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A-kinase-anchoring proteins coordinate inflammatory responses to cigarette smoke in airway smooth muscle

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CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD) is characterized by airway inflammation, neutrophils, mucus hypersecretion, airway remodeling, and parenchymal tissue destruction (emphysema), all contributing to irreversible airflow limitation and accelerated lung function decline (1). Cigarette smoking is the main risk factor for the development of COPD (7). Smoking cessation is the most effective strategy to slow down the accelerated decline in lung function observed in the disease (2, 23), but it does not reverse the structural changes in the lungs.

Pharmacological COPD management is mainly focused on the treatment of symptoms, including airway obstruction and inflammation. In COPD, mainstays for eliciting bronchodilation include inhaled anticholinergics or β2-agonists (23, 30). β2-Agonists primarily act on airway smooth muscle (ASM) cells, increasing the intracellular production of the second messenger cyclic AMP (cAMP) to induce ASM relaxation and bronchodilation (19). In addition to their role in regulating airway diameter, ASM cells also possess secretory and proinflammatory functions and through this contribute to local chronic airway inflammation in COPD (3, 6, 28, 29). Although anti-inflammatory effects of β2-agonists in vitro are established (13, 20), the evidence for this in vivo is still under debate (34).

cAMP induces its biological actions by activating various effectors, including protein kinase A (PKA) and exchange protein directly activated by cAMP (Epac) (36, 40). CAMP and its effectors are under tight spatiotemporal control by a family of scaffolding proteins with over 50 members called A-kinase-anchoring proteins (AKAPs) (32, 37, 38). AKAPs regulate diverse cellular processes, including release of inflammatory cytokines from cardiomyocytes and alveolar macrophages (8, 21). For example, in alveolar macrophages, AKAP10 is required for the potentiation of LPS-induced IL-6 and IL-10 production by the cAMP-elevating agonist prostaglandin E2 (PGE2) (21). Although less pronounced, AKAP11 also plays a modulatory role in the above-mentioned processes (21).

Human ASM cells are known to express AKAP1-AKAP3, AKAP5, AKAP9-AKAP13, MAP2B, and Ezrin (15, 24). Inhibition of AKAP-PKA interactions using the PKA-anchoring

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disrupter peptides, Ht31 and AKAP-JS, in human ASM cells extended the duration of the β2-agonist-induced cAMP signal at the plasma membrane (15). The functional role of AKAP-mediated compartmentalization in the lung, the effects of cigarette smoke exposure on AKAP expression, and the expression profile of AKAPs in lung diseases such as COPD are unknown.

AKAP5 and AKAP12 directly interact with the β2-adrenoceptor (5, 11, 16, 32, 39). Thus we hypothesized that alterations in AKAP5 and AKAP12 expression may affect the anti-inflammatory effects of β2-agonists on cigarette smoke-induced inflammatory responses. In the current study, we investigated AKAP expression in human ASM cells after exposure to cigarette smoke extract (CSE) as well as in lung tissue from patients with COPD patients. We observed changes in AKAP5 and AKAP12 expression in COPD lungs as well as in cultured, CSE-exposed human ASM cells. Importantly, disruption of AKAP-PKA interactions prevented the anti-inflammatory properties of the β2-agonist fenoterol via disturbance of ERK phosphorylation.

**MATERIALS AND METHODS**

**Cell culture.** Human bronchial ASM cells obtained from three different healthy donors during lung transplant procedures and immortalized by stable ectopic expression of human telomerase reverse transcriptase (hTERT) as described previously (12) were used at passages 20–29. Primary human ASM cells obtained as described previously (35) were used at passages 1–5. The cells were maintained in high-glucose Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated fetal calf serum supplemented with HEPES (25 mM), l-glutamine (2 mM), amphotericin B (1.5 μg/ml), and penicillin (100 U/ml)/streptomycin (100 μg/ml) in a humidified atmosphere at 37°C in air-CO2 (95:5% vol/vol). For primary ASM cells, the medium was supplemented with sodium pyruvate (1 mM), Gibco MEM nonessential amino acids, and gentamicin (45 μg/ml).

**Human lung tissue.** To study possible differences in AKAP expression between patients with moderate and severe COPD, we used tissue from COPD Global Initiative for Chronic Obstructive Lung Disease (GOLD) stage II and stage IV, respectively. Lung tissue was collected from current and ex-smoking patients, using asymptomatic current and ex-smokers as a control group (for patient characteristics see Tables 1 and 2). Tissue from the control group and patients with COPD GOLD stage II was derived from nondiseased lung tissue of patients undergoing resective surgery for pulmonary carcinoma, in controls without airway obstruction or chronic airway symptoms, such as cough and sputum production. Classification of COPD severity was based on the GOLD criteria (33). Tissue from patients with GOLD IV was obtained from subjects undergoing surgery for lung transplantation. All tissue was collected according to the Research Code of and approved by the University Medical Center Groningen (https://www.umcg.nl/EN/Research/Researchers/General/ResearchCode/Pages/default.aspx), and it conformed to national ethical and professional guidelines ("Code of conduct; Dutch federation of biomedical scientific societies"; http://www.federa.org).

**CSE preparation.** CSE was prepared using 3R4F research cigarettes (Reference Cigarette Program, University of Kentucky) (4) as described previously (25, 26). In short, two cigarettes were combusted using a peristaltic pump (Watson Marlow 323 E/D) in to 25 ml of serum-free medium; this was designated 100% CSE and was diluted to 15% CSE using serum-free medium.

**Cell stimulation.** hTERT ASM cells were grown to confluence, and growth was halted by exchange of the complete medium for serum-free medium for 24 h. Cells were pretreated with 50 μM st-Ht31 (Promega) to disrupt AKAP-PKA interactions (4, 18), for 20 min before stimulation with 15% CSE in serum-free medium. Control cultures were pretreated with vehicle alone. When used, fenoterol (0.001–0.1000 μM), the PKA activator 6-Bnz-cAMP (6-Bnz; 500 μM), or the Epac activator 8-pCPT-2′-O-Me-cAMP (8-pCPT; 100 μM) were added 10 min after the addition of st-Ht31. The action of these cAMP analogs has previously been characterized in this system (29). Cells were stimulated with 15% CSE for different time points: 10 min for vasodilator-stimulated phosphoprotein (VASP) phosphorylation, 1 h for ERK phosphorylation, 2 h for NF-κB translocation, and 24 h for AKAP mRNA and protein expression and ELISA measurements of IL-8. Serum-free medium (vehicle) served as control for all experiments, as st-Ht31P, the commonly used control for st-Ht31, has been reported to be a direct inhibitor of PKA (24). In addition, st-Ht31P decreased cell viability in a dose-dependent manner (Fig. 6E), rendering st-Ht31P invalid as a control in this setup and in line with previous observations from our group (28).

**RII overlay.** RII overlay assay was performed as described previously (14, 18). In brief, proteins were subjected to SDS-PAGE, transferred to PVDF membranes, and incubated in RII blocking buffer. Membranes were incubated with [γ-32P]-labeled PKA RIIα subunits in fresh RII blocking buffer overnight at room temperature. Membranes were washed twice with blocking buffer and twice with PBS (10 min each). Binding of [γ-32P]-RIIα subunits was visualized by autoradiography using a STORM 830 Scanner (Molecular Dynamics).

**Table 1. Characteristics of the study objects used for the gene expression**

<table>
<thead>
<tr>
<th>Subject Groups</th>
<th>Control</th>
<th>COPD Stage II</th>
<th>COPD Stage IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Age, yr</td>
<td>57 (46–67)</td>
<td>71 (49–80)</td>
<td>58 (55–61)</td>
</tr>
<tr>
<td>Male/female</td>
<td>1/4</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td>Ex-smoker/current smoker</td>
<td>3/2</td>
<td>4/1</td>
<td>5/0</td>
</tr>
<tr>
<td>Pack yr</td>
<td>32 (10–40)</td>
<td>23 (8.5–30)</td>
<td>33 (30–54)</td>
</tr>
<tr>
<td>FEV1% predicted</td>
<td>98.3 (86.1–102.6)</td>
<td>59.1† (54.6–61.1)</td>
<td>16.8† (14.0–23.6)</td>
</tr>
<tr>
<td>FEV1/FVC</td>
<td>80.9 (70.6–85.3)</td>
<td>63.9* (45.7–71.1)</td>
<td>25.7† (19.2–41.8)</td>
</tr>
</tbody>
</table>

All values except number of subjects, sex, and smoking status are expressed as median values with minimum and maximum range in parentheses. COPD, chronic obstructive pulmonary disease; Ex-smoker, nonsmoker for at least 1 yr; FEV1% predicted, forced expiratory volume in 1 s as percentage of predicted value; FVC, forced vital capacity. *P < 0.001 compared with control group. 1 data point unknown, 2 data points unknown.

**Table 2. Characteristics of the control persons and patients whose lung tissues were used for the immunohistochemistry**

<table>
<thead>
<tr>
<th>Subject Groups</th>
<th>Control</th>
<th>COPD Stage II</th>
<th>COPD Stage IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>10</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>Age, yr</td>
<td>67 (55–74)</td>
<td>71* (58–80)</td>
<td>58‡ (50–66)</td>
</tr>
<tr>
<td>Male/female</td>
<td>4/6</td>
<td>6/2</td>
<td>6/3</td>
</tr>
<tr>
<td>Pack yr</td>
<td>15 (1.5–65)</td>
<td>36.5 (10–44)</td>
<td>26 (18–38)</td>
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<tr>
<td>FEV1% predicted</td>
<td>97.1 (63.7–121)</td>
<td>65* (41.7–71)</td>
<td>20.6† (13–36)</td>
</tr>
<tr>
<td>FEV1/FVC</td>
<td>73.6 (64–86.2)</td>
<td>63* (45.7–64.1)</td>
<td>36.8† (25–66)</td>
</tr>
</tbody>
</table>

All values except number of subjects and sex are expressed as median values with minimum and maximum range in parentheses. Only ex-smokers were included. *P < 0.05, †P < 0.001 compared with control group, §P < 0.001 compared with COPD stage II (Mann Whitney U-test). 1 data point unknown, 2 data points unknown.
Western blot. Cells were lysed in RIPA lysis buffer [containing 0.5 mM phenylmethylsulphonyl fluoride, 0.1 mM sodium orthovanadate, 1 mM sodium fluoride, 3.5 μM β-glycerolphosphate, 1 mM EDTA, 1% Triton X-100 (vol/vol), 0.1% sodium dodecyl sulphate (wt/vol), 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 μg/μl soybean trypsin inhibitor, 1.43 μg/μl aprotinin, and 0.8 mM benzamidin] followed by sonication. Samples for detection of AKAP9 (611518; BD Biosciences), AKAP12 (ab87067; Abcam), Ezrin (Ab4069; Abcam), AKAP5 (sc-6442; Santa Cruz Biotechnology), AKAP8 (sc-10766; Santa Cruz Biotechnology), (phospho)VASP (3112; Cell Signaling Technology), or (phospho)ERK (9101 and 9102; Cell Signaling Technology) expression were subjected to SDS-PAGE. Immunoblotting with primary antibodies (dilution 1:500 for AKAPs antibodies, 1:1,000 for the VASP antibody, 1:2,000 for ERK antibodies) was done overnight at 4°C. Blots were incubated with secondary antibody (1:2,000 A9044 or A0545; Sigma-Aldrich). Signals were detected with chemiluminescence reagents according to the manufacturer’s protocol. GAPDH (1:2,000; sc-47724; Santa Cruz Biotechnology) served as a control for equal loading. Blots were quantified using ImageJ.

Real-time PCR. After 24 h, total RNA was isolated using the NucleoSpin RNA II isolation kit (Machery-Nagel), and cDNA was obtained using the Reverse Transcription System (A3500, Promega) following the manufacturers’ instructions. This cDNA was used as a control for equal loading. Blots were quantified using ImageJ.
template for quantitative real-time PCR for AKAP5 (forward: 5'-GACGCCCTACGTTGATCT-3'; reverse: 5'-GAAATGCCCAGTTTCTCTATG-3') and AKAP12 (forward: 5'-CAAGCACAGGAGGAGTTACAG-3'; reverse: 5'-CTGGTCTTCCAAACAGACAATG-3') using the PIXO Real-Time PCR (Helixis). Relative quantifications of gene expression were normalized against 18S expression (forward: 5'-CGCCGCTAGAGGTGAAATTC-3'; reverse: 5'-TTGGCAAATGCTTTCGCTC-3').

**Immunohistochemistry.** Three-micron sections from paraffin-embedded lung tissue from patients with COPD and control subjects (Table 2) were deparaffinized, and antigen retrieval was performed in a Pascal pressure cooker (DakoCytomation) using preheated 0.1 mM Tris·HCl buffer for 15 min at 125°C. Endogenous peroxidase was blocked with 0.3% H2O2 in PBS for 30 min. Mouse monoclonal antibodies against AKAP5 (sc-17772, Santa Cruz Biotechnology; 1:25), AKAP12 (ab87067, Abcam; 1:25), or Ezrin [3C12] (ab4069, Abcam; 1:25) in PBS containing 1% BSA were used as a first antibody and incubated overnight at 4°C. Incubation time of the secondary antibody (rabbit-H9251-mouse, 1:100) and third antibody (goat-H9251-rabbit, 1:100) in 1% human antibody serum in 1% BSA/PBS was 30 min. Visualization was performed by 0.1% 3,3'-diaminobenzidine staining for 10 min. Sections were counterstained with hematoxylin for 3 min. Semiquantitative evaluation of the intensity of the staining in ASM or total tissue was performed by two persons by independent identification of the intensity of the staining in four classes: 0, no; 1, low; 2, medium; and 3, strong.

**Measurements of IL-8 and cell viability.** IL-8 was measured in cell supernatants using an ELISA kit (PeliKine compact; Sanquin) according to the manufacturer’s instructions. After removal of the media, the cells were washed twice with PBS, and viability was determined using the AlamarBlue method (36, 44).

**Immunofluorescence.** Cells were seeded on coverslips, after 24 h put to serum-free conditions overnight, and stimulated for 2 h. After stimulation, cells were fixed in 3% paraformaldehyde (PFA) for 15 min; subsequently 3% PFA and 0.3% Triton X-100 were added. Blocking was performed (1% BSA and 2% donkey serum in CytoTBS-T for 1 h) followed by incubation in a humidifying chamber with the primary antibody against p65 (1:20 in blocking solution) at 4°C. Nuclei were stained with a Hoechst staining (1:10,000). ProLong Gold antifade reagent was added to mount the cells before cells were visualized. Colocalization of p65 with nuclear staining was quantified using Tissuefaxs (TissueGnostics).

**Data processing and statistical analysis.** Normal distribution was determined using the Shapiro-Wilk test, and, if normal distribution was determined, differences between 2 groups were compared by paired sampled t-test.
was determined, differences between two groups were compared by paired sampled \( t \)-test, when looking at cell culture experiments, unless stated otherwise. A Kruskal-Wallis one-way ANOVA followed by Mann-Whitney \( U \)-test was used when comparing patient material. A two-tailed \( P \) value \(<0.05\) was considered statistically significant. All tests were performed using SPSS 22.0 for Windows.

**RESULTS**

To investigate AKAP expression in hTERT ASM cells, we initially carried out RII overlay assays. Protein samples from the cells were separated by SDS-PAGE, blotted onto nitrocellulose, and overlaid with radioactively labeled regulatory RII subunits of PKA that bind to AKAPs on the nitrocellulose membranes (14, 17, 18) (Fig. 1A). Preincubation of RII subunits with the peptide AKAP18-L314E (L314E), which, like the st-Ht31 peptide (17), binds with subnanomolar affinity to the AKAP-binding site of RII subunits to abolish AKAP-RII interactions, abrogated binding of RII to AKAPs (Fig. 1A, right). This confirms that AKAP detection carried out in the

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**Fig. 4.** Expression of AKAP12 in patients with COPD and control subjects. AKAP12 protein expression was analyzed by immunohistochemistry. A representative picture from a control patient’s lung is shown (A). Bars indicate 100 \( \mu \)m. B: semiquantification of expression in the ASM of COPD vs. control patients. See Table 2 for details of the patients; all individuals were ex-smokers. White arrows indicate ASM. Results are expressed as individual data points for each patient with the median of the separate experiments.

**Fig. 5.** Expression of Ezrin expression in lung tissue of patients with COPD and control subjects. Ezrin protein expression was analyzed by immunohistochemistry in lung tissue from patients with COPD and control subjects. A: representative images are shown. Bars indicate 200 \( \mu \)m. B: semiquantification of the signals for patients with COPD vs. control subjects was independently performed in duplicate by 2 persons. See Table 2 for details of the patients; all individuals were ex-smokers. Results are expressed as individual data points for each patient with the median of the separate experiments, \( *P < 0.05\), control patients vs. patients with COPD using a Kruskal-Wallis 1-way ANOVA followed by Mann-Whitney \( U \)-test.
presence of the inactive control peptide, AKAP186-PP, was specific (Fig. 1A, left). The molecular weights of the detected proteins correspond to those of several known AKAPs, and subsequent Western blotting identified AKAP5, AKAP8, AKAP9, AKAP12, and Ezrin in hTERT ASM cells (Fig. 1B). The RII overlay showed that exposure to CSE altered the AKAP protein pattern with most AKAP signals being reduced (Fig. 1A). Using immunoblotting, the effects of CSE exposure on the protein expression of the identified AKAPs were compared (Fig. 1C). AKAP5, AKAP9, and AKAP12 were decreased, whereas Ezrin expression was increased, and AKAP8 was not affected. Immunofluorescence microscopic analyses revealed similar effects of CSE on AKAP5, AKAP12, and Ezrin levels (Fig. 1D).

To assess whether similar changes in AKAP expression are also seen upon long-term cigarette smoke exposure in patients with COPD, we studied mRNA expression of the different AKAPs in lung tissue. We observed that the mRNA levels of...
AKAP5 (Fig. 2A) and AKAP12 (Fig. 2B) were lower in patients with COPD with both GOLD stages II and IV compared with control subjects. No significant differences in AKAP5 or AKAP12 expression were seen between patients with GOLD stage II and IV. To study whether the reduced mRNA levels of AKAP5 and AKAP12 in lung tissue of patients with COPD could be the result of cigarette smoke exposure, we studied the effect of CSE on AKAP5 and AKAP12 mRNA levels in primary ASM cells. Indeed, in all primary ASM cell lines tested, AKAP5 and AKAP12 mRNA was reduced upon CSE exposure (Fig. 2C).

We next assessed AKAP protein immunoreactivity in lung sections from patients with stage II and stage IV COPD and control subjects, all of which are ex-smokers (Table 2). Similar to AKAP9 expression (25), AKAP5, AKAP12 (Figs. 3A and 4A), and Ezrin (Fig. 5) exhibited prominent immunoreactivity in the epithelium. In the ASM layer, Ezrin was not detected (received a score of 0), but strong immunoreactivity for AKAP5 and AKAP12 was evident (Figs. 3A and 4A, white arrows). AKAP5 in the ASM showed a decrease in the COPD stage II vs. control tissue, whereas no significant difference was observed between control specimens and COPD stage IV tissue (P < 0.05 and P = 0.09 respectively, Fig. 4A). AKAP12 was not significantly different in the COPD stages II and IV vs. controls (Fig. 4B).

We next examined functional implications for altered AKAP expression in CSE-exposed ASM cells and ASM from lungs of subjects with COPD. In agreement with our previous findings (26), CSE induced IL-8 release from hTERT ASM cells, with the levels in cell culture supernatant being increased by 77 ± 21% compared with basal levels in untreated cultures (P < 0.01, Fig. 6, A and B). Cell viability under these conditions was unaffected (Fig. 6, C and D). We found that CSE-induced IL-8 release was dose dependently attenuated by the β2-agonist, fenoterol, with a maximum reduction of 50% being reached with 0.1 μM fenoterol (P < 0.01; Fig. 6A). The PKA activator 6-Bnz (500 μM) also reduced CSE-induced IL-8 release by about 50% (Fig. 6B). Similarly, as we observed previously (26), selective pharmacological activation of Epac with 8-pCPT (100 μM) decreased CSE-induced IL-8 release (P = 0.07, Fig. 6B).

To determine the role of AKAP-PKA interactions in CSE-induced IL-8 release and in the inhibitory effects of the β2-agonist fenoterol, the PKA activator 6-Bnz, and the Epac activator 8-pCPT, we next measured the impact of the cell-permeant PKA-anchoring disruptor peptide, st-Ht31. The control peptide st-Ht31P could not be used as a control because it caused death of the ASM cells (Fig. 6E), which is in line with observations in human bronchial epithelial cells (25). Treatment with st-Ht31 (50 μM) did not affect viability of the

Fig. 7. Disruption of AKAP-PKA interactions does not affect the NF-κB pathway. With the use of immunofluorescence, nuclear translocation of p65 was visualized after 2 h of exposure to 15% CSE in the absence or presence of st-Ht31 using TNF-α as a positive control. Representative pictures of 3 independent experiments are shown. Bars indicate 10 μm.
hTERT ASM under any condition (Fig. 6, C and D), whereas it caused a small but significant increase of IL-8 release under basal conditions and markedly augmented IL-8 release from CSE-exposed ASM cells \( (P < 0.05 \text{ both; Fig. 6, A and B}) \). In addition, disrupting AKAP-PKA interactions with st-H31 significantly reversed the inhibitory effects of the \( \beta_2 \)-agonist fenoterol on CSE-induced IL-8 release by about 50\% (Fig. 6A), and the presence of fenoterol could not prevent the augmentation of IL-8 release that was induced by st-H31 in CSE-exposed cultures. We also studied the effect of the peptide in the presence of the PKA activator 6-Bnz, which fully activates PKA throughout the cell beyond any compartmental restrictions. 6-Bnz significantly reduced IL-8 release, and disruption of AKAP-PKA interactions with st-H31 did not reverse the suppressive effects of 6-Bnz on CSE-induced IL-8 release (Fig. 6B); this further supports the notion that AKAP-PKA interactions are required for the inhibitory effect of cAMP on IL-8. In contrast, we observed that increased IL-8 release induced by st-H31 in CSE-exposed cells was refractory to treatment with the Epac activator, 8-pCPT (Fig. 6B).

IL-8 release induced by CSE involves nuclear translocation of the NF-κB subunit, p65, which is regulated by Epac (26). In a manner similar to TNF-α (used as positive control), CSE caused nuclear translocation of p65 (Fig. 7). As seen for IL-8 release, CSE-induced p65 nuclear translocation was unaffected by the addition of st-H31 (Fig. 7, quantifications not shown). PKA activity, as measured by VASP phosphorylation (26), was not affected by st-H31, suggesting that disruption of AKAP-PKA complexes by st-H31 does not profoundly alter basal or CSE-induced total cellular PKA activity (Fig. 8A). Interestingly, CSE alone did induce a significant increase in VASP phosphorylation (Fig. 8A). There is evidence that direct activation of PKA attenuated CSE-induced IL-8 release by inhibiting ERK1/2 phosphorylation (26). In line with the effect of st-H31 to increase basal IL-8 release, we also observed that st-H31 increased basal phosphorylation of ERK1/2 (Fig. 8B). CSE-induced phosphorylation of ERK1/2 was not statistically significantly affected by st-H31. However, the peptide did give a trend toward preventing the inhibitory effect of fenoterol on CSE-induced ERK1/2 phosphorylation (Fig. 8B, \( P = 0.078 \)). This was similar to the effect of st-H31 without CSE stimulation (Fig. 8B). Collectively, these data suggest an important role for AKAP-PKA interactions in the regulation of ASM-mediated inflammatory responses.

**DISCUSSION**

In the present study, we describe the expression of a subset of AKAPs that are differently affected by CSE exposure in ASM cells. In particular, the expression level of AKAP5, which is an important regulator of \( \beta_2 \)-AR sensitivity \( (5, 11, 16, 32) \), was significantly reduced, suggesting a mechanism that could link cigarette smoking and COPD pathogenesis (39). We show that mRNA for AKAP5 and AKAP12 is reduced in lung tissue obtained from patients with COPD, and the use of immunohistochemistry allowed us to observe a significant decrease of AKAP5 protein in COPD stage II lung specimens. Disruption of AKAP-PKA interactions with st-H31 increased basal and CSE-induced IL-8 release from ASM cells and prevented the inhibitory effect of the \( \beta_2 \)-agonist fenoterol, on IL-8 secretion, i.e., prevented the anti-inflammatory effect of fenoterol. This indicates that the inhibitory effect of fenoterol at least in part depends on AKAP-PKA interactions. Inhibition of AKAP-PKA interactions is associated with a disruption of the inhibitory action of fenoterol on ERK phosphorylation. The data indicate that CSE-induced downregulation of AKAPs may promote airway inflammation and may reduce the regulatory effect of \( \beta_2 \)-agonists on CSE-induced airway inflammation. Our study is the first to link AKAPs to cigarette smoke-evoked inflammatory responses in ASM cells.

Previously, we had demonstrated that CSE-induced IL-8 release from human ASM cells is prevented by treatment with fenoterol as well as by selective activation of the cAMP effectors PKA and Epac (26). Epac activation had less pronounced effects on CSE-induced IL-8 release compared with PKA activation. The more limited effectiveness of Epac activation with 8-pCPT is most likely attributable to a reduction of
Etap1 expression that can be induced by CSE (26, 27). We reported earlier that activation of PKA inhibits CSE-induced IL-8 release by suppressing ERK1/2 phosphorylation (26). To analyze the potential involvement of AKAPs in the reduction of CSE-induced IL-8 release by fenoterol/PKA, we used the AKAP-PKA interaction inhibitor st-Ht31, demonstrating that AKAPs are required for spatial coordination of PKA activity that suppresses ERK1/2 via β2-adrenoceptors. CSE-induced phosphorylation of ERK1/2 was not affected by st-Ht31, possibly because the phospho-ERK1/2 level was already at its maximum. With the observation that, in contrast to ERK1/2 phosphorylation, CSE-induced IL-8 release is further enhanced with the addition of st-Ht31, it is possible that another AKAP-PKA-sensitive pathway is involved in IL-8 release. For example, other studies have identified that p38 and JNK-induced signaling pathways can regulate the release of IL-8 (42) and that these kinases could also be affected by PKA.

A role for AKAPs in coordinating the duration of cAMP production after stimulation of the β2-adrenoceptor has been shown in several cell types, including ASM cells transfected with a cyclic nucleotide gated ion channel-based reporter (15). In that study, disruptors of AKAP-PKA interactions, Ht31 and AKAP-JS, did not cause a change in whole cell cAMP accumulation after stimulation with the β2-agonist isoproterenol or forskolin, a direct activator of cAMP-producing adenyl cyclases (31). However, the duration of the transient local cAMP signal measured at the plasma membrane was significantly sustained by AKAP-PKA interaction disruptors (15). It was shown that AKAP12 is responsible for resensitization and recycling of the β2-adrenoceptor after treatment with isoproterenol, whereas AKAP5 is responsible for PKA-mediated β2-adrenoceptor phosphorylation and subsequent switching from the cAMP pathway to β-arrestin-ERK1/2 signaling (5, 11, 22, 32, 39). Therefore, the altered expression of AKAP5 after exposure to CSE and in COPD might have important consequences for ERK1/2 in IL-8 signaling.

We observed that CSE causes a downregulation of AKAP5 and AKAP12 mRNA and protein in both immortalized and primary ASM cells, suggesting a novel mechanism for CSE influences on β2-adrenoceptor-directed cell functions. Because st-Ht31 reduces the anti-inflammatory effects of fenoterol, CSE-induced alterations in AKAP expression may contribute to increased IL-8 release from ASM. We also observed the downregulation of AKAP5 and AKAP12 mRNA in lung tissue from patients with COPD, suggesting roles of these AKAPs in β2-adrenoceptor responses. Previously, it was observed in BeWo trophoblast cells that a common pathway may regulate expression of AKAP5 and AKAP12 protein and mRNA, which is different from that of AKAP6 mRNA regulation (9). Along these lines, in the present study, we observed that CSE reduced AKAP5 and AKAP12 mRNA expression, whereas AKAP8 was not affected.

In addition to ASM, we observed high expression of AKAP5 and AKAP12 in airway epithelium. Recently, we showed that (25), in contrast to our present findings in ASM cells, expression of AKAP5 and AKAP12 is not significantly altered by CSE in bronchial epithelial cells in vitro. In bronchial epithelial cells, AKAPs (presumably AKAP9) are involved in maintaining cell-cell contacts and the epithelial barrier function by interaction with the adhesion molecule E-cadherin (25). The observed dysregulation of AKAP expression after CSE exposure could contribute to an increased inflammatory response, as seen in COPD (1). In line with this, we report here that disturbing AKAP functioning using st-Ht31 increased the IL-8 release.

Dysfunction of cAMP compartimentalization and local PKA signaling occurs in various diseases including cardiac and neurological diseases (10, 32, 41), and here we show that this also may play a role in COPD. The observed dysregulation of the expression of AKAPs that are important in β2-adrenoceptor regulation may pave the way to novel pharmacological approaches for the treatment of COPD. In summary, the present study demonstrates that AKAPs, in particular their interactions with PKA, are involved in the regulation of proinflammatory responses, specifically IL-8 release by ASM. In addition, we show that AKAP5 and AKAP12, which regulate β2-adrenoceptor sensitivity, are dysregulated upon CSE exposure and in patients with COPD. Therefore, there is a potential for regulating inflammatory responses and possibly β2-adrenoceptor functioning by pharmacological targeting of AKAPs.

**REFERENCES**


