Cyclic AMP modulates cigarette smoke-induced MMP9/TIMP1 balance in human bronchial epithelial cells

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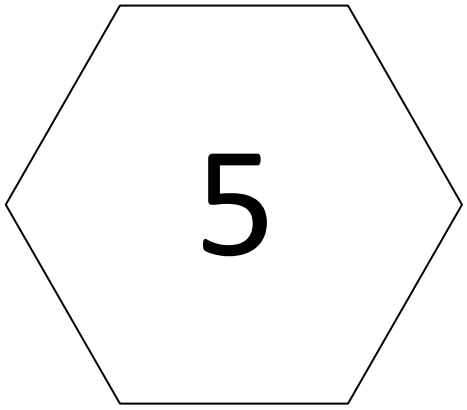

Abstract

Matrix metalloproteases (MMPs) are enzymes involved in the degradation of extracellular matrix (ECM) proteins such as fibronectin and collagen. Tissue inhibitors of metalloproteinases (TIMPs) dampen MMP activity. Lungs of chronic obstructive pulmonary disease (COPD) patients show remodeling including airway fibrosis and emphysema in which MMP and TIMP activity may play a role. The main risk factor for COPD, cigarette smoke, may provoke an imbalance between MMPs and TIMPs. Currently, COPD patients are treated with β_2-agonists known to elevate cyclic AMP (cAMP). As cAMP modulates MMP9 and TIMP1 expression, activity and secretion, we investigated the potential role of the cAMP effectors protein kinase A (PKA) and the exchange protein directly activated by cAMP (Epac) in these effects. Exposure of the human bronchial epithelial cell line 16HBE14o- to cigarette smoke extract (CSE) elevated MMP9 and also the MMP9/TIMP1 ratio. In addition, CSE induced pro-MMP9 as measured by zymography. Direct pharmacological activation of PKA by 6-Bnz-cAMP reduced CSE-induced MMP9 mRNA, whereas the β_2-agonist fenoterol was less effective and no effect was observed when Epac was selectively activated by 8-pCPT-2'-*O*-Me-cAMP. The level of pro-MMP9 was reduced by fenoterol. Pharmacological activation of PKA tended to decrease pro-MMP9, whereas Epac activation was not effective. Our findings suggest that cAMP and its effector PKA reduce CSE-induced pro-MMP9, a process accompanied by downregulation of CSE-induced MMP9 mRNA expression by PKA.

Introduction

In the lung, the extracellular matrix (ECM) is the main constituent of the basement membrane and interstitial connective tissue which provide structural support and act as a physical barrier in tissue compartmentalization (1; 2). The majority of ECM proteins, including collagens, fibronectin, elastin and proteoglycans, are produced by fibroblasts, but can also be produced by other structural lung cells like smooth muscle cells and epithelial cells (3; 4). An imbalance between production and degradation of ECM proteins may result in structural changes in the lung such as emphysema, characterized by excessive degradation of parenchymal ECM (5), and small airway fibrosis characterized by excessive deposition of ECM proteins (6). Both features are characteristics of chronic obstructive pulmonary disease (COPD) (2).

Proteases such as matrix metalloproteases (MMPs) are importantly involved in the tight regulation of the balance between production and degradation of ECM proteins (7). The 24 members of the MMP family are zinc-containing proteases each known to degrade a specific subset of matrix proteins. MMPs are secreted as a pro-MMP and are activated by release of the pro-domain (8). Different MMPs are able to degrade different subsets of protein. MMP9 for example degrades gelatin and all collagens (8; 9). To appropriately control ECM degradation, inhibitors of MMP activity like tissue inhibitors of metalloproteinase (TIMPs) are of central importance (7). An imbalance between MMP and TIMP activities may underpin airway fibrosis as well as emphysema. In COPD patients, some MMPs, including MMP9, are elevated (10), which could explain emphysema. However, the main inhibitor of MMP9, TIMP1 is also elevated in COPD patients (11) making the biological net effect of increased MMP9 more difficult to predict. Next to ECM degradation, MMP9 secretion enhances or inhibits inflammatory cytokine and enzyme levels and may therefore regulate both repair and destruction processes (12). Taken together, it is difficult to predict the overall contribution of the altered expression pattern of MMP9 and TIMP1 in the pathophysiology of COPD, a process which may depend on local expression patterns.

Part of the pharmacotherapy of COPD patients is treatment with β_2-agonists and phosphodiesterase (PDE) inhibitors both of which elevate cAMP by activation of adenylyl cyclase or inhibition of cAMP hydrolysis, respectively (13; 14). In turn, cAMP activates protein kinase A (PKA) or exchange protein directly activated by cAMP (Epac) (15), which may have bronchodilatory and anti-inflammatory effects (1; 16).

Interestingly, cAMP may modulate the balance between MMPs and TIMPs. (Non-) specific PDE inhibitors, adenosine or the cAMP analog 8-Br-cAMP reduce MMP9 and TIMP1 activity, expression or seceretion in several cell types (17-20). In human bronchial epithelial cells, however, a potential link between cAMP and its effectors on one end, and MMP9 as well as TIMP1 on the other end has not been studied yet. As epithelial cells are a source for the secretion of MMP9 and TIMP1 (21), we analyzed in this study the effect of the β_2-agonist fenoterol, the pharmacological PKA activator 6-Bnz-cAMP (22) and the pharmacological Epac activator 8-pCPT-2'-*O*-Me-cAMP (22) on cigarette smoke extract-induced changes in MMP9 (activity and mRNA expression) and in TIMP1 (mRNA expression) in the human bronchial epithelial cell line 16HBE14o-.

Materials & Methods

Materials

Minimual Essential Medium, L-glutamine, fungizone, gentamicin, penicillin/straptavidin and trypsin were obtained from Gibco Life Technologies (Paisley, UK). 3R4F research cigarettes were from University of Kentucky (Lexington, KY, USA). The cAMP analogues, 8-pCPT-2'-*O*-Me-cAMP and 6-Bnz-cAMP were from BIOLOG Life Science Institute (Bremen, Germany). Fenoterol was obtained from Boehringer Ingelheim (Ingelheim, Germany). cDNA synthesis kit was purchased from Promega (Madison, WI, USA). SYBR Green Reagent was from Qiagen (Venlo, the Netherlands). Amicon Ultra-0.5 Centrifugal Filter Devices were from Merck Millipore (Tullagreen Carrigtwohill, Cork, Ireland). Bovine gelatin and Triton X-100 were purchased from Sigma Aldrich (St-Louis, MO, USA).

Human lung tissue

Human lung tissue from healthy controls and COPD patients 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"; htttp://www.federa.org). Patient characteristics are described in Supplement Table 1.

Supplement Table 1: Patient characteristics

	Control	COPD
Number of subjects	4	7
Age (years)	68.8 (55-78)	62.3 (44-81)
Male/female	2/2	4/3
Ex-smoker/current smoker	4/0	6/1
Pack years	23.9 (3-52.5)	41.3 (27-65)
FEV1% predicted	95.3 (75.9-134.0)	27.8 (14.4-53.1)
FEV1%/FVC%	73.5 (71.4-77.0)	35.3 (26.7-53.1)

Cell culture and stimulation

The human bronchial epithelial cell line 16HBE14o- (16HBE) were cultured in Minimal Essential Medium (MEM) supplemented with L-glutamine, fungizone, penicilin/streptavidin and gentamicin as previously described (23; 24). Cells were grown to confluence, trypsinized and equal numbers of cells were plated on collagen and fibronectin coated plates (23; 24). After reaching cell confluence, cells were serum deprived overnight, followed by exposure to different percentages of cigarette smoke extract (CSE) for 6 hrs. 100% CSE was prepared by combusting 2 research cigarettes without a filter through 25 ml of serum deprived medium (24). Prior to CSE exposure, cells were pretreated for 30 minutes with the β_2-agonist fenoterol (0.1 and 1 μM), the specific Epac activator 8-pCPT-2'-*O*-Me-cAMP (100 μM) or the specific PKA activator 6-Bnz-cAMP (500 μM).

PCR

After 6 hrs of cell stimulation, messenger RNA (mRNA) was isolated according to manufacturers instructions (Nucleospin RNA II, Machery Nagel, Düren, Germany). cDNA was prepared from 1 μg RNA and gene expression was analyzed by quantitative real-time PCR (MyiQ™ Single-Color detection system; Bio-Rad Laboratories Inc. Life Science Group, Hercules, CA, USA) and normalized to the ribosomal subunit 18S (see Supplement Table 2 for the primers used).

Supplement Table 2: Primers used for RT-PCR

Primer		Sequence 5'-3'
18S	Forward	CGCCGCTAGAGGTGAAATTC
	Reverse	TTGGCAAATGCTTTCGCTC
Collagen Iα1	Forward	AGCCAGCAGATCGAGAACAT
	Reverse	TCTTGTCCTTGGGGTTCTTG
Fibronectin	Forward	TCGAGGAGGAAATTCCAATG
	Reverse	ACACACGTGCACCTCATCAT
MMP-9	Forward	GAGACCGGTGAGCTGGATAG
	Reverse	TACACGCAGTGAAGGTGAG
TIMP-1	Forward	AATTCCACCTCGTCATCAG
	Reverse	TGCAGTTTTCCAGCAATGAG

Zymography

Gelatin was added to a SDS-Page gel, which is degraded by MMP2 and MMP9 (8). After 6 hrs of cell stimulation, 800 µl of cell supernatant was concentrated to 25 µl using an Amicon Ultra-0.5 Centrifugal Filter Device. Equal volumes were loaded on a 8% SDS Page gel containing 1mg/ml bovine gelatin. Electrophoresis was performed and afterwards gels were washed three times in 2.5 % Trition X-100 for a total of 2.5 hrs. Gels were washed three times in MMP assay buffer (50 mM Tris-HCl pH 7.4, 200 mM NaCl, 5 mM $CaCl_2$, 0.005% Brij35) for an additional 2.5 hrs. Subsequently, gels were incubated in MMP assay buffer for 16 hrs at room temperature to re-activate both pro-MMP9 and active MMP9. Staining of the gel was performed by incubation of the gel in staining solution (0.5% Coomassie Blue G-250 in 30% methanol and 10% acetic acid) for 3 hrs. Gels were washed in destaining solution (30% methanol, 10% acetic acid) until clear bands became visible on the gel (25). As illustrated in Fig. 1E, due to differences in protein weight both pro-MMP9 and active MMP9 are visible on the gel.

Matrix metalloprotease and TIMP ELISA

Quantibody Human MMP array 1 obtained from Ray Biotech, Inc (Norcross, GA, USA) was used to analyze MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, MMP-10, MMP-13, TIMP-1, TIMP-2 and TIMP-4 protein in cell supernatants of human bronchial epithelial cells exposed to CSE (1 or 10%) for 30 min, 20 hrs or 24 hrs. The assay was performed according to manufacturer's instruction using undiluted cell supernatants.

LC-MS/MS

Active MMP9, pro-MMP9 and TIMP1 protein concentrations in cell supernatants were determined by LC-MS/MS as previous prescribed (26).

Statistics

Results are expressed as individual data points. The median is indicated for each group. For all experiments, statistical analysis was performed using a Mann-Whitney Rank Sum test to compare the groups of interest.

Results

CSE increases the MMP9/TIMP1 ratio

Gene expression analysis of MMP9 after stimulation of 16HBE cells with different CSE concentrations revealed that MMP9 mRNA expression is significantly increased after stimulation with 5% CSE (Fig. 1A, $p<0.05$). Although TIMP1 mRNA expression is not significantly altered by CSE exposure, a trend towards a reduction of TIMP1 by 5% CSE is observed (Fig.1B, $p=0.10$). As a consequence, the ratio between MMP9 and TIMP1 mRNA expression is increased after exposure to 5% CSE (Fig. 1C, $p<0.001$). Using an MMP ELISA, we also observed an MMP9 increase and a TIMP1 decrease in cell supernatants after exposure to CSE (data not shown). LC-MSMS was used to determine the amount of TIMP1, pro-MMP9 and active MMP9 protein in cell supernatants. No active MMP9 levels were found in the supernatants under basal conditions or after CSE exposure. However, CSE reduced TIMP1 protein levels (Fig 1D). Consequently, in line with the mRNA results, LC-MSMS revealed an increased pro-MMP9/TIMP1 ratio (Fig. 1D). We identified both active MMP9 and pro-MMP9 by zymography (Fig. 1E). The level of pro-MMP9 was more pronounced compared to the level of active MMP9. While the level of active MMP9 was under the detection level, pro-MMP9 was increased by CSE (Fig. 1F).

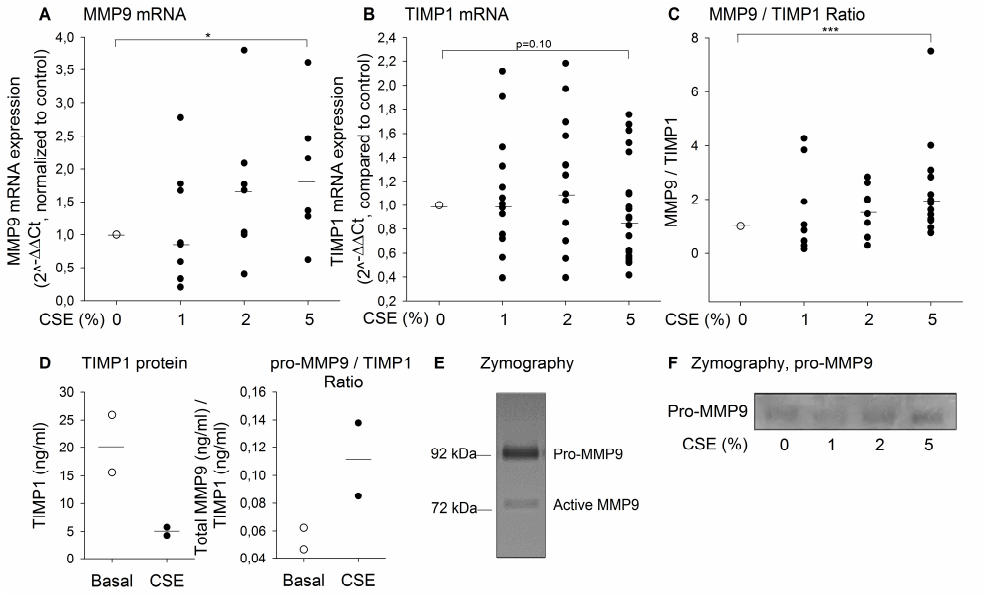

Figure 1: CSE increases the MMP9/TIMP1 ratio. 16HBE cells were exposed to the indicated concentrations of CSE for 6 hrs, followed by analysis of MMP9 and TIMP1 mRNA expression by RT-PCR (A,B), protein expression by LC-MS/MS (D) and zymography (E&F). Calculated MMP9/TIMP1 ratio is shown in (C). In E, both pro-MMP9 and active MMP9 are shown. For A, B, C & E data were presented as separate data points of 7-13 experiments. In D, data of 2 experiments are shown. $^{*}p<0.05$, $^{***}p<0.001$ compared to basal control.

COPD patients

Total lung tissue of control and COPD patients was also analyzed for mRNA expression of MMP9 and TIMP1. Patient characteristics are shown in Supplement Table 1. No statistical differences were observed in MMP9 and TIMP1 mRNA expression between control and COPD patients (Fig. 2A&B). The MMP9/TIMP1 ratio, however, showed a weak trend to a reduction in COPD patients (Fig. 2C, p=0.10).

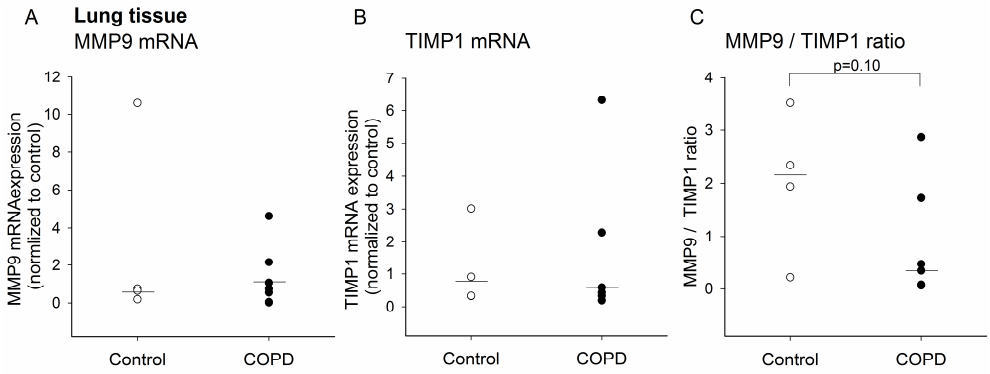

Figure 2: MMP9 (A) and TIMP1 (B) mRNA expression was measured by RT-PCR in lung tissue from control and COPD patients. Calculated MMP9/TIMP1 ratio is shown in (C). Average of all controls was set to 1. Data were presented as separate data points of 3-7 experiments.

Fenoterol reduces pro-MMP9

To analyze the effect of cAMP on CSE-induced MMP9 and TIMP1 alterations in 16HBE cells, the β_2-agonist fenoterol was used. CSE-induced MMP9 mRNA expression tended to decrease by 1 μM fenoterol (Fig. 3A). Fenoterol alone or in combination with CSE did not alter the mRNA expression of ECM proteins fibronectin and collagen I (data not shown), TIMP1 mRNA expression (Fig. 3B) or the MMP9/TIMP1 ratio (Fig. 3C). Interestingly, however, the induction of pro-MMP9 by CSE was reduced by fenoterol, an effect most prominent at a concentration of 1 μM (Fig. 3D).

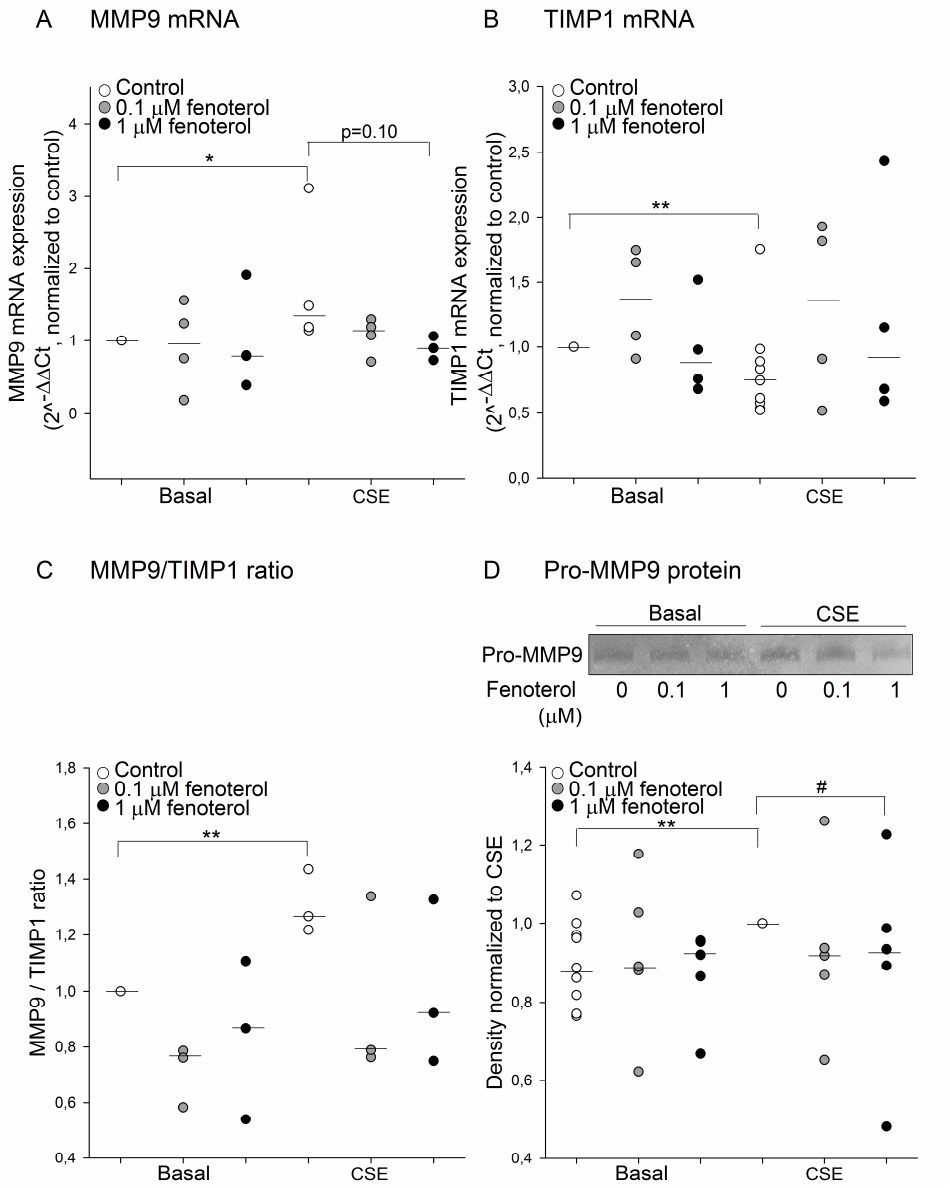

Figure 3: Fenoterol prevents induction of pro-MMP9 by CSE. 16HBE cells were exposed to 5% CSE alone or in combination with the indicated concentrations fenoterol for 6 hrs, followed by analysis of MMP9 (A) and TIMP1 (B) mRNA expression by RT-PCR. MMP9/TIMP1 ratio was calculated (C). Pro-MMP9 was measured by zymography (D). mRNA expression and zymography were presented as separate data points of 4-5 experiments. $^{*}p<0.05$, $^{**}p<0.01$ compared to basal control. $^{\#}p<0.05$ compared to CSE.

Activation of PKA reduces MMP9 mRNA and pro-MMP9 levels

The specific activator of PKA, 6-Bnz-cAMP, significantly prevented the CSE-induced level of MMP9 mRNA and even significantly reversed it towards a reduction (Fig. 4A, $p<0.05$). CSE-induced reduction of TIMP1 mRNA expression was not altered by pharmacological Epac activation by 8-pCPT-2'-*O*-Me-cAMP or by the specific PKA activator 6-Bnz-cAMP (Fig. 4B). The MMP9/TIMP1 ratio was also not altered by both compounds (Fig. 4C). No alterations in fibronectin and collagen I mRNA expression were found after stimulation with CSE, 8-pCPT-2'-*O*-Me-cAMP or 6-Bnz-cAMP (data not shown). Using zymography, 6-Bnz-cAMP tended to decrease CSE-induced pro-MMP9 (Fig. 4D) while 8-pCPT-2'-*O*-Me-cAMP did not alter CSE-induced pro-MMP9.

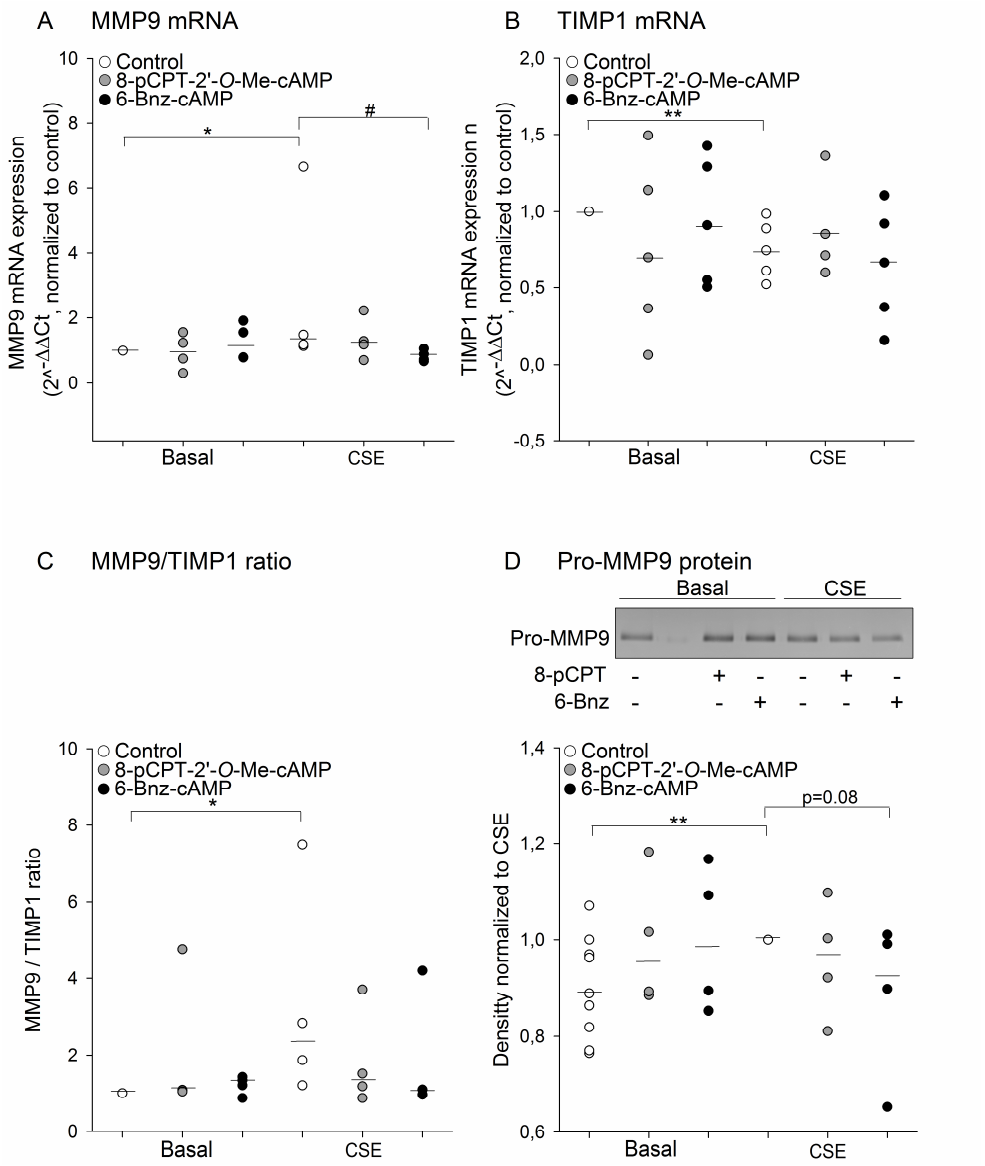

Figure 4: PKA tends to prevent induction of pro-MMP9 by CSE. 16HBE cells were exposed to 5% CSE alone and in combination with 100 μM 8-pCPT-2'-*O*-Me-cAMP or 500 μM 6-Bnz-cAMP 6 hrs, followed by analysis of MMP9 (A) and TIMP1 (B) mRNA expression by RT-PCR. MMP9/TIMP1 ratio was calculated (C). Pro-MMP9 was determined by zymography (D). mRNA expression and zymography were presented as separate data points of 4-5 experiments. $^{*}p<0.05$, $^{**}p<0.01$ compared to basal control. $^{\#}p<0.05$ compared to CSE.

Discussion

In our study in the human bronchial epithelial cell line 16HBE14o- (16HBE), CSE induces MMP9 over TIMP1 as evident by the increased MMP9/TIMP1 ratio. We also found indications that cAMP may prevent this CSE-induced MMP9/TIMP1 imbalance presumably via PKA as specific activation of PKA mimicked the effect of the β_2-agonist fenoterol. In addition, CSE-induced elevation in pro-MMP9 was prevented by fenoterol. Direct activation of PKA tended to prevent induction of pro-MMP9; only PKA activation reduced CSE-induced MMP9 mRNA expression. Taken together, PKA seems to reduce both MMP9 mRNA and pro-MMP9 in 16HBE cells exposed to CSE.

MMPs are not only secreted by epithelial cells (21), but also by neutrophils (27), airway smooth muscle cells (28), monocytes and fibroblasts (29). The following rational led to our choice to perform our study in a human bronchial epithelial cell line: a) in pilot studies we observed a more pronounced release of MMP9 protein from epithelial cells compared to human airway smooth muscle cells, and b) epithelial cells represent the first layer of defense against exposure of the lung to cigarette smoke, the main risk factor for COPD. In addition, elevation of MMP9 by CSE in bronchial epithelial cells has been reported before (30). Using different techniques, our data indicate that in 16HBE cells CSE enhances the level of MMP9 mRNA and pro-MMP9, whereas a trend towards a reduction of TIMP1 was observed. Thus, in these cells CSE shifts the balance of MMP9 and TIMP1 towards MMP9, which could contribute to an ECM protein turnover imbalance.

In human epithelial cells various effects of CSE exposure on TIMP1 expression have been described. Exposure of *primary* bronchial epithelial cells from central airways to cigarette smoke condensate for 24 hrs increased the level of TIMP1 protein (31). However, exposure of human epithelial cells derived from *small* airways to CSE for 24 hrs did not alter TIMP1 protein expression (32). The observed differences in TIMP1 expression might be related to differences in CSE composition, concentrations and exposure times as well as to the epithelial cell types used (primary vs cultured, central-bronchial vs small airways). The increase in TIMP1 protein observed by others was obtained after 24 hrs (31; 32), which

might represent a long term response to environmental stimuli to counterbalance the induction of MMP9, although the ratio was unchanged. As we exposed 16HBE cells to CSE for only 6 hrs, we may have observed acute effects in response to CSE. In addition, we observed an increased cellular MMP9/TIMP1 ratio to CSE, which is in line with increased MMP9/TIMP1 ratio in sputum of COPD patients (33).

In total lung tissue of COPD patients, we did not observe an increase in the mRNA expression of MMP9 and TIMP1. Others studies reported on an increase of MMP9 mRNA expresion in lung tissue of severe COPD patients compared to non-smokers (34). Smoking status itself may also alter MMP expression and activity despite of the COPD status. Alveolar macrophages of healthy smokers exposed to CSE expressed a higher MMP9 level compared to cells from non-smokers (35). Interestingly, Finlay et al. reported on elevated gelatinase activity in BALF of patients with emphysema but also in current smokers of the control group (36). Both studies, albeit in specific lung compartments, point to an effect of cigarette smoke on MMP expression and activity. Even 6 months after smoking cessation, sputum MMP9 levels were still elevated in ex-smokers (37). As our control patients were all ex-smokers and 6 out of 7 COPD patients were ex-smokers, the apparent lack of an increased MMP9 level in lung tissue of COPD patients observed in our study might be explained by the smoking status of the control group, or by the fact that changes in specific compartments in the lung can be obscured by the effects (or lack of) in other compartments.

In lung tissue of COPD patients, we did not observe changes in TIMP1 level either. In alveolar macrophages of COPD patients exposed to cigarette smoke conditioned medium, elevated TIMP1 expression was observed compared to healthy smokers (35). COPD patients show the same amount of TIMP1 compared to non-smokers (35). Also in primary epithelial cells from COPD patients, an increase in TIMP1 expression was found compared to non-smoking controls, whereas no differences were found between current smokers and COPD patients (38). These findings indicate that smoking status is not only important for MMP9 expression, but also for TIMP1 expression. Therefore, the lack of TIMP1 alterations observed in our study might be due to the fact that all our control patients and 6 out of 7 COPD patients were ex-smokers and therefore did not

differ in smoking status. In support of our findings, other studies also did not observe increased TIMP1 levels in COPD patients (35; 38).

To summarize this part, we report here on a different MMP9 expression in COPD lung tissue compared to CSE-exposed16HBE cells. Our experimental findings should be envisioned in the context that both an increase or a decrease of MMP9 might be of benefit in certain areas or tissues of the diseased lung. It is likely to assume that areas with emphysema may benefit from a reduction of MMP, while areas with airway fibrosis may benefit from an increase in MMP activity. In addition, the total lung tissue of COPD patients we used also included other cell types, including fibroblasts, airway smooth muscle cells and leukocytes. Therefore, the differences in MMP9 and TIMP1 expression between the experiments in 16HBE cells and the lung tissue could be explained by the presence of different compartments and cell types.

An important component of the management of COPD patients is treatment with β_2-agonists and PDE inhibitors, both of which elevate cAMP albeit by distinct mechanisms (13; 14). In 16HBE cells, we found that cAMP and its effector PKA prevent CSE-induced pro-MMP9. In the monocytic cell line THP-1and in neutrophils, it has been shown that LPS-induced MMP9 mRNA and protein is reduced by adenosine as well as the PDE inhibitor cilostazol (19; 39). In monocytes, however, adenosine did also not reduce MMP9 protein (19). In 16HBE, we did not observe a change in basal TIMP1 levels by cAMP or by activation of the cAMP effectors PKA and Epac. In a fibrosarcoma cell line, TIMP1 protein levels were increased by the cAMP analog 8-Bromo-cAMP (20), whereas elevation of cAMP by cilostazol enhanced TIMP1 expression in a monocytic cell line (17). These findings may point to cell type specific mechanisms of cAMP-mediated effects on MMP9 and TIMP1 expression and that its regulation in epithelial cells is largely cAMP independent. A cAMP-independent regulation of TIMP1 expression was also observed in monocytes (17).

Interestingly, specific activation of PKA and to a lesser extent fenoterol prevented CSE-induced MMP9 mRNA expression. In contrast, the level of pro-MMP9 was reduced by fenoterol and by PKA, but not Epac. It is tempting to speculate that

β_2-agonists and the cAMP effector PKA may normalize an imbalance in the MMP9/TIMP1 ratio and subsequent ECM turnover induced by environmental agents such as cigarette smoke. Another explanation for the reduction of the pro-MMP9 level by fenoterol and specific PKA activation may involve elevation of other proteinases, such as MMP3 or MMP2, which favour the cleavage of the pro-domain of MMP9 (12). However, in certain areas of the lung a reduction of the MMP9/TIMP1 ratio may enhance fibrotic processes. Development and site of deposition of cAMP-elevating drugs should take the balance between emphysema and fibrosis into account.

In conclusion, we report here that cAMP and primarily its effector PKA prevent the increased MMP9/TIMP1 ratio induced by CSE in the human bronchial epithelial cell line 16HBE14o-. Thus, PKA may represent an interesting target for drug development against remodeling in lung diseases. Inhibition of PKA may enhance the MMP9/TIMP1 ratio and thereby reduce airway wall fibrosis. In contrast, activation of PKA may reduce the MMP9/TIMP1 ratio and thereby reduce progression of emphysema. Further research in primary epithelial cells and in other structural cell types is needed to identify the exact role of PKA and the mechanisms involved in CSE induced MMP9 activity.

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