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Research Article

Regulation of endothelial protein C receptor shedding by cytokines is mediated through differential activation of MAP kinase signaling pathways

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ABSTRACT

The endothelial protein C receptor (EPCR) plays a pivotal role in coagulation, inflammation, cell proliferation, and cancer, but its activity is markedly changed by ectodomain cleavage and release as the soluble protein (sEPCR). In this study we examined the mechanisms involved in the regulation of EPCR shedding in human umbilical endothelial cells (HUVEC). Interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), but not interferon-γ and interleukin-6, suppressed EPCR mRNA transcription and cell-associated EPCR expression in HUVEC. The release of sEPCR induced by IL-1β and TNF-α correlated with activation of p38 MAPK and c-Jun N-terminal kinase (JNK). EPCR shedding was also induced by phorbol 12-myristate 13-acetate, ionomycin, anisomycin, thiol oxidants or alkylators, thrombin, and disruptors of lipid rafts. Both basal and induced shedding of EPCR was blocked by the metalloproteinase inhibitors, TAPI-0 and GM6001, and by the reduced non-protein thiols, glutathione, dihydrolipoic acid, dithiothreitol, and N-acetyl-L-cysteine. Because other antioxidants and scavengers of reactive oxygen species failed to block the cleavage of EPCR, a direct suppression of metalloproteinase activity seems responsible for the observed effects of reduced thiols. In summary, the shedding of EPCR in HUVEC is effectively regulated by IL-1β and TNF-α, and downstream by MAP kinase signaling pathways and metalloproteinases.

Introduction

Numerous cell surface molecules such as adhesion molecules, cytokines, chemokines, growth factors, and receptors for cytokines are proteolytically cleaved by cell membrane-associated metalloproteinases [1–8]. As a consequence, these molecules are released into the extracellular space as soluble forms with altered functions. Metalloproteinase-mediated shedding is controlled by different mechanisms depending on cell type and the nature of processed cell surface proteins (for review see [2,3]). These mechanisms

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Abbreviations: ADAM, a disintegrin and metalloproteinase; α-LA, α-lipoic acid; APC, activated protein C; APMA, 4-aminophenylmercuric acetate; BSA, bovine serum albumin; CV, crystal violet; DHLA, dihydrolipoic acid; DMSO, dimethyl sulfoxide; DPI, diphenylene iodonium; DTT, dithiothreitol; EPCR, endothelial protein C receptor; ERK 1/2, extracellular signal-regulated kinase; FCS, fetal calf serum; GSH, reduced glutathione; GSSG, oxidized glutathione; HUVEC, human umbilical vein endothelial cells; IFN-γ, interferon-γ; IL-1β, interleukin-1β; IL-6, interleukin-6; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MβCD, methyl-β-cyclodextrin; MEK, mitogen-activated/ERK kinase; MMP, matrix metalloproteinase; NAC, N-acetyl-L-cysteine; NEM, N-ethyl maleimide; PBS, phosphate-buffered saline; PC, protein C; PDTC, pyrrolidine dithiocarbamate; PI3K, phosphoinositol-3-kinase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; ROS, reactive oxygen species; sEPCR, soluble endothelial protein C receptor; TNF-α, tumor necrosis factor-α

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include different cell signaling pathways; for example, receptor tyrosine kinase, ERK 1/2 and p38 mitogen-activated protein kinases (MAPK) [9,10].

The endothelial protein C receptor (EPCR) is one of the cell surface components whose activities and functions are regulated through metalloproteinase-mediated shedding. In connection with thrombomodulin, this receptor plays a pivotal role in the protein C (PC) pathway controlling thrombosis, limiting inflammatory responses and decreasing endothelial cell apoptosis in response to inflammation and ischemia (for review [11]). Furthermore, new data support an important role for the EPCR exposition on endothelial surfaces in cancer metastasis via inhibition of tumor cell adhesion and transmigration [12]. In murine melanoma cell metastasis models, transgenic EPCR overexpressing mice exhibited marked reductions in liver and lung metastases compared to wild-type animals. These findings highlight the importance of mechanisms regulating the EPCR exposition on the surface of endothelial cells.

In addition to protein expression, the exposition of EPCR depends strongly from the cleavage of EPCR and its release in the soluble form (sEPCR) [5,13–16]. A number of *in vitro*-treatments potently up-regulate the shedding of EPCR and reduce the rate of protein C activation [6,16–21]. The mechanisms involved in the regulation of this shedding process, however, are not clearly defined. On one hand, high levels of plasma sEPCR have been detected in patients with inflammatory diseases [18,20,22,23]. This may support the concept that increased shedding of EPCR is associated with the hypercoagulopathy frequently observed in inflammatory conditions [24]. On the other hand, a contribution of polymorphisms in the *EPCR* gene to the levels of sEPCR in patients with venous thromboembolism was shown [25].

Released sEPCR itself inhibits the function of protein C and its activated form (APC) through competition with APC/PC binding on membrane-associated EPCR [26]. Recently, the interaction of sEPCR with factor VII (FVII) and its activated form (FVIIa) was demonstrated providing evidence that EPCR and its soluble form serve as binding sites for FVII/FVIIa and inhibit the procoagulant activity of FVIIa-tissue factor complex [27,28].

All these data indicate that EPCR shedding is of pathophysiological relevance to the regulation of coagulation and possibly in the progression of cancer diseases. The importance of pro-inflammatory cytokines and downstream signal transduction cascades in EPCR shedding has not yet been systematically evaluated.

Materials and methods

Materials

Phorbol-12-myristate 13-acetate (PMA), PD-98059, SB-203580, SP600125, wortmannin, TAPI-0, ionomycin, and bovine thrombin (specific activity 1800-2200 NIH units/mg protein) were from Calbiochem (Schwalbach, Germany). Recombinant human IFN-γ, IL-1β, IL-6, and TNF-α were from Roche Diagnostics GmbH (Mannheim, Germany) and the goat HRP-conjugated anti-rat IgG_1 was from Chemicon International, Inc (Hampshire, United Kingdom). Other reagents were purchased from Sigma-Aldrich (Deisenhofen, Germany). Cytokines, reduced and oxidized forms of glutathione (GSH and GSSG, respectively), methyl-β-cyclodextrin (MβCD), N-acetyl-L-cysteine (NAC), dithiothreitol (DTT) and pyruvate were prepared as aqueous solutions. Other reagents were dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 0.3% or less; controls using DMSO alone were run in all these cases.

Cell culture and incubation

Human umbilical vein endothelial cells (HUVEC) were purchased from Promocell (Heidelberg, Germany) and maintained in Endothelial Growth Medium MV (Promocell) supplemented with 5% FCS. For all experiments, exponentially growing subconfluent cells were used at passages 5 to 8.

Analyses of EPCR expression in HUVEC

EPCR expression in HUVEC was determined at the mRNA level using reverse transcriptase-(RT)-PCR and at the protein level using 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 of the supplier (Sigma-Aldrich, Deisenhofen, Germany). Isolated RNA was converted to cDNA using the GeneAmp RNA-PCR kit (Applied Biosystems, Foster City, CA, USA). A portion of RT-reaction products was then amplified for PCR-based quantification of EPCR mRNA, using glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA as the house-keeping gene [29,30]. The applied primer pair was for EPCR (409 bp): 5′-GGC AGT TTC ATC ATT GCT GG (sense) and 5′-TTG AAC GCC TCA GGT GAT TC (antisense). Primers were used in a final concentration of 0.8 μM. The amplification included 25 cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min with the final cycle at 72 °C for 10 min. Buffers and reagents were from GeneAmp Kit (PerkinElmer LAS GmbH, Jügesheim, Germany). The PCR products were subjected to agarose gel electrophoresis and photographed on a G:BOX Chemi, GelVue UV Transilluminator device (SynGene, USA). Images were analyzed using GeneTools software from SynGene. Each PCR was performed at least twice.

Measurements of EPCR expression at the protein level were performed according to a protocol employing a cell-based ELISA format [31]. After an incubation period, cells were fixed with 4% formaldehyde in phosphate-buffered saline (PBS) at pH 7.4. The primary rat anti-human EPCR antibody RCR-379 was diluted at a 1:250 concentration with 5% BSA in PBS and incubated with fixed cells overnight at 4 °C, followed by washing with 0.1% Triton X-100/PBS. The secondary antibody, goat HRP-conjugated anti-rat IgG_1 diluted 1:250 in 5% BSA in PBS, was incubated for 1 h at room temperature in the dark. Peroxidase activity was measured at 450 nm using FACE™ Maker kit (Active Motif, Carlsbad, CA, USA), according to the manufacturer's instructions. Data were normalized for cell number estimated by crystal violet staining.

ELISA-based determination of released sEPCR

The amount of sEPCR released by endothelial cells was determined using a specific Asserachrom sEPCR kit (Diagnostica Stago, Asnieres, France) according to the manufacturer's instructions. For this purpose, cells were grown to confluence in 96-well microplates in complete medium. Cells were then incubated for 3 h in a starvation medium without FCS and supplements and further incubated for various time periods (1–6 h) in the same medium