Human Vascular Smooth Muscle Cells Express Functionally Active Endothelial Cell Protein C Receptor

Ellen Bretschneider, Barbara Uzonyi, Artur-Aron Weber, Jens W. Fischer, Robert Pape, Katharina Lotzter, Karsten Schroër

Abstract—The endothelial cell protein C receptor (EPCR) is expressed on endothelial cells and regulates the protein C anticoagulant pathway via the thrombin-thrombomodulin complex. Independent of its anticoagulant activity, activated protein C (APC) can directly signal to endothelial cells and upregulate antiapoptotic and antiinflammatory genes. Here we show that vascular smooth muscle cells (SMCs) also express EPCR. EPCR protein on SMCs was detected by flow cytometry and Western blotting. EPCR mRNA was identified by quantitative RT-PCR. To examine the functionality of EPCR, intracellular signaling in APC-stimulated SMCs was analyzed by determination of intracellular free calcium transients using confocal laser scanning microscopy. Phosphorylation of extracellular signal–regulated kinases 1 and 2 (ERK-1/2) was detected by immunoblotting. APC-induced ERK-1/2 phosphorylation was inhibited by an anti-EPCR antibody and by a cleavage site blocking anti–PAR-1 antibody, indicating that binding of APC to EPCR and cleavage of protease-activated receptor-1 (PAR-1) were involved. APC elicited an increase in [³H]-thymidine incorporation. The mitogenic effect of APC was significantly enhanced in the presence of thrombin. EPCR expression was also detected in SMCs in the fibrous cap of human carotid artery plaques. The present data demonstrate functionally active EPCR in SMCs and suggest that EPCR-bound APC might modulate PAR-1-mediated responses of SMCs to vascular injury. (Circ Res. 2007;100:255-262.)

Key Words: endothelial cell protein C receptor ■ activated protein C ■ vascular smooth muscle cells ■ intracellular signaling

The endothelial cell protein C receptor (EPCR) is an integral membrane protein that belongs to the major histocompatibility complex class I/CD1 family. EPCR was detected in endothelial cells (ECs) with prominent expression in the aorta and other large arteries. EPCR regulates the protein (PC) anticoagulant pathway by binding PC and augmenting PC activation by the thrombin–thrombomodulin complex on the endothelial surface. After dissociation from EPCR and binding to protein S, activated PC (APC) inactivates factors Va and VIIIa, thereby inhibiting further thrombin generation. The plasma levels of PC and APC were reported to be 65 nmol/L and 38 pmol/L, respectively. However, local APC concentration might be increased at sites of vascular injury where thrombin is generated and in complex with thrombomodulin activates EPCR-bound PC. APC retains its ability to bind EPCR. Similar to free APC, EPCR-bound APC can be inactivated by the plasma protease inhibitors α1-antitrypsin and PC inhibitor with a half life of approximately 15 minutes. This slow inactivation allows EPCR-bound APC to signal cells. In human umbilical vein ECs (HUVECs), APC triggers mobilization of intracellular free calcium ([Ca²⁺]), activates extracellular signal regulated kinases 1 and 2 (ERK-1/2), and upregulates antiinflammatory and antiapoptotic genes. Moreover, APC inhibits hypoxia- or staurosporine-induced apoptosis in human brain ECs. Because the cytosolic domain of EPCR is rather short, it is unlikely to be capable of signaling directly. Fibroblasts transfected with EPCR were responsive to APC only when protease activated receptor-1 (PAR-1) or PAR-2 were coexpressed with EPCR. In HUVECs, which express PAR-1 and PAR-2 constitutively, proteolytic cleavage of PAR-1 but not PAR-2 by EPCR-bound APC appeared to be required to elicit cellular responses.

The PAR family consists of 4 members (PAR-1, PAR-2, PAR-3, and PAR-4) that are differently expressed in a variety of cells and tissues. PARs are cellular receptors for trypsin-like serine proteases such as thrombin, factor Xa, and tissue factor/factor VIIa complex (review previously). PARs bind different G proteins, thereby triggering signaling pathways that are distinct for each of the PAR members. Moreover,
PARs are differentially regulated. Each of the PARs can be selectively activated by synthetic peptides (PAR-APs). These peptides are useful tools to distinguish between distinct PARs (for review, see Hollenberg and Compton). In smooth muscle cells (SMCs), PARs have been shown to mediate cellular effects including contraction, synthesis of extracellular matrix, and mitogenesis. Recently, we have shown that SMCs of human saphenous vein express functionally active PAR-1, PAR-3, and PAR-4. SMCs also express PAR-2 (K. Schroer, unpublished results, 2005). The present study indicates that cultured SMCs express functionally active EPCR, that EPCR mediates APC signaling through activation of PAR-1 but not PAR-2 or PAR-3, and that APC acts as a mitogen. EPCR expression on SMCs was also detected in vivo in human carotid artery plaques.

Materials and Methods

Materials

APC (Hemochrom Diagnostica, Essen, Germany); synthetic PAR-APs (PAR-1-AP: TFLLRN-NH2; PAR-2-AP: SLIGKV-NH2; PAR-3-AP: TFRGAP-NH2; Biosyntan, Berlin, Germany); pertussis toxin (PTX Sigma, Taufkirchen, Germany) and PD 98059 (Calbiochem); p-amidino phenylmethylsulfonyl fluoride (p-APMSF) (Calbiochem, Bad Soden, Germany); recombinant hirudin (h-hirudin) (Hoechst, Frankfurt, Germany); mouse anti-human CD31 and mouse IgG1 (DakoCytomation, Hamburg, Germany); rat anti-human EPCR (Sanbio, Beutelsbach, Germany); rat IgG1 (R&D Systems, Wiesbaden-Nordenstadt, Germany); goat anti-mouse phycoerythrin and goat anti-rat fluorescein isothiocyanate (Dianova, Hamburg, Germany); mouse anti-human PAR-1 antibody (ATAP2), anti-human PAR-2 antibody (SAM11), anti-human PAR-3 antibody (H-103), and horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA); fluo-4-acetomethyl ester (Molecular Probes, Leiden, The Netherlands); [H]-thymidine (PerkinElmer Life and Analytical Sciences, Boston, MA); and a-thrombin was kindly provided by Dr J. Stuerzebecher (Institut fuers Vaskulare Medizin, Klinikum der Friedrich-Schiller-Universitat Jena, Germany). Cell culture reagents and reagents for quantitative RT-PCR were purchased from Invitrogen (Karlsruhe, Germany).

Cell Culture

Specimens of saphenous veins were obtained from patients undergoing aortocoronary bypass surgery. SMCs were obtained by outgrowth from lamina media explants, cultured as described previously, and used at passages 4 to 8. Human coronary artery ECs were purchased from PromoCell (Heidelberg, Germany) and cultured in EC growth medium MV2 (C-22121) according to the protocol of the supplier. HUVECs were prepared and cultured as described. Cells were preincubated with antibodies and inhibitors 30 minutes before application of the agonist.

Flow Cytometry

Cells were harvested with PBS containing 0.2% EDTA. Cells were incubated with anti-EPCR or anti-CD31, and after washing with fluorescein isothiocyanate–conjugated goat anti-rabbit antibody (Ab) or phycoerythrin-conjugated goat anti-mouse Ab. Measurement was performed with a FACScan Calibur flow cytometer (BD Biosciences). Cells (10 000) were routinely counted, and cell population was defined based on forward-side scatter characteristics. Data were analyzed with the CellQuest program.

Determination of EPCR and CD31 Transcripts

RNA was reverse transcribed, external standards were prepared, and sequence was confirmed as described previously. RT-PCR parameters were established using SYBR Green as detection dye on a Rotor-Gene 2000 amplification system (Corbett Research, Mortlake, Australia) with primers for EPCR (5‘, CACCTTGACAGGCT-CATTGCC; 3‘, ACATGGCCGTCCACCTGTGC; product size, 226 bp; annealing temperature, 65°C) and CD31 (5‘, GCGCATGGGCT-GGTAGAGG; 3‘, TCACAGGGGTGTCCACAGG; product size, 184 bp; annealing temperature 65°C). Primers were selected using Primer3 software. Specificity of PCR products was confirmed by melting curve analysis.

Measurement of [Ca2+]i

SMCs were grown to confluence on 87-mm2 culture plates, loaded with fluo-4-acetomethyl ester (2 μmol/L) for 30 minutes at 37°C, and rinsed with HEPES-buffered salt solution (145 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgSO4, 1.5 mmol/L CaCl2, 10 mmol/L HEPES, 10 mmol/L glucose, and 0.25% human serum albumin). The cells were mounted on a 37°C heated cell culture unit. Mobilization of [Ca2+]i, was determined by using an Axiovert 200M inverted microscope equipped with a confocal laser scanning head LSM510, x 20/0.5 Plan Neofluar objective, and an argon laser (488 nm) (C. Zeiss, Jena, Germany). Data for graphs were recorded and processed using LSM510 software.

Immunoblotting

Phosphorylated ERK-1/2 and EPCR protein were determined in cell lysates by Western blotting. For detection of ERK-1/2 phosphorylation, cells were grown to confluence and made quiescent by serum removal for 24 hours. SMCs were pretreated with inhibitors or antibodies for 30 minutes before the stimulant was added. Cell extracts were prepared in sodium dodecyl sulfate lysis buffer (2% wt/vol sodium dodecyl sulfate, 10% glycerol, 62.5 mmol/L Tris-HCl [pH 6.8], 50 mol/L dithiothreitol, 0.1% wt/vol bromphenol blue). Proteins were separated by SDS-PAGE and blotted onto polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, Mass). Membranes were blocked in TBS-T (20 mol/L Tris-HCl [pH 7.6], 137 mol/L NaCl, 0.1% Tween) containing 5% wt/vol nonfat dry milk. For detection of phosphorylated ERK-1/2, membranes were incubated with a phospho-specific antibody directed against ERK-1/2 (1:1000) for 60 minutes. To detect EPCR protein, membranes were incubated with a polyclonal antihuman EPCR antibody (R&D Systems, Minneapolis, Minn; 1:2000 overnight). After washing in TBS-T, the membranes were incubated with a horseradish peroxidase–conjugated secondary antibody (1:3000) for 60 minutes. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL; Amersham Biosciences, Buckinghamshire, UK). Finally, membranes were stained with 0.1% amido black solution to control loading and transfer of proteins.

Determination of [H]-Thymidine Incorporation into DNA

Cells were seeded into 24-well plates (4×104 cells/mL, 0.5 mL/well) and grown to confluence. After 24 hours, growth arrest in serum-free culture medium was stimulated for 24 hours with the indicated agents. Four hours before the end of the stimulation period, the culture medium cells were stimulated for 24 hours with the indicated agonists. Finally, membranes were stained with 0.1% amido black solution to control loading and transfer of proteins.

Statistics

Data are mean±SEM of n independent experiments. Statistical analysis was performed by i-way ANOVA, followed by Bonferroni Multiple Comparisons test using GraphPad InStat version 3.01 for Windows 95 (GraphPad Software, San Diego, Calif). Data are mean±SEM. P values of <0.05 were considered significant.

Results

SMCs Express EPCR mRNA and EPCR Protein

EPCR mRNA was detected by quantitative RT-PCR in cultured SMCs (40 transcripts per 1000 copies of GAPDH)
and in HUVECs (218 transcripts per 1000 copies of GAPDH), which were used as a positive control for EPCR expression. To exclude that detection of EPCR mRNA in SMCs was attributable to contaminating ECs, mRNA of the EC marker CD31 was measured in both cell types. CD31 was strongly expressed in HUVECs (1370 transcripts per 1000 copies of GAPDH) but negligible in SMCs (4 transcripts per 1000 copies of GAPDH). Thus, the SMC cultures contained essentially no CD31 transcripts, indicating that there was no EC contamination. EPCR protein expression on the surface of SMCs was demonstrated by flow cytometry analysis (Figure 1A), whereas CD31 was not (Figure 1B), demonstrating the absence of EC contamination. HUVECs were used as a positive control for establishment of EPCR and CD31 stainings (Figure 1A and IB in the online data supplement, available at http://circres.ahajournals.org). EPCR protein in SMCs was additionally verified by Western blotting. ECs from the human coronary artery were used as a positive control (Figure 1C).

APC Mobilizes $[\text{Ca}^{2+}]_i$ in Cultured SMCs

We determined whether APC elicits a rise in $[\text{Ca}^{2+}]_i$ in cultured SMCs. In most cell lines, 65 nmol/L APC triggered a transient $[\text{Ca}^{2+}]_i$ signal (Figure 2A). When SMCs were stimulated twice with thrombin, a $[\text{Ca}^{2+}]_i$ signal was observed after the first application but not after the second application, indicating homologous receptor desensitization (not shown). The $[\text{Ca}^{2+}]_i$ signal to thrombin (100 nmol/L) was maintained after the prior application of APC (Figure 2A). In contrast, after a preceding challenge with thrombin, the $[\text{Ca}^{2+}]_i$ response to APC was abolished (Figure 2B). The $[\text{Ca}^{2+}]_i$ signal to APC was also abolished after prior application of the PAR-1-AP (10 $\mu$mol/L) (Figure 2C). These data indicate that activation and following desensitization of PAR-1 completely prevented the calcium response to APC.

APC Phosphorylates ERK-1/2

Stimulation of SMCs with APC induced ERK-1/2 phosphorylation at low nanomolar concentrations (6.5 to 65 nmol/L) (Figure 3A). Phosphorylation of ERK-1/2 by APC (65 nmol/L) was time dependent and reached a maximum after 10 to 20 minutes (Figure 3B). ERK phosphorylation by thrombin (10 nmol/L) at 10 minutes is shown for comparison. PAR-1 is known to couple to several heterotrimeric G proteins, including $G_q$ and the PTX-sensitive $G_s$. ERK phosphorylation 10 minutes after APC (65 nmol/L) stimulation was markedly attenuated when the SMCs were preincu-
bated with PTX (100 nmol/L) for 20 hours, indicating that Gi was involved (Figure 3C).

In further experiments, SMCs were stimulated with APC pretreated with \( p \)-APMSF (200 \( \mu \)mol/L), an inhibitor that blocks the active site of serine proteases. Active site–blocked APC failed to phosphorylate ERK-1/2, indicating that the proteolytic activity of APC was required for signaling (Figure 4A). By contrast, r-hirudin (200 nmol/L), a specific inhibitor of the serine protease thrombin, did not influence APC-induced ERK phosphorylation, ruling out contamination of APC by thrombin (Figure 4B). To clarify the role of EPCR in APC signaling, SMCs were incubated with an anti-human EPCR antibody (RCR-252, 10 \( \mu \)g/mL) for 30 minutes before APC application. Figure 5A demonstrates the inhibitory effect of the antibody. The APC-induced signal was also prevented by cleavage-site blocking anti–PAR-1 antibody ATAP2 (25 \( \mu \)g/mL) (Figure 5B), whereas anti–PAR-2 antibody SAM11 (25 \( \mu \)g/mL) (Figure 5C) and PAR-3 antibody (25 \( \mu \)g/mL) (Figure 5D) were ineffective. \( p \)-APMSF, anti-EPCR-antibody, and anti–PAR-1 antibody did not influence ERK-1/2 phosphorylation by TFLLRN (100 \( \mu \)mol/L), demonstrating that inhibitory effects were specific for APC responses (supplemental Figure IIA through IIC).

The involvement of PAR-1 was further studied in desensitization experiments. PAR-1-AP (10 \( \mu \)mol/L) phosphorylated ERK-1/2 time dependently, with maximum at 5 to 10 minutes; PAR-2-AP (100 \( \mu \)mol/L) and PAR-3-AP (100 \( \mu \)mol/L) caused maximum ERK phosphorylation at 10 to 20 minutes (not shown). Cells were pretreated with PAR-1-AP to activate and desensitize PAR-1. In comparison with unstimulated controls, (Figure 6A, lane a), stimulation of the cells with the PAR-1 AP (10 \( \mu \)mol/L) caused significant phosphorylation of ERK-1/2 after 10 minutes (Figure 6A, lane b), which returned nearly to control level within 80 minutes (Figure 6A, lane c). A second stimulation of the same cells with the PAR-1-AP resulted in attenuated ERK phos-
In contrast, pretreatment of the SMCs with the PAR-2-AP for 80 minutes did not influence the APC-induced signal (Figure 6, lane c). Similar results were obtained when desensitization experiments were performed with PAR-3-AP (100 μmol/L). After stimulation with PAR-3-AP, ERK-1/2 phosphorylation was detectable after 10 minutes (Figure 6C, lane b) and returned to baseline at 80 minutes (Figure 6C, lane c). In PAR-3-pretreated SMCs, second application of the peptide did not elicit a further calcium signal (Figure 6C, lane d), whereas APC-induced ERK-1/2 phosphorylation was not affected by pretreatment with the PAR-3-AP (Figure 6C, lane e). These data provide further evidence that APC phosphorylated ERK-1/2 via activation of PAR-1 but not PAR-2 or PAR-3.

**APC Stimulates [3H]-Thymidine Incorporation into DNA**

Functionality of APC-induced signaling in SMCs was studied by determination of mitogenesis, ie, [3H]-thymidine incorporation into DNA. APC, at concentrations that caused ERK phosphorylation, induced concentration-dependent increases in DNA synthesis (Figure 7A). APC-induced increases in DNA synthesis was diminished by PTX, indicating that Gi was involved in signaling by APC (Figure 7B). DNA synthesis was also inhibited by the mitogen-activated protein kinase kinase (MEK) inhibitor PD 98059 (10 μmol/L). r-Hirudin did not influence DNA synthesis by APC (Figure 7B).

**APC Induces ERK-1/2 Phosphorylation and DNA Synthesis in the Presence of Thrombin**

To clarify whether SMCs were still responsive to APC in the presence of the strong PAR-1 activator thrombin,²⁶ cells were stimulated with 3 nmol/L thrombin and 65 nmol/L APC simultaneously. Under these conditions, ERK-1/2 phosphorylation was more pronounced compared with the separate effects of thrombin or APC alone (Figure 8A). Increase in DNA synthesis by 65 nmol/L APC was comparable with that induced by 10 nmol/L thrombin or 100 μmol/L PAR-1-AP. PAR-2-AP (100 μmol/L) did not induce a mitogenic effect (Figure 8B). Simultaneous stimulation of SMCs with thrombin (10 nmol/L) and APC (65 nmol/L) resulted in an additive increase in DNA synthesis (Figure 8B). Because thrombin can activate PARs other than PAR-1, DNA synthesis was examined after simultaneous stimulation with the PAR-1-AP (100 μmol/L) and APC (65 nmol/L). Similar to the effect seen with thrombin, a synergistic effect was observed (Figure 8B). These data suggest that in SMCs, APC can contribute to PAR-1 activation in the presence of thrombin. Interestingly, simultaneous stimulation of SMCs with thrombin (10 nmol/L) and the PAR-1-AP (100 nmol/L) did not result in a synergistic effect on [3H]-thymidine incorporation.

**Discussion**

The EPCR is known to be highly expressed in HUVECs and other ECs and, together with PAR-1, mediates signaling and gene regulation by APC.⁶⁻¹⁰ The present study demonstrates that SMCs also express functionally active EPCR. Contamination of SMC cultures with ECs was unlikely as mRNA of the EC marker CD31²⁴ in SMCs was essentially nonexistent,
whereas EPCR mRNA levels in SMC cultures were lower by a factor of 5 compared with HUVECs. In agreement with quantitative RT-PCR results, flow cytometry revealed expression of EPCR protein in SMCs and HUVECs, whereas CD31 was expressed in HUVECs but not in SMCs.

Functionality of EPCR was examined by measuring mobilization of \([\text{Ca}^{2+}]_i\), phosphorylation of ERK-1/2, and DNA synthesis. Similar to thrombin, APC elicited a transient rise in \([\text{Ca}^{2+}]_i\). When PAR-1 was desensitized by preceding stimulation of SMCs with thrombin or the PAR-1-AP, calcium responses to APC were abolished. This is in agreement with the findings of Dömőtőr et al on human brain ECs and HUVECs, whereas CD31 was expressed in HUVECs but not in SMCs.

SMCs as compared with HUVECs. At low nanomolar concentrations, APC caused phosphorylation of ERK-1/2. This effect was prevented by antibodies that block binding of APC to EPCR and cleavage site of PAR-1, respectively. Antibodies that prevented cleavage of PAR-2 or PAR-3 were ineffective. In mouse cortical neurons, both PAR-1 and PAR-3 were found to be involved in APC-mediated protection from apoptosis. It is not known whether APC in these cells directly activates PAR-3, whether PAR-3 is a cofactor for PAR-1 activation by APC, or whether PAR-3 in neurons acts by a hitherto unknown mechanism. In mouse platelets, PAR-3 does not itself mediate transmembrane signaling but instead functions as a cofactor for cleavage of PAR-4 by thrombin. In contrast, we have shown recently that the peptide corresponding to the N terminus of cleaved PAR-3 induced transmembrane signaling in SMCs independently of another PAR. Desensitization experiments on ERK-1/2 phosphorylation provided further evidence that PAR-1 but not PAR-2 or PAR-3 was involved in APC-mediated signaling. Pretreatment of the SMCs with the PAR-1-AP resulted in diminished ERK phosphorylation by APC, apparently because of homol-

Figure 7. \(^{[3]H}\)-Thymidine incorporation into SMCs and of its modification by PTX, PD98059, and r-hirudin. A, Concentration-dependent increase in DNA synthesis by APC. Data represent mean±SEM of 6 to 8 independent experiments. *P<0.05 vs control (CON). B, Influence of PTX (100 nmol/L), PD98059 (10 μmol/L), and r-hirudin (200 nmol/L) on \(^{[3]H}\)-thymidine incorporation by APC. Data are mean±SEM of 6 independent experiments. *P<0.05 vs control (CON), #P<0.05 vs APC.

Figure 8. Cellular responses after simultaneous stimulation of SMCs with different PAR-1 agonists (CON indicates unstimulated SMCs). A, Phosphorylation of ERK-1/2 after simultaneous application of APC (65 nmol/L) and thrombin (THR) (3 nmol/L) and after separate stimulation with APC or thrombin. B, Increase in \(^{[3]H}\)-thymidine incorporation into DNA after stimulation with APC (65 nmol/L), thrombin (10 nmol/L), thrombin (10 nmol/L) plus APC (65 nmol/L), PAR-1-AP (100 μmol/L), PAR-1-AP (100 μmol/L) plus APC (65 nmol/L), thrombin (10 nmol/L) plus PAR-1-AP (100 μmol/L), and PAR-2-AP (100 μmol/L). Data are mean±SEM of 6 independent experiments. *P<0.05 thrombin+APC vs thrombin, #P<0.05 PAR-1-AP+APC vs PAR-1-AP.
ogous receptor desensitization. In contrast, desensitization of PAR-2 or PAR-3 by pretreatment with the PAR-2-AP or PAR-3 AP did not influence the APC response. Activation of PAR-1 required proteolytic cleavage of the receptor. In SMCs, active site–blocked APC failed to phosphorylate ERK-1/2. Thus, in agreement with the findings on ECs, binding of APC to EPCR and proteolytic cleavage of PAR-1 were required to trigger signaling in SMCs.

PAR-1 is known to bind several G proteins. Mobilization of [Ca\(^{2+}\)], in APC-stimulated SMCs implies activation of G\(_i\). In addition to G\(_i\), PAR-1 has been shown to couple also to G\(_\text{q}\). Concordantly, APC-induced ERK phosphorylation and DNA synthesis were inhibited by PTX. Our data support the finding on ECs, indicating that coupling to the PTX sensitive G\(_{\text{q}}\) and subsequent inhibition of adenylyl cyclase promotes SMC proliferation. Inhibition of APC-induced DNA synthesis in the SMCs by the ERK pathway inhibitor PD 98059 indicated that ERK activation was involved in the mitogenic effect.

Ludeman et al addressed the question of whether APC might play an important role for PAR-1 signaling, by comparing the kinetics and potencies of thrombin versus APC to activate PAR-1. They showed that APC was ~1000-fold less potent than thrombin for cleavage of PAR-1 and triggering of PAR-1–mediated responses in ECs. The authors concluded that APC is a poor agonist for PAR-1 relative to thrombin and therefore unlikely to contribute to PAR-1 cleavage beyond that affected by thrombin. In the present study, simultaneous stimulation of SMCs with thrombin or PAR-1-AP and APC induced a synergistic effect on ERK-1/2 phosphorylation and DNA synthesis. Whether this effect is still PAR-1 dependent, or whether there are additional mechanisms involved, remains to be elucidated. Interestingly, simultaneous stimulation of SMCs with thrombin and the PAR-1 AP did not result in any additive increase in [\(\text{H}\)]-thymidine incorporation. On cytokine-perturbed HUVECs, it has been demonstrated that PAR-1 signaling by APC is distinct from thrombin signaling. Thus, although thrombin and APC activate the same receptor, they can mediate opposite biological effects. The authors concluded that EPCR cosignaling may modify PAR-1–dependent APC signaling. Thus, one may speculate that on SMCs, binding of APC to EPCR facilitates cleavage of PAR-1 by APC, even in the presence of thrombin. These findings might be relevant at sites of vascular injury. EPCR was found to be expressed in SMC-rich areas in the fibrous cap of human carotid artery plaques. Double immunostaining (supplemental Figure IIIA through IIIC) and confocal laser-scanning microscopy (supplemental Figure IIIa through IIIc) clearly revealed colocalization of EPCR and \(\alpha\)-smooth muscle actin. These data support the hypothesis that EPCR is involved in the phenotypic control of SMCs during human atherosclerosis. Additionally, SMCs can initiate tissue factor–dependent thrombin generation. Generated thrombin activates PAR-1 on SMCs. On the other hand, SMCs also express thrombomodulin that, in complex with thrombin, can activate PC. Thus, while activating PAR-1 on its own, thrombin creates a further PAR-1 activator, namely APC. Involvement of PAR-1 in SMC proliferation is supported by findings in a balloon angioplasty model in rats. Administration of a potent PAR-1 antagonist attenuated vascular restenosis without inhibiting thrombus formation.

In summary, the present study demonstrates that SMCs express functionally active EPCR, that EPCR-bound APC activates PAR-1, and that APC is also active in the presence of thrombin. Detection of EPCR in SMCs of human carotid artery plaques suggests the relevance of EPCR in vivo. More work is required to substantiate the biological significance of EPCR expression on SMCs and to clarify the precise mechanisms by which APC activates PAR-1 and induces signaling.

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Disclosures

None.

References


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Legends to Supplemental Figures 1-3:

**Figure 1A+B:**
FACS analysis was performed with SMC and with HUVEC as positive control for EPCR expression. EPCR protein was detected on SMC (Figure 1A in the MS) and on HUVEC (supplemental Figure 1A). CD31 was detected in HUVEC (supplemental Figure 1B) but was undetectable in SMC (Figure 1B in the MS).

**Figure 2A-C:**
The specificity of *p*-APMSF (200 µmol/L), anti-EPCR antibody (Ab) (10 µg/ml) and anti-PAR-1 antibody (25 µg/ml) were tested in PAR-1-AP-stimulated cells. As shown in the supplemental Figures 2A–C, ERK-1/2 phosphorylation was not modified by any of these substances.

**Figure 3A-C, 3a-d:**
**Analysis of human atherosclerotic lesions.** Atherectomy specimens from the internal carotid artery that had been operated for symptomatic occlusive disease of one of the internal carotid arteries were collected retrospectively from the files of the Institute of Pathology, University of Düsseldorf, Germany. The specimens were fixed in 4% buffered formaldehyde, cut transversally and embedded in paraffin. EPCR was detected using anti-hEPCR antibody (affinity purified goat IgG, R&D Systems, 1:100) and donkey anti-goat IgG-FITC (mouse/human adsorbed, Santa Cruz, 1:200). Alpha SM-actin was detected using the mouse anti-human alpha SM-actin antibody (Dako, 1:200) and sheep anti-mouse Cy3-conjugated secondary antibody (Sigma, 1:600). Nuclei were stained using Hoechst 33342 and microscopic analysis was performed using the Olympus BX50 fluorescence microscope and the confocal laser scanning microscope Zeiss LSM 510.

**Results.** In the fibrous cap of atherectomy specimens SMC-rich areas were detected which clearly expressed EPCR. Double-immunostaining revealed co-localization of EPCR and alpha-SM actin on the cellular level (Figure 3C). The expression of EPCR
in SMC was further verified by confocal laser scanning microscopy. These data prove that EPCR is expressed by SMC in the fibrous cap of human carotid artery plaques (Figure 3d) and thus support the hypothesis that EPCR-mediated signaling is involved in the phenotypic control of SMC during human carotid artery plaques.

**Legend.** Double immunostaining for EPCR and alpha-SM actin of an atherectomy specimen derived from human internal carotid artery. A) EPCR immunostaining staining in the fibrotic cap; B) alpha SM-actin staining of the same section; C) overlay of EPCR and alpha SM-actin staining; a-d) confocal images of the cell cluster framed in A-C, a) nuclei, b) EPCR, c) alpha-SM actin, d) overlay showing co-expression of EPCR and alpha-SM actin on the cellular level. Original magnification 200-fold in A-D and 1120-fold for confocal imaging in a-d.
Suppl. Figure 1 A

Suppl. Figure 1 B

Counts

Fluorescence intensity

Counts

Fluorescence intensity

- rat IgG
- anti-EPCR

- mouse IgG
- anti-CD31
Suppl. Figure 2A-C

**Figure 2A:** Western blot indicating that p-APMSF (200 μmol/L) did not influence ERK-1/2 phosphorylation by PAR-1-AP (100 μmol/L)

**Figure 2B:** Western blot indicating that anti-PAR-1 antibody (25 µg/ml) did not influence ERK-1/2 phosphorylation by PAR-1-AP (100 μmol/L)

**Figure 2C:** Western blot indicating that anti-EPCR antibody (10 μg/ml) did not influence ERK-1/2 phosphorylation by PAR-1-AP (100 μmol/L)
Suppl. Figure 3A-C and 3a-d

EPCR-Expression by SMC in human atherosclerotic lesions