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

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Running head: Anti-inflammatory role of AKAPs in airway smooth muscle

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**Abstract**

β₂-agonists inhibitors can relieve chronic obstructive pulmonary disease (COPD) symptoms by stimulating cyclic AMP (cAMP) signaling. A-kinase anchoring proteins (AKAPs) compartmentalize cAMP signaling by establishing protein complexes. We previously reported that the β₂-agonist fenoterol, direct activation of protein kinase A (PKA) and exchange factor directly activated by cAMP (Epac) decrease cigarette smoke extract (CSE)-induced release of neutrophil attractant interleukin-8 (IL-8) from human airway smooth muscle (ASM) cells. In the current study we tested the role of AKAPs in CSE-induced IL-8 release from ASM cells and assessed the effect of CSE on the expression levels of different AKAPs. We also studied mRNA and protein expression of AKAPs in lung tissue from COPD patients. Our data show that CSE exposure of ASM cells decreases AKAP5 and AKAP12, both capable of interacting with β₂-adrenoceptors. In lung tissue of COPD patients, mRNA levels of AKAP5 and AKAP12 were decreased compared to lung tissue from controls. Using immunohistochemistry, we detected less AKAP5 protein in ASM of COPD GOLD stage II patients compared to control subjects. St-Ht31, which disrupts AKAP-PKA interactions, augmented CSE-induced IL-8 release from ASM cells and diminished its suppression by fenoterol, an effect mediated by disturbed ERK signaling. The modulatory role of AKAP-PKA interactions in the anti-inflammatory effects of fenoterol in ASM cells and the decrease in expression of AKAP5 and AKAP12 in response to cigarette smoke and in COPD patient lungs suggests that cigarette smoke-induced changes in AKAP5 and AKAP12 in COPD patients may affect efficacy of pharmacotherapy.
Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by airway inflammation, with among others neutrophils, mucus hypersecretion, airway remodeling and by 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 pro-inflammatory functions, and through this contribute to local chronic airway inflammation in COPD (3, 6, 28, 29). Though anti-inflammatory effects of β₂-agonists in vitro are established (13, 20), the evidence for this in vivo is still under debate (34).

Cyclic AMP induces its biological actions by activating various effectors, including protein kinase A (PKA) and exchange protein directly activated by cAMP (Epac) (36, 40). Cyclic AMP 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 disruptor peptides, Ht31 and AKAP-IS, in human ASM cells extended the duration of the $\beta_2$-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 $\beta_2$-adrenoceptor (5, 11, 16, 32, 39). Thus we hypothesized that alterations in AKAP5 and AKAP12 expression may affect the anti-inflammatory effects of $\beta_2$-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 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 $\beta_2$-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, immortalized by stable ectopic expression of human telomerase reverse transcriptase (hTERT) as described previously (12) were used at passages 20-29. Primary human airway smooth muscle cells obtained as described previously (35) were used at passage 1-5. The cells were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM) 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 Non-Essential 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 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 COPD GOLD stage II patients was derived from non-diseased 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 Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria (33). Tissue from GOLD IV patients was obtained from subjects undergoing surgery for lung transplantation. All tissue was collected according to the Research Code of the University Medical Center Groningen (http://www.rug.nl/umcg/onderzoek/researchcode/index) and 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, Rotterdam, The Netherlands) 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 hours. Cells were pre-treated with 50 μM st-Ht31 (Promega, Madison, MT, USA) to disrupt AKAP-PKA interactions (4, 18), for 20 min prior to stimulation with 15% CSE in serum-free medium. Control cultures were pre-treated with vehicle alone. When used, fenoterol (0.001 – 0.1 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 minutes after the addition of st-Ht31. The action of these cAMP analogues has previously been characterized in this system (29). Cells were stimulated with 15% CSE for different time points: 10 min for VASP phosphorylation, 1 h for ERK phosphorylation, 2 h for nuclear factor-κB (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 (Figure 6E), rendering st-Ht31P invalid as a control in this set-up, 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 (5% milk powder, 0.1% BSA, 0.1% sodium azide in PBS) for three hours at room temperature. The membranes were incubated with [γ^{32}P]-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^{32}P]-RII subunits was visualized by autoradiography using a STORM 830 Scanner (Molecular Dynamics).
**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 (v/v), 0.1% sodium dodecyl sulphate (w/v), 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, 1:1000 for the VASP antibody, 1:2000 for ERK antibodies) was done overnight at 4 °C. Blots were incubated with secondary antibody (1:2000 A9044 or A0545; Sigma-Aldrich). Signals were detected with chemiluminescence reagents according to the manufacturer's protocol. GAPDH (1:2000; sc-47724; Santa Cruz Biotechnology) served as a control for equal loading. Blots were quantified using ImageJ.

**Real time PCR** - After 24 hours, 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 template for quantitative real time PCR for AKAP5 (Forward; 5'-GACGCCCTACGTTGATCT-3', Reverse; 5'-GAAATGCCCAGTTTCTCTATG-3'), and AKAP12 (Forward; 5'-CAAGCACCAGGAGTTACAG-3', Reverse; 5'-CTGGTCTTCCAAACAGACAAATG-3') using the PIXO™ Real-Time PCR (Helixis, Carlsbad, CA, USA). Relative quantifications of gene expression were normalized against 18S expression (Forward; 5'-CGCCGCTAGAGGTGAAATTC-3', Reverse; 5'-TTGGCAAATGCTTTCGCTC-3').

**Immunohistochemistry** – 3 micron sections from paraffin embedded lung tissue from COPD patients and control subjects (Table 2) were deparaffinized and antigen retrieval was performed in a Pascal pressure cooker.
(DakoCytomation, Inc. Carpinteria, USA) using preheated 0.1 mM Tris/HCl buffer for 15 minutes at 125 °C. Endogenous peroxidase was blocked with 0.3% H₂O₂ in PBS for 30 minutes. Mouse monoclonal antibodies against AKAP5 (sc-17772, Santa Cruz; 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-α-mouse 1:100) and third antibody (goat-α-rabbit 1:100) in 1% Human AB serum in 1% BSA/PBS was 30 minutes. Visualization was performed by 0.1% 3,3'-diaminobenzidine staining for 10 minutes. Sections were counterstained with haematoxylin for 3 minutes. Semi-quantitative 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, Amsterdam, The Netherlands) 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, and after 24 hours put to serum free conditions overnight, they were stimulated for 2 hours. After stimulation cells were fixed in 3% paraformaldehyde (PFA) for 15 minutes, 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 incubating 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 (1:10000). ProLong Gold antifade reagent was added to mount the cells before cells were visualized. Co-localization of p65 with nuclear staining was quantified using Tissuefaxes (TissueGnostics GmbH).

**Data processing and statistical analysis** - Normal distribution was determined using the Shapiro Wilk Shapiro–Wilk test and if normal distribution 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 analysis of variance followed by Mann-Whitney U test was used when comparing patient material. A two tailed \( p \)-value <0.05 was considered as statistically significant. All tests were performed using SPSS 22.0 for Windows.
Results

In order 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 labelled regulatory RIIα subunits of PKA that bind to AKAPs on the nitrocellulose membranes (14, 17, 18); Figure 1A). Pre-incubation of RIIα subunits with the peptide AKAP18δ-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 RIIα to AKAPs (Figure 1A, right panel). This confirms that AKAP detection carried out in the presence of the inactive control peptide, AKAP18δ-PP, was specific (Figure 1A, left panel). 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 (Figure 1B). The RII overlay showed that exposure to CSE altered the AKAP protein pattern with most AKAP signals, being reduced (Figure 1A). Using immunoblotting, the effects of CSE exposure on the protein expression of the identified AKAPs were compared (Figure 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 (Figure 1D).

To assess whether similar changes in AKAP expression are also seen upon long-term cigarette smoke exposure in COPD patients, we studied mRNA expression of the different AKAPs in lung tissue. We observed that the mRNA levels of AKAP5 (Figure 2A) and AKAP12 (Figure 2B) were lower in COPD patients with both GOLD stages II and IV compared to control subjects. No significant differences in AKAP5 or AKAP12 expression were seen between GOLD stage II and IV patients. To study whether the reduced mRNA levels of AKAP5 and AKAP12 in lung tissue of COPD patients 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 (Figure 2C).

We next assessed AKAP protein immunoreactivity in lung sections from stage II and stage IV COPD patients and control subjects, all of which are ex-smokers (Table 2). Similar to AKAP9 expression (25), AKAP5, AKAP12 (Figure 3A & 4A) and Ezrin (Figure 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 (Figure 3A & 4A, white arrows). AKAP5 in the ASM showed a decrease in the COPD stage II versus control tissue, whereas no significant difference was observed between control specimens and COPD stage IV tissue (p<0.05 and p=0.09 respectively, Figure 4A). AKAP12 was not significantly different in the COPD stages II and IV versus controls (Figure 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 to basal levels in untreated cultures (P<0.01, Figure 6A & B). Cell viability under these conditions was unaffected (Figure 6C & 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; Figure 6A). The PKA activator 6-Bnz (500 μM) also reduced CSE-induced IL-8 release by about 50% (Figure 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, Figure 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, since it caused death of the ASM cells (Figure 6E), which is in line with observations in human bronchial epithelial cells.
Treatment with st-Ht31 (50 μM) did not affect viability of the hTERT ASM under any condition (Figure 6C & D), while 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 both; Figure 6A & B). In addition, disrupting AKAP-PKA interactions with st-Ht31 significantly reversed the inhibitory effects of the β2-agonist fenoterol on CSE-induced IL-8 release by about 50% (Figure 6A), and the presence of fenoterol could not prevent the augmentation of IL-8 release that was induced by st-Ht31 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-Ht31 did not reverse the suppressive effects of 6-Bnz on CSE-induced IL-8 release (Figure 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-Ht31 in CSE-exposed cells was refractory to treatment with the Epac activator, 8-pCPT (Figure 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 TNFα (used as positive control), CSE caused nuclear translocation of p65 (Figure 7). As seen for IL-8 release, CSE-induced p65 nuclear translocation was unaffected by the addition of st-Ht31 (Figure 7, quantifications not shown). PKA activity, as measured by VASP phosphorylation (26) was not affected by st-Ht31 suggesting that disruption of AKAP-PKA complexes by st-Ht31 does not profoundly alter basal or CSE-induced total cellular PKA activity (Figure 8A). Interestingly, CSE alone did induce a significant increase in VASP phosphorylation (Figure 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-Ht31 to increase basal IL-8 release, we also observed that st-Ht31 increased basal phosphorylation of ERK1/2 (Figure 8B). CSE-induced phosphorylation of ERK1/2 was not statistically significantly affected by st-Ht31. However, the
peptide did give a trend towards preventing the inhibitory effect of fenoterol on CSE-induced ERK1/2 phosphorylation (Figure 8B, \( p=0.078 \)). This was similar to the effect of st-Ht31 without CSE stimulation (Figure 8B). Collectively, these data suggest an important role for AKAP-PKA interactions in the regulation of ASM mediated inflammatory responses.

**Discussion**

In the current 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 COPD patients, and using immunohistochemistry observed a significant decrease of AKAP5 protein in COPD stage II lung specimens. Disruption of AKAP-PKA interactions with st-Ht31 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 fenoterol's anti-inflammatory effect. 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 to PKA activation. The more limited effectiveness of Epac activation with 8-pCPT is most likely due to a reduction of Epac1 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-IS, did not cause a change in whole cell cAMP accumulation after stimulation with the β2-agonist isoprenaline or forskolin, a direct activator of cAMP producing adenylyl 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.
Since 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 COPD patients, 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 AKAP8 mRNA regulation (9). Along these lines, in the current study, we observed that CSE reduced AKAP5 and AKAP12 mRNA expression, while AKAP8 was not affected.

In addition to ASM, we observed high expression of AKAP5 and AKAP12 in airway epithelium. Recently, we showed (25), in contrast to our current findings in ASM cells, that 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 compartmentalization and local PKA signaling occurs in various diseases including cardiac and neurological diseases (10, 32, 41), and here we show 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 current study demonstrates that AKAPs, in particular their interactions with PKA, are involved in the regulation of pro-inflammatory 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 COPD patients. Therefore, there is a potential for regulating inflammatory responses and possibly β2-adrenoceptor functioning by pharmacological targeting of AKAPs.
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Disclosures

None.

Author contributions

WJP designed and performed experiments, drafted the manuscript and contributed to the experimental and conceptual design. IHH contributed to the design of the study, scientific discussion and helped to draft the manuscript. LJH performed experiments. PS assisted in designing experiments. EK contributed to the experimental and the conceptual design. AJH provided the immortalized cells used for this study. WT provided the patient material used for this study and contributed to the scientific discussion. HM contributed to the experimental and conceptual design. MS contributed to the experimental and conceptual design and helped to draft the manuscript. All authors have critically reviewed the manuscript and approved its submission.

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on inflammatory gene expression in human ASM cells: a role for protein kinase A.


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**Figure legends:**

**Figure 1:** Cigarette smoke extract (CSE) changes the expression of several AKAPs in human ASM. (A) RII-overlay for the detection of AKAPs before and after 24 h of CSE treatment (15 %). Pre-incubation with peptide AKAP18δ-L314E (L314E), which binds to regulatory RII subunits of PKA and thereby blocks the interaction of the radioactive RII subunits with AKAPs on the membrane, served as a control to confirm the specificity of the signals. The peptide AKAP18δ-PP (PP), which does not bind RII subunits, does not interfere with the interaction of the labeled RII subunits and AKAPs on the membrane. (B) Expression of AKAP5 (AKAP79), AKAP12 (AKAP250, Gravin), AKAP8 (AKAP95), AKAP9 (AKAP450,
AKAP350, Yotiao) and Ezrin (AKAP78) was detected by immunoblotting. (C, D)

AKAP expression was studied by immunoblotting and immunofluorescence before and after treatment with 15% CSE for 24 h. Results are expressed as means ±SEM, n = 3-6 independent experiments. *=p<0.05, **=p<0.01 compared to unstimulated controls. Normal distribution was determined using the Shapiro Wilk Shapiro–Wilk test and if normal distribution was determined, differences between two groups were compared by paired sampled T-test.

**Figure 2: Expression of AKAP5 and AKAP12 mRNA is altered in COPD patients and by CSE in vitro.** Expression of (A) AKAP5 and (B) AKAP12 was measured in lung tissue of subjects without COPD, COPD GOLD stages II or IV (n=5 each group). See Table 1 for characteristics of the subjects. (C) Gene expression of AKAP5 and AKAP12 were measured in primary airway smooth muscle cells after exposure to 15% CSE for 24 h, each data point reflects a separate donor. Results are expressed as individual data points with the median of the separate experiments. *=p<0.05, **=p<0.01 compared to unstimulated control subjects (A & B) or basal controls (C). Normal distribution was determined using the Shapiro Wilk Shapiro–Wilk test and if normal distribution was determined, differences between two groups were compared by paired sampled T-test.
Figure 3: Expression of AKAP5 in lung tissue from COPD patients and control subjects. AKAP5 protein expression was analyzed by immunohistochemistry. A representative image from a control patient's lung is shown (A). Bars indicate 100 µm. (B) Semi-quantification of expression in the ASM of COPD versus control patients. See Table 2 for details of the patients, all individuals were ex-smokers. White arrows indicate airway smooth muscle. Results are expressed as individual data points for each patient with the median of the separate experiments, *=p<0.05, control patients versus COPD patients using a Kruskal–Wallis one-way analysis of variance followed by Mann-Whitney U test.

Figure 4: Expression of AKAP12 in COPD patients 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 µm. (B) Semi-quantification of expression in the ASM of COPD versus control patients. See Table 2 for details of the patients, all individuals were ex-smokers. White arrows indicate airway smooth muscle. Results are expressed as individual data points for each patient with the median of the separate experiments.

Figure 5: Expression of Ezrin expression in lung tissue of COPD patients and control subjects. Ezrin protein expression was analyzed by immunohistochemistry in lung tissue from COPD patients and control subjects. (A) Representative images are shown. Bars indicate 200 µm. (B) Semi-quantification of the signals for COPD patients versus control subjects was independently performed in duplicate by two 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 versus COPD patients using a Kruskal–Wallis one-way analysis of variance followed by Mann-Whitney U test.
Figure 6: AKAPs coordinate CSE-induced IL-8 release from hTERT ASM. (A and B) IL-8 release was measured using ELISA after 24 h exposure to 15% CSE in the absence or presence of st-Ht31, fenoterol or 6-Bnz-cAMP or 8-pCPT-2’-O-Me-cAMP as indicated. (C & D) Cell viability was tested after 24 h using alamarBlue®. (E & F) The cells were incubated with the peptide st-Ht31P in the indicated concentrations for 24 h (n=3). The AKAP-PKA interaction disruptor peptide st-Ht31 (50 µM) was added 20 min. before the addition of fenoterol, 8-pCPT or 6-Bnz, CSE was added 25 min. after this (n=4-15). *=p<0.05, **=p<0.01, ***=p<0.001 compared to unstimulated control or as indicated. Normal distribution was determined using the Shapiro Wilk Shapiro–Wilk test and if normal distribution was determined, differences between two groups were compared by paired sampled T-test.

Figure 7: Disruption of AKAP-PKA interactions does not affect the NF-κB pathway. Using 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.

Figure 8: Disruption of AKAP-PKA interactions inhibits fenoterol-induced inhibition of ERK phosphorylation without affecting PKA activation. (A) PKA activity was determined using the phosphorylation of VASP at Ser157 after 10 min exposure to 15% CSE in the absence or presence of st-Ht31. (B) Phosphorylation of ERK1/2 was visualized after 1 h of 15% CSE exposure in the absence or presence of st-Ht31. Protein phosphorylation was corrected for total protein loaded in each lane either by taking into account the ratio of the density of the phosphorylated-protein against the sum of the phosphorylated and non-phosphorylated protein (A) or the ratio of the phosphorylated protein against total protein (B). *=p<0.05, **=p<0.01 statistically significant differences, compared to
unstimulated control. Normal distribution was determined using the Shapiro Wilk
Shapiro–Wilk test and if normal distribution was determined, differences between
two groups were compared by paired sampled T-test.
**Table 1: Characteristics of the study objects used for the gene expression.**

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<0.01, ***=p<0.001 compared to control group. *=1 data point unknown, ++2 data points unknown. Values 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.

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<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>
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<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>
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Table 2: Characteristics of the control persons and patients whose lung tissues were used for the immunohistochemistry. All values except number of subjects and gender are expressed as median values with minimum and maximum range in parentheses. Only ex-smokers were included. FEV1% predicted: forced expiratory volume in 1 second as percentage of predicted value. FVC: forced vital capacity. *p<0.05, ***p<0.001 compared to control group, $p<0.05, $$$p<0.001 compared to COPD stage II (Mann-Whitney U test). + 1 data point unknown, ++ 2 data points unknown

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<td>63$^{+++}$ (45.7-64.1)$^+$</td>
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Figure 1:

A  RII overlay

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PP

L314E

B  Immunoblot

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C  Protein expression after CSE exposure

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D  Immunofluorescence

Basal

CSE
Figure 4:

AKAP12

A

B  ASM quantification

AKAP12 intensity class

Control  COPD II  COPD IV

100 μm
Figure 6:

A IL-8 release – β₂-agonist

B IL-8 release – cAMP analogues

C Cell viability – β₂-agonist

D Cell viability – cAMP analogues

E Cell viability – st-Ht31P