Epac as a novel regulator of airway smooth muscle phenotype and function
Roscioni, Sara Silvia

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2010

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
Distinct interaction between cigarette smoke and cAMP effectors Epac and PKA in airway smooth muscle synthetic function in COPD

Sara S. Roscioni, Anouk Oldenburger, Esther Jansen, Mark H. Menzen, Andrew J. Halayko, Wim Timens, Herman Meurs, Harm Maarsingh, Martina Schmidt

Submitted (2010)
Abstract
Cigarette smoke-induced release of pro-inflammatory cytokines such as interleukin-8 (IL-8) from airway smooth muscle (ASM) cells may contribute to the development of chronic obstructive pulmonary disease (COPD). Here, we investigated the role of the cAMP-effectors Epac and PKA on cigarette smoke extract (CSE)-induced IL-8 release by human ASM cells as well as the potential signaling mechanisms involved. Additionally, the impact of CSE on Epac and PKA expression was evaluated. CSE-induced IL-8 release from ASM was reduced by the β2-agonist fenoterol, the Epac activator 8-pCPT-2’-O-Me-cAMP and the PKA activator 6-Bnz-cAMP. CSE induced IκBα degradation and p65 nuclear translocation, processes that were primarily reversed by the Epac activator 8-pCPT-2’-O-Me-cAMP. In addition, CSE increased extracellular signal-regulated kinase (ERK) phosphorylation, which was selectively reduced by the PKA activator 6-Bnz-cAMP. Furthermore, CSE decreased Epac1 expression, but had no effects on Epac2 and PKA expression. Importantly, we observed reduced Epac1 expression in lung tissue from COPD patients. In conclusion, our data indicate that Epac and PKA differentially decrease CSE-induced IL-8 release by ASM cells, via inhibition of NF-κB and ERK signaling, respectively. Our findings further indicate that cigarette smoke exposure may reduce anti-inflammatory effects of cAMP in the airways via down-regulation of Epac1.

Introduction
Chronic obstructive pulmonary disease (COPD) is a chronic inflammatory disorder characterized by a massive infiltration of leukocytes in the airways. Cigarette smoke-induced inflammation is known to be involved in the development of COPD in a subset of smokers [1, 2]. Neutrophils are an important component of the inflammation as they release inflammatory mediators and proteases, which are believed to play a role in the progressive lung destruction (emphysema) in COPD [3] and have been associated with COPD severity [2, 4] and exacerbations [5]. Interleukin-8 (IL-8) is a potent neutrophil chemoattractant and activator [2], and its levels correlate positively with neutrophil counts in COPD [6]. IL-8 levels are increased in sputum [7], in broncho-alveolar lavage fluid [8] and in the bronchiolar epithelium from COPD patients [9] and mRNA expression of IL-8 in bronchial biopsies correlate with COPD progression [10]. Cigarette smoke induces release of IL-8 from inflammatory cells [11, 12] and structural cells in the lung [9, 13], including airway smooth muscle (ASM) cells [14, 15]. In vitro, IL-8 release by cigarette smoke extract (CSE) involves activation of NF-κB [12, 14] and extracellular signal-regulated kinase (ERK) [11, 14]. Activation of NF-κB requires phosphorylation and degradation of IκBα and subsequent nuclear translocation of the NF-κB subunit p65 [16, 17], whereas ERK activation occurs via phosphorylation and subsequent nuclear translocation [18].
Currently, the most effective intervention for COPD is smoking cessation [19], but no preventive or curative pharmacological treatment exists. Despite their immunosuppressant effects in asthmatics, corticosteroids do not exhibit significant anti-inflammatory properties in patients with COPD [20]. COPD therapy is importantly based on symptomatic treatment, using bronchodilator drugs, including β₂-agonists [1]. β₂-agonists have also been shown to inhibit cytokine release in vitro [21-23]. However, evidence of anti-inflammatory effectiveness of β₂-agonists in vivo is lacking, which may be due to the β₂-adrenergic receptor desensitization in both airway inflammatory and structural cells [24, 25]. Hence, activation of post-β₂-adrenergic receptor mechanisms, could be advantageous to maintain the beneficial effects of β₂-agonists without the risk of receptor desensitization.

Among the structural cells in the airways, ASM cells represent a promising therapeutical target in chronic obstructive diseases, due to their intrinsic contractile ability. Moreover, ASM release IL-8 and express β₂-adrenergic receptors that couple with cAMP-signaling effectors [26]. Binding of β₂-agonists to the Gₛ-protein coupled β₂-adrenergic receptor results in the elevation of intracellular cyclic adenosine monophosphate (cAMP) and subsequent activation of protein kinase A (PKA) and exchange proteins activated by cAMP (Epac). Recently, we have shown that both effectors regulate IL-8 release in ASM cells via a ERK-dependent mechanism [27] (chapter 4). Moreover, cAMP elevation inhibits CSE-induced IL-8 release by human neutrophils, although the contribution of the different cAMP effectors in this process is unknown [11]. Based on these considerations, we investigated the role of Epac and PKA in CSE-induced IL-8 release by ASM cells and the underlying molecular mechanisms of these effects. Modulation of the expression profile pattern of Epac and PKA by CSE was also evaluated, as well as the expression of these cAMP effectors in lung samples derived from COPD patients.

Methods

*Human lung tissue.* Human lung tissue was collected from COPD patients and asymptomatic smokers used as a control group (Table I). Classification of COPD severity was based on the Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria [1]. Tissue from the control group and from GOLD stage II patients was derived from noninvolved lung tissue of patients undergoing resective surgery for pulmonary carcinoma; the control group had no airway obstruction or chronic airway symptoms, such as cough and sputum production. Tissue from GOLD stage IV patients was collected from subjects undergoing surgery for lung transplantation. The COPD-patients did not have clinical signs of chronic bronchitis and were not suffering from alpha-1-antitrypsin deficiency. The clinical characteristics of the patients are given in Table 1. Informed consent was obtained; the study protocol was consistent with national ethical and professional guidelines (“Code of conduct; Dutch federation of biomedical scientific societies”; http://www.federa.org).
Table 1. Characteristics of the study subjects.

<table>
<thead>
<tr>
<th>Subject groups</th>
<th>Control</th>
<th>COPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>9</td>
<td>20</td>
</tr>
<tr>
<td>Age (years)</td>
<td>62 (46-78)</td>
<td>60.5 (44-81)</td>
</tr>
<tr>
<td>Male/female</td>
<td>3/6</td>
<td>12/8</td>
</tr>
<tr>
<td>Ex-smoker/current smoker</td>
<td>7/2</td>
<td>16/4</td>
</tr>
<tr>
<td>Pack years</td>
<td>30.0 (3.0-52.5)</td>
<td>31.5 (8.5-65.0)</td>
</tr>
<tr>
<td>FEV1% predicted</td>
<td>96.9 (71.9-134.0)</td>
<td>37.8 (14.0-75.8) ^***</td>
</tr>
<tr>
<td>FEV1/FVC</td>
<td>727 (70.6-85.3)</td>
<td>43.7 (19.2-71.1) ^***</td>
</tr>
</tbody>
</table>

All values except number of subjects, gender and smoking status are expressed as median values with minimum and maximum range in parentheses. Ex-smoker: non smoker for at least one year. FEV1% predicted: forced expiratory volume in 1 second as percentage of predicted value. FVC: forced vital capacity. ^***P<0.001 compared to control group.

Cigarette smoke extract. Cigarette smoke extract was prepared as previously described [28]. Briefly, 2 research cigarettes (University of Kentucky 2R4F; filters removed) were combusted using a peristaltic pump (Watson Marlow 323 E/D, Rotterdam, The Netherlands) and passing the smoke through 25 ml of FBS-free DMEM supplemented with antibiotics at a rate of 5 minutes/cigarette. The obtained solution is referred to as 100% strength.

Cell culture. Human bronchial smooth muscle cell lines, immortalized by stable ectopic expression of human telomerase reverse transcriptase enzyme (hTERT-ASM), passages 1-30 were used. All procedures were approved by the human Research Ethics Board of the University of Manitoba [29]. Primary human ASM cells were isolated from human tracheal sections from anonymized lung transplantation donors (obtained from the Department of Cardiothoracic Surgery, University Medical Center Groningen) as previously described [30]. Passages 1-5 were used. Prior to the experiments, cells were growth arrested overnight and treated with fenoterol, 8-pCPT-2'-O-Me-cAMP or 6-Bnz-cAMP for 20 min before stimulation with CSE. The PKA inhibitor H89 was added 30 min before the other stimuli. Alamar blue was used to determine cell viability.
Interaction between cigarette smoke and cAMP in COPD

ELISA. 24 hrs after stimulation of ASM cells with CSE, culture medium was collected for the determination of IL-8 concentrations by ELISA according to the manufacturer’s instructions (Sanquin, the Netherlands).

Silencing of Epac1 and Epac2 expression. Epac1 and Epac2 knock-down was achieved by transfection of hTERT-ASM cells (90% confluency) with 200 pmol of siRNA (Table 2). ON-TARGETplus Non-targeting Pool (D-001810-10-20) was used as a negative control (control siRNA). Lipofectamine 2000 (1mg/ml) was used as a vehicle. 6 hrs after transfection, cells were washed. The day after, cells were seeded in 24 wells plates and serum-deprived overnight. 24 hrs after stimulation with CSE in the absence or presence of 8-pCPT-2’-O-Me-cAMP or 6-Bnz-cAMP, supernatants were collected for analysis of IL-8 levels. Furthermore, transfected cells were lysed for analysis of Epac1 and Epac2 protein or mRNA.

Table 2. Sequences of siRNA probes against Epac1 and Epac2.

<table>
<thead>
<tr>
<th>Epac1</th>
<th>ON-TARGETplus SMART pool siRNA</th>
<th>Target sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>J-007676-05</td>
<td></td>
<td>CGUGGGAACUCAUGAGAUG</td>
</tr>
<tr>
<td>J-007676-06</td>
<td></td>
<td>GGACCGAGAUGGCCAAUUC</td>
</tr>
<tr>
<td>J-007676-07</td>
<td></td>
<td>GAGCGUCUCUUUGUUGUCA</td>
</tr>
<tr>
<td>J-007676-08</td>
<td></td>
<td>CGUGGUACAUUAUCUGGAA</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Epac2</th>
<th>ON-TARGETplus SMART pool siRNA</th>
<th>Target sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>J-009511-05</td>
<td></td>
<td>GAACACACCUCUCAUUGAA</td>
</tr>
<tr>
<td>J-009511-06</td>
<td></td>
<td>GGAGAAUAUCGACAGUAU</td>
</tr>
<tr>
<td>J-009511-07</td>
<td></td>
<td>GCUCAAAACCUAUGUGUU</td>
</tr>
<tr>
<td>J-009511-08</td>
<td></td>
<td>CAAGUUGACUCUAGUGAA</td>
</tr>
</tbody>
</table>

Western analysis. Cell lysates were prepared and subjected to protein determination using a Pierce BSA protein assay (Thermo Scientific, Rockford, IL, USA). Similar protein amounts were loaded on a SDS-Page gel (8-15%) for electrophoresis and transferred to a nitrocellulose membrane. Incubation with the specific primary and secondary antibodies was performed overnight (Table 3). After the addition of western lighting plus-ECL, determination of protein bands was achieved using the G-BOX iChem (Syngene, Cambridge, UK). Bands were normalized to GAPDH, β-actin or ERK, depending on the protein under investigation.
Table 3. List of antibodies used in western analysis.

<table>
<thead>
<tr>
<th>Target</th>
<th>Source (catalog number)</th>
<th>Dilution</th>
<th>Secondary antibody (dilution 1:2000)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VASP</td>
<td>Cell Signaling (3112)</td>
<td>1:500</td>
<td>rabbit</td>
</tr>
<tr>
<td>p-ERK</td>
<td>Cell Signaling (9101S)</td>
<td>1:1000</td>
<td>rabbit</td>
</tr>
<tr>
<td>ERK</td>
<td>Cell Signaling (9102)</td>
<td>1:500</td>
<td>rabbit</td>
</tr>
<tr>
<td>Epac1</td>
<td>Cell Signaling (41555)</td>
<td>1:500</td>
<td>mouse</td>
</tr>
<tr>
<td>Epac2</td>
<td>Cell Signaling (41565)</td>
<td>1:500</td>
<td>mouse</td>
</tr>
<tr>
<td>PKA-C</td>
<td>BD Transduction Laboratories (610980)</td>
<td>1:500</td>
<td>mouse</td>
</tr>
<tr>
<td>PKA-RII</td>
<td>BD Transduction Laboratories (558244)</td>
<td>1:500</td>
<td>mouse</td>
</tr>
<tr>
<td>IκBα</td>
<td>Santa Cruz (sc-203)</td>
<td>1:200</td>
<td>rabbit</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Santa Cruz (sc-47724)</td>
<td>1:2000</td>
<td>mouse</td>
</tr>
<tr>
<td>β-actin</td>
<td>Sigma (A 5441)</td>
<td>1:2000</td>
<td>mouse</td>
</tr>
</tbody>
</table>

RNA isolation. mRNA from cultured human ASM cells was extracted by using a Nucleospin RNA II kit (Macherey Nagel, Düren, Germany). mRNA from human lung tissue was extracted using trizol reagent and chloroform to separate the RNA containing phase (aqueous). RNA was precipitated with propanol and washed with ethanol. After drying the pellet, RNA was resuspended in RNase free water. The eluted mRNA was quantified using spectrophotometry (Nanodrop, ThermoScientific, Wilmington, USA). 1μg of mRNA was converted in cDNA by reverse transcriptase using Promega tools (Madison, WI, USA).

RT-PCR. cDNA was subjected to real-time PCR (RT-PCR) using a MyiQ™ Single-Color detection system (Bio-Rad laboratories Inc. Life Science Group, Hercules, CA, USA) and specific primers (Table 4). The amount of target gene was normalized to ribosomal subunit 18S (designated as ΔCT). Relative differences were determined using the equation 2^-ΔΔCT (2^-ΔΔCT=1 in control). When necessary, a 1.2 % gel was used to separate the RT-PCR products.
Table 4. Primers used for RT-PCR.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence 5'-3'</th>
</tr>
</thead>
</table>
| Epac1            | Forward: GACCGAGATGCCCAATTCTA  
                             Reverse: TTTGAAAGTGCCACAAGTGAG  |
| Epac2            | Forward: ATTCGAATACGGCCAGAAC  
                             Reverse: TCTATGGTCGACGAGGCTCT  |
| Ribosomal subunit 18S | Forward: CGCCGCTAGAGGTGAAATTC  
                             Reverse: TTGGCAAATGCTTTCGCTC  |

Immunofluorescence. 10,000 cells per well were plated in coverslips and serum deprived for 24 hrs before stimulation. Cells were fixed by addition of 3% paraformaldehyde (PFA) for 15 min, followed by incubation in 3% PFA plus 0.3% Triton X-100 for 5 min. After blocking, cells were incubated overnight with p65 antibody (Santa Cruz, CA, USA, dilution 1:20) and donkey-anti rabbit FITC (dilution: 1:50) was added. Nuclei were stained with a Hoechst staining (1:10000). After this, coverslips were mounted using ProLong Gold antifade reagent and analyzed using an Olympus AX70 microscope equipped with digital image capture system (ColorView Soft System with Olympus U CMAD2 lens).

Materials. 6-Bnz-cAMP and 8-pCPT-2'-O-Me-cAMP were purchased from BIOLOG Life Science Institute (Bremen, Germany). Fenoterol was obtained from Boehringer Ingelheim (Ingelheim, Germany). Protease inhibitors, albumin bovine serum, Triton X-100, H89 were from Sigma-Aldrich (St-Louis, MO). Cell medium components were from GIBCO-BRL Life Technologies (Paisley, UK). Donkey serum and donkey anti-rabbit FITC fluorescent antibody was obtained from Jackson Immuno Research (West Grove, PA). Hoechst nuclear staining and anti-fade solution and lipofectamine 2000 (1mg/ml) were purchased from InVitrogen (Carlsbad, CA) as well as the PCR primers. ECL solution for western blot detection was purchased from PerkinElmer Inc. (Waltman, MA). siRNA probes were obtained from Dharmaco Inc. (Lafayette, CO).

Statistical analysis. Results are expressed as mean±SEM of separate experiments. In Table 1 results represent median values (minimal and maximal values between parenthesis). To evaluate differences between stimulated and control samples, statistical analysis was performed by unpaired or paired two-tailed student’s t-test, as appropriate. A nonparametric Mann Whitney test was used to compare features of control versus COPD patients. P values <0.05 were considered to be statistically significant.
cAMP signaling attenuates CSE-induced IL-8 release from human ASM cells

Stimulation of hTERT-ASM cells with 15% CSE for 24 hrs induced a significant increase in IL-8 release ($P<0.001$, Fig. 1), without affecting the cell number as assessed by Alamar blue assay (not shown). The CSE-induced IL-8 release was almost fully inhibited by co-treatment with the β2-agonist fenoterol (0.1 and 1 µM; $P<0.001$ both; Fig. 1A), in a concentration-dependent manner.

To study the role of individual cAMP effectors Epac and PKA in the inhibition of CSE-induced release of IL-8, we applied the selective PKA activator 6-Bnz-cAMP.
Interaction between cigarette smoke and cAMP in COPD

(100 and 500 µM) and the selective Epac activator 8-pCPT-2'-O-Me-cAMP (30 and 100 µM). Treatment with 6-Bnz-cAMP concentration-dependently and fully inhibited CSE-induced IL-8 release (P<0.001; Fig. 1B), whereas only the highest concentration of 8-pCPT-2'-O-Me-cAMP (100 µM) inhibited IL-8 release, and to a lesser degree compared to PKA (P<0.001; Fig. 1C). None of the stimuli significantly altered basal IL-8 levels (Fig. 1). Similarly, treatment with fenoterol (1 µM), 6-Bnz-cAMP (500 µM) and 8-pCPT-2'-O-Me-cAMP (100 µM) reduced CSE-induced IL-8 release from primary human ASM cells, without affecting basal IL-8 levels (Fig. 1D).

The effects of the two cAMP effectors Epac and PKA on IL-8 release by hTERT-ASM cells were validated using different experimental approaches. Due to the lack of isoform-specific Epac inhibitors, siRNA probes against Epac1 and Epac2 were used to silence Epac expression [27]. Treatment with siRNA for either Epac1 or Epac2 only slightly reduced the inhibitory effect of 8-pCPT-2'-O-Me-cAMP on CSE-induced IL-8 release (not shown). However, simultaneous knock-down of both Epac1 and Epac2 mRNA and protein (Fig. 2A), significantly impaired the inhibitory effect of 8-pCPT-2'-O-Me-cAMP on CSE-induced IL-8 release from hTERT-ASM cells (P<0.001, Fig. 2B), leaving the basal and CSE-induced IL-8 release unaffected (not shown). As expected, the effect of the PKA activator 6-Bnz-cAMP was not
affected by the siRNA silencing of Epac1 and Epac2 (Fig. 2B). The specificity of the cAMP analogs was confirmed by evaluation of the phosphorylation of the PKA-specific substrate VASP (vasodilator-activated phosphoprotein). Thus, PKA activation with 6-Bnz-cAMP, but not Epac activation with 8-pCPT-2'-O-Me-cAMP, induced VASP phosphorylation, an effect that could be reduced by the PKA inhibitor H89 (1 µM; Fig. 3A). Moreover, the inhibitory effect of 6-Bnz-cAMP, but not 8-pCPT-2'-O-Me-cAMP, on CSE-induced IL-8 release was largely reduced by H89 (P<0.001, Fig. 3B).

Fig. 3. Inhibition of PKA attenuates the effect of 6-Bnz-cAMP on CSE-induced IL-8 release. Phosphorylation of VASP in hTERT-ASM cells treated with 100 µM 8-pCPT-2'-O-Me-cAMP or 500 µM 6-Bnz-cAMP in the absence or presence of the PKA inhibitor H89 (300 nM) was analysed by using an antibody, which recognizes both the phosphorylated VASP (phospho-VASP) and the non phosphorylated VASP (VASP) (A). VASP was normalized to GAPDH. Representative immunoblots of 3 experiments are shown. hTERT-ASM were pre-treated without (white bars) or with (black bars) 300 nM H89 for 30 min before stimulation with 15% CSE, 100 µM 8-pCPT-2'-O-Me-cAMP, 500 µM 6-Bnz-cAMP or their combinations (B). Data are presented as means±SEM of 3-9 separate experiments. ***P<0.001 compared to basal control. #P<0.05, ###P<0.001 compared to CSE. ‡‡‡P<0.001.
Fig. 4. 8-pCPT-2′-O-Me-cAMP prevents CSE-induced breakdown of IκBα and p65 nuclear translocation. p65 nuclear translocation was determined by immunofluorescence on hTERT-ASM cells stimulated without (control) or with 15% CSE for 2 hrs, alone or in combination with 100 µM 8-pCPT-2′-O-Me-cAMP or 500 µM 6-Bnz-cAMP. Results of 3 experiments are shown (A) with the quantification of p65 nuclear staining (B). hTERT-ASM cells were treated with 15% CSE, 100 µM 8-pCPT-2′-O-Me-cAMP, 500 µM 6-Bnz-cAMP or their combinations for 1 hr. IκBα levels were determined by western blot (C). Bands were normalized to GAPDH. Representative immunoblots are shown. Data are presented as means±SEM of 6-7 experiments. ***P<0.001 compared to basal control. ###P<0.001 compared to CSE.
Role of NF-κB in CSE-induced IL-8 release
NF-κB activation has been shown to be crucial for CSE-induced IL-8 production [11, 14]. Accordingly, treatment of hTERT-ASM cells with CSE for 2 hrs increased nuclear staining of p65 (P<0.001, Figs 4A and 4B, see appendix), indicating increased translocation of the transcriptionally active NF-κB subunit from the cytosol to the nucleus. Basal p65 cellular localization was not altered by either the Epac activator 8-pCPT-2′-O-Me-cAMP or the PKA activator 6-Bnz-cAMP (Fig. 4A). Importantly, p65 nuclear translocation by CSE was significantly inhibited by 8-pCPT-2′-O-Me-cAMP (P<0.001) but not by 6-Bnz-cAMP (Figs 4A and 4B). p65 nuclear translocation was preceded by loss (degradation) of the NF-κB inhibitory protein IκBα after 1 hr treatment with CSE (P<0.001, Fig. 4C). As we observed for p65 nuclear translocation, this response was significantly prevented by the Epac activator (P<0.001, Fig. 4C), and only slightly reduced by PKA activation (Fig. 4C). Taken together, these findings indicate that stabilization of the IκBα-p65 complex underlies the inhibitory effect of Epac on NF-κB activation.

Role of ERK in CSE-induced IL-8 release
In line with previous studies from our group [14, 28], CSE induced a significant increase in ERK phosphorylation in hTERT-ASM cells after 1 hr stimulation (P<0.001, Fig. 5). Importantly, CSE-induced ERK activation was completely normalized by the PKA activator 6-Bnz-cAMP (P<0.001, Fig. 5), whereas activation of Epac had no effect on ERK phosphorylation (Fig. 5). These findings clearly show a differential effect of PKA and Epac on CSE-induced ERK activation.

Fig. 5. 6-Bnz-cAMP prevents CSE-induced ERK phosphorylation. hTERT-ASM cells were lysed after being stimulated with 15% CSE, 100 µM 8-pCPT-2′-O-Me-cAMP, 500 µM 6-Bnz-cAMP or their combinations for 1 hr followed by Western blot analysis of phospho-ERK (p-ERK). Total ERK (ERK) was used as a loading control. Representative immunoblots of p-ERK and ERK are shown. Data are presented as means±SEM of 5-7 separate experiments. ***P<0.001 compared to basal control. ###P<0.001 compared to CSE.
Modulation of Epac expression by CSE
Interestingly, in hTERT-ASM cells the expression of Epac1 protein was significantly decreased after 24 hrs treatment with CSE ($P<0.05$) (Fig. 6A). By contrast, no change was observed in the protein abundance for Epac2 or the catalytic (PKA-C) and regulatory type II (PKA-RII) subunits of PKA (Figs 6A and 6B). CSE also down-regulated Epac1 mRNA in both hTERT-ASM and primary human ASM cells, after 24 hrs of stimulation (not shown).

Fig. 6. Epac1 is down-regulated by CSE. hTERT-ASM cells were treated for 24 hrs with 15% CSE. Then, cells were lysed for protein determination. Expressions of Epac1 and Epac2 (A) and catalytic (PKA-C) and regulatory type II (PKA-RII) subunits of PKA (B) were normalized to $\beta$-actin (for Epac) and GAPDH (for PKA). Data represent mean±SEM of 3-4 independent experiments. *$P<0.05$ compared to time point 0.

Expression patterns of Epac and PKA in lung tissue from COPD patients
Given the effect of CSE on cultured human ASM cells, we evaluated the expression of Epac and PKA in lung tissue from COPD patients and from asymptomatic smokers, whose characteristics are described in Table 1. Remarkably, the expression of Epac1 protein was significantly lower in COPD patients, whereas no differences were observed for Epac1 mRNA (not shown), indicating that smoking affects the translation of Epac1. In addition, in line with the data in human ASM cells (Figure 6), no change in the protein (Fig. 7C and 7D) and mRNA expression (not shown) of Epac2 and PKA was observed. These findings may indicate that chronic exposure to cigarette smoke might affect Epac-mediated function in COPD.
Fig. 7. Epac and PKA expression in COPD patients. Epac1 (A), Epac2 (B), PKA-C (C) and PKA-RII (D) protein expression from lung homogenates of controls and COPD patients. Data are derived from 9 controls and 15-19 COPD patients. *P<0.05.
Interaction between cigarette smoke and cAMP in COPD

Discussion

In this study, we show a differential inhibitory role for the cAMP effectors Epac and PKA on CSE-induced release of IL-8 from human ASM cells. Thus, it was demonstrated that Epac activation inhibits CSE-induced IL-8 release by blocking NF-κB activation, whereas activation of PKA inhibits ERK activation. Moreover, CSE significantly reduces Epac1 mRNA and protein expression in ASM cells, leaving Epac2 and PKA unaffected. Importantly, Epac1 protein expression was also significantly reduced in COPD patients, which may translate our *in vitro* findings to a pathophysiological context.

Cigarette smoke contributes to the development of COPD by inducing a chronic inflammation known to be associated with irreversible damage of the airways and lung parenchyma (emphysema) [31, 32]. The observed pathogenic potential of cigarette smoke correlates in part with the increased release of the neutrophil chemoattractant IL-8 by inflammatory and structural cells [6, 7, 11-13] including ASM cells [14, 15]. Current objectives of therapy are to reduce episodes of airway obstruction and improve airflow limitation as a means of improving quality of life. However, no current treatment effectively inhibits inflammation-driven progressive decline in lung function [1]. Hence, there is a need for novel targets of anti-inflammatory therapy in this disease.

Beside its beneficial acute bronchodilatory effects, cAMP also exhibit anti-inflammatory properties *in vitro*, by inhibiting the release of cytokines by several cell types in the airways [21-23], a response usually associated with activation of PKA [23]. However, our prior data have shown that both cAMP effectors Epac and PKA modulate bradykinin-induced IL-8 release from human ASM cells [27] (chapter 4). Only few studies addressed the role of cAMP in CSE-induced IL-8 release [11, 33], whereas the effects of Epac and PKA on this response have not been investigated at all. Thus, CSE-induced IL-8 release by human neutrophils was decreased by the β2-agonist salmeterol [11], whereas salmeterol only reduced IL-8 release by human ASM cells in the presence of fluticasone [33]. In contrast, here we report that the β2-agonist fenoterol alone can reduce CSE-induced IL-8 release from human ASM cells by itself, an effect that is mimicked by specific activation of Epac and PKA. Differences in cell culture (tracheal vs bronchial ASM or cell passages used), treatment (salmeterol vs fenoterol and incubation time) might account for different observed effects. Importantly, our study represents the first description that Epac and PKA inhibit CSE-induced inflammatory cytokine release.

Epac and PKA appear to have similar effects with regard to ASM synthetic, proliferative and contractile capacities [27, 30, 34] (chapters 4, 7-9). As no cross-inhibition of both cAMP effectors was observed, we can exclude any contribution of co-operativity between Epac and PKA in our findings. Importantly, our results indicate that Epac and PKA exert their inhibitory effects towards CSE-induced IL-8 release via distinct signaling routes. It has been shown that CSE activates both NF-κB and ERK to increase IL-8 release [11, 12, 14]. In the current study, we show that
activation of Epac specifically inhibited CSE-induced NF-κB activation, whereas activation of PKA specifically reduced ERK phosphorylation. Thus, our study unravelled novel distinct, but complementary, immunosuppressive mechanisms of Epac and PKA in human ASM and defines NF-κB and ERK as specific targets of Epac and PKA, respectively. Despite the fact that both Epac and PKA are expressed in cultured human (primary and immortalized) ASM cells [27, 30] and reduce CSE-induced IL-8 release, by using effective concentrations of their selective activators we could observe a stronger inhibitory effect of PKA compared to Epac. The lower inhibitory efficacy of the Epac activator 8-pCPT-2’-O-Me-cAMP towards CSE may relate to the observed reduced Epac1 abundance after CSE treatment. Interestingly our data using siRNA reveal that the combined knock-down of both Epac1 and Epac2 is required to prevent the suppressive effects of Epac on CSE-induced IL-8, suggesting a functional interaction between the two Epac isoforms. Importantly, we translated our findings to a pathophysiological setting as reduced Epac1 expression was observed in lung homogenates from COPD patients. These findings might have important clinical implications towards a better understanding of COPD pathogenesis and the improvement of its pharmacological treatment. Indeed, despite its anti-inflammatory effects in vitro, clinical studies only show modest beneficial effects of β2-agonists in the treatment of airway inflammation [35, 36]. Such a discrepancy has been assigned to variable β2-adrenergic receptor abundance or its potential polymorphisms [37] and additionally to alterations of β2-adrenergic receptor signaling by homologous or heterologous desensitization in both inflammatory and structural cells in the airways [25, 38, 39]. As a potential effector in cAMP-driven and β2-adrenergic receptor-induced signaling and a newly discovered inhibitor of NF-κB-dependent inflammatory response, Epac1 down-regulation by cigarette smoke may provide an additional explanation for the variable anti-inflammatory capacities of β2-agonists in the treatment of COPD. Reduced expression of Epac1 by the COPD-associated pathogenic factor transforming growth factor-β in human lung fibroblasts has been previously associated with the profibrotic role of this growth factor in the lung [40]. As decreased expression levels of Epac1 in CSE-treated cultured human ASM cells and samples from COPD patients were observed in this study, our data may indicate that besides fibrosis, Epac1 down-regulation may also be of importance in the development of other clinical features of COPD triggered by cigarette smoke. In conclusion, Epac and PKA inhibit CSE-induced IL-8 release by human ASM by preventing the activation of NF-κB and ERK, respectively. These findings indicate Epac and PKA hold potential as targets for anti-inflammatory therapy in COPD. Moreover, CSE-induced reduction in Epac1 expression might point to a divergent contribution of cAMP effectors in mediating immunosuppressive potential of current drug therapies. Hence, studying the mechanisms by which cigarette smoke drives Epac1 down-regulation could unveil alternative ways for intervention, as targeting...
specific cAMP effectors could allow a more effective control of cAMP signaling driven by Gs-coupled receptors.

References


34. Roscioni SS, Dekkers BG, Prins AG, Menzen MH, Meurs H, Schmidt M, Maarsingh H: cAMP inhibits modulation of airway smooth muscle phenotype via the exchange protein activated by cAMP (Epac) and protein kinase A. Br J Pharmacol 2010,


