory diseases [7–10].

Recently, a zymogen product of limited proteolysis of protein C, called activated protein C (APC) and capable to exert a variety of anti-inflammatory activities [11], was proposed to be an agent in the treatment of sepsis [12–14]. It has been demonstrated that APC inhibits the binding of transcription factors of the NF-κB family at target sites [15–17]. This, in turn, may result in a down-regulation of pro-inflammatory gene clusters including cytokines and chemokines, adhesion proteins, tissue factor, and nitric oxide synthase.

Since the induction of $sPLA_2$-IIA in various cells depends on the activation of NF-κB [3,18–20], we hypothesized that APC may affect the expression of $sPLA_2$-IIA. Our present testing of this hypothesis showed for the first time that APC, but not protein C or an APC-derived anticoagulant peptide, is capable to inhibit the $sPLA_2$-IIA production in HASMC.

## Materials and methods

### Materials

Human plasma-derived APC (11–13 Units/mg) was purchased from Hematological Technologies Inc. (Cell Systems, Biotechnologie Vertrieb GmbH, St. Katharinen, Germany). Human protein C was from Enzyme Research Laboratories Ltd. (South Bend, IN, USA) and H-1152, PD-98059, and bovine thrombin (specific activity 1800–2200 NIH units/mg protein) were from Calbiochem (Schwalbach, Germany). SCH-79797 was purchased from BIOTREND Chemikalien GmbH (Köln, Germany). Monoclonal anti-human PAR-1 antibodies ATAP2 and WEDE15 were obtained from Santa Cruz Biotechnology (CA, USA) and Beckman Coulter (Fullerton, CA, USA), respectively. Forskolin, bovine serum albumin (BSA), the monoclonal anti-EPCR antibodies RCR-252 and RCR-379, and an APC-derived peptide (fragment 390–404 of heavy chain that is essential for anticoagulant activity of APC) were purchased from Sigma–Aldrich (Deisenhofen, Germany). The goat HRP-conjugated anti-rat $IgG_1$ was from Chemicon International, Inc (Hampshire, United Kingdom) and recombinant human interferon-γ (IFN-γ) was from Roche Diagnostics (Mannheim, Germany). PD-98059 and SCH-79797 were dissolved in dimethyl sulfoxide (DMSO). The final concentrations of DMSO were 0.3% or less, and controls using DMSO alone were run in all cases. Other agents were used as aqueous solutions.

### Cell culture and incubation

HASMC and human coronary artery endothelial cells (HCAEC) were purchased from Promocell (Heidelberg, Germany). HASMC were cultivated as described previously [21]. Briefly, the cells were grown in smooth muscle cell growth medium-2 supplemented with 5% fetal calf serum (FCS), 0.5 ng/ml human epidermal growth factor, 2 ng/ml human basic fibroblast growth factor, 5 μg/ml bovine insulin, 50 μg/ml gentamycin sulfate and 50 ng/ml amphotericin B at 37 °C in a humidified atmosphere of 5% $CO_2$. In two days after plating, the medium was replaced by low-serum medium containing 0.5% FCS to synchronise the cell growth, and after 48 h the medium was again replaced by medium containing 5% FCS. The cell culture medium supplemented with 5% FCS was originally used in experiments where the mechanisms controlling the $sPLA_2$-IIA expression in HASMC were studied [21]. In the present study, it was applied but 0.2% bovine serum albumin (BSA) was used instead of FCS in experiments where ERK 1/2 activation and EPCR expression were analyzed and the effect of APC was compared with that found in 5% FCS-containing medium, respectively. HCAEC were maintained in endothelial growth medium supplemented with 5% FCS. For all experiments, exponentially growing subconfluent cells were used at passages 5 to 8.

### Measurement of EPCR expression

EPCR expression in HASMC was determined at the mRNA level with reverse transcriptase-(RT)-PCR and at the protein level with cell-based ELISA. For RT-PCR analyses, cells were plated into 25 $cm^2$ dishes at a cell density of $10^4$ cells/$cm^2$ and cultured for 48 h in FCS-supplemented medium. RNA was isolated after lysis of cells in TRI reagent according to the instructions (Sigma–Aldrich,