New avenues for Epac in inflammation and tissue remodeling in COPD

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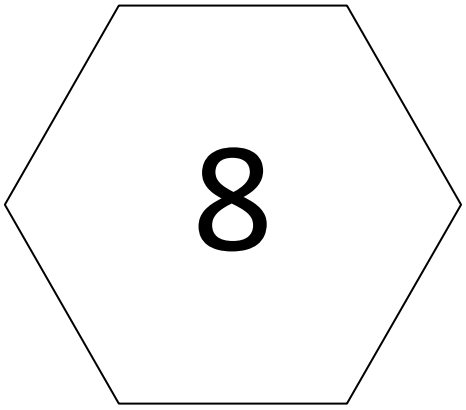
General discussion



Chronic obstructive pulmonary disease (COPD) is a chronic inflammatory disease of the airways and the lung parenchyma, further characterized by airway obstruction and remodeling (1). Currently, COPD is the third leading cause of death worldwide (2). Chronic inflammation and tissue remodeling, including loss of epithelial barrier, small airway fibrosis, mucus hypersecretion, increased airway smooth muscle mass and parenchymal destruction (emphysema), contribute to the progressive and irreversible decline in lung function in COPD (1; 3-5). No curative treatment for COPD exists and symptoms are treated with glucocorticosteroids, anticholinergics, β_2-agonists or phosphodiesterase (PDE)-4 inhibitors being used either alone or in combination (6; 7). The mechanism of action of both β_2-agonists and PDE4 inhibitors involves elevation of the second messenger cyclic AMP (cAMP), although by distinct mechanisms (8; 9). Whereas binding of β_2-agonists to the G_s protein-coupled β_2-adrenergic receptor activates AC subsequently leading to the formation of cAMP, PDE4 inhibitors prevent the breakdown of cAMP into the inactive 5'-AMP (8-10). cAMP has been implicated in the development and progression of chronic diseases due to its ability to modulate gene transcription and secretion (9; 11; 12). The two main effectors of cAMP are protein kinase A (PKA) and the exchange protein directly activated by cAMP (Epac), which consists of the two isoforms Epac1 and Epac2 (11). Several recent studies have focused on the role of cAMP in cell biology in general and in pulmonary disease in particular, which has resulted in intriguing, unexpected findings. In this thesis, novel findings on the role of Epac are presented, which may lead to the development of new drugs to optimize the treatment of COPD.

Epac1 and Epac2: Inflammation

A major characteristic of COPD is inflammation, a process characterized by infiltration and activation of diverse inflammatory cells including macrophages, monocytes, lymphocytes and particularly neutrophils, and subsequently leading to the secretion of cytokines by these cells (1). An important cytokine that is increased in COPD is interleukin-8 (IL-8) (13). The secretion of IL-8 correlates with pulmonary neutrophil levels in COPD, underscoring the importance of this cytokine in COPD (14). The effect of cigarette smoke, the main risk factor for

COPD, on IL-8 release has been investigated *in vitro*. Bronchial epithelial cells, macrophages, fibroblasts and airway smooth muscle cells all secrete IL-8 after stimulation with cigarette smoke (15-20). A previous study from our group demonstrated that Epac1, Epac2 and PKA act in concert to modulate the release of IL-8 from airway smooth muscle cells via signaling to the main Epac effector Rap1 and extracellular signal-regulated kinases (ERK1/2) (21). Importantly, however, the roles of Epac and PKA in cigarette smoke-induced IL-8 release were not investigated yet. In **chapter 3**, we demonstrated that cigarette smoke extract (CSE)-induced release of IL-8 from human airway smooth muscle was almost fully inhibited by the β_2-agonist fenoterol. Direct pharmacological activation of either Epac or PKA mimicked the effect of fenoterol on CSE-induced IL-8 release. Silencing of both Epac1 and Epac2 resulted in a reduction of the anti-inflammatory effects of Epac activation. We investigated the underlying mechanisms, and reported that Epac and PKA decreased CSE-induced IL-8 release via inhibition of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and ERK1/2, respectively (**chapter 3**). In addition Epac1, but not Epac2 or PKA, protein expression was down regulated in CSE-exposed human airway smooth muscle cells as well as in lung tissue of COPD patients (**chapter 3**). The down-regulation of Epac1 protein in COPD patients might be due to irreversible protein structure alterations caused by cigarette smoke-induced oxidative stress which is also responsible for lung damage and chronic inflammation (22). Interestingly, it has also been reported that the pro-fibrotic cytokine transforming growth factor (TGF)-β_1 decreases the cellular level of Epac1 (23). These findings might have important clinical implications towards a better understanding of COPD pathogenesis and the improvement of its pharmacological treatment. Indeed, despite its anti-inflammatory effects as shown in this thesis in *in vitro* experiments, clinical studies only show modest beneficial effects of β_2-agonists in the treatment of airway inflammation (24; 25). Such a discrepancy has been assigned to variable β_2-adrenergic receptor abundance, β_2-adrenergic receptor gene polymorphisms and alterations of β_2-adrenergic receptor signaling by desensitization in both inflammatory and structural cells in the airways (26-29). As a potential effector in cAMP-driven and β_2-adrenergic receptor-induced signaling and a newly discovered inhibitor of NF-κB-dependent inflammatory response, Epac1 down-

regulation by cigarette smoke may provide an additional explanation for the variable anti-inflammatory capacities of β_2-agonists in the treatment of COPD.

To investigate the potential cause for the downregulation of Epac1, we studied the possible involvement of microRNAs (miRNAs). MicroRNAs are epigenetic regulators involved in fine-tuning of cellular activities by posttranscriptional repression of mRNA, by direct degradation of mRNA and by inhibition of the translation process (30). In COPD, miRNAs have been implicated in the regulation of inflammatory processes (30) and miRNA-7 is increased in serum of COPD patients (31). Interestingly, *in silico* analysis revealed that Epac1 might represent a putative target of miRNA-7 (targetscan.org). In **chapter 4**, we have reported on a potential interaction of Epac1 and miRNA-7. CSE induced miRNA-7 specifically in human airway smooth muscle cells. In line with the specific induction of miRNA-7 in human airway smooth muscle cells by CSE, miRNA-7 was increased in bronchial smooth muscle of COPD patients isolated by laser dissection compared to controls. Importantly, Epac1 expression tended to be reduced in miRNA-7 overexpressing human airway smooth muscle cells. Our data implicate that upregulation of miRNA-7 by cigarette smoke correlates with the downregulation of Epac1 in COPD. This interaction might be involved in Epac related inflammation. Both Epac1 and Epac2 seem to be implicated in the reduction of CSE-induced IL-8 release from human airway smooth muscle cells as shown by pharmacological activation of Epac by 8-pCPT-2-*O*-Me-cAMP and silencing of both Epac1 and Epac2 (**chapter 3**). The Rap-activated phospholipase Cε (PLCε), a direct effector of Epac (32; 33), has been linked to the production of pro-inflammatory mediators including keratinocyte-derived chemokine (KC), the murine functional homolog of interleukin (IL)-8 (34), IL-1β and tumor necrosis factor (TNF) (35). The PLCε-mediated increase in KC was accompanied by neutrophilia (34), an important feature of COPD (1; 3). Interestingly, it has been reported that PLCε is highly expressed in the mouse lung (36). To translate our findings on inflammation *in vitro* to the *in vivo* situation and to make a distinction between the effects of Epac1, Epac2 and the Epac effector PLCε, Epac1-/-, Epac2-/- and PLCε-/- mice were exposed to cigarette smoke for 5 days (**chapter 7**). An acute model of cigarette smoking was used, as recently reported by our group (37). Using this model we were able to induce inflammation and remodeling after acute cigarette

smoke exposure (37). In this model we demonstrated in **chapter 7** that compared to wild-type (WT) mice exposed to cigarette smoke, the number of total inflammatory cells, macrophages, and neutrophils as well as IL-6 release were lower in Epac2-/- mice, which was also the case for neutrophils and IL-6 in PLCε-/- mice. Again compared to WT mice exposed to cigarette smoke, the number of macrophages was reduced in Epac1-/- mice. Whereas, the numbers of lymphocytes, only present in low numbers in bronchoalveolar lavage fluid (BALF) of air-exposed WT mice, were increased in Epac1-/- mice compared to WT mice exposed to either fresh air or cigarette smoke (**chapter 7**). Together our data indicated that particularly Epac2 acts pro-inflammatory *in vivo*. In line with our findings presented in this thesis showing either a pro- or an anti-inflammatory role for Epac1, it has been reported in other studies that the effect of Epac1 on inflammation seems to be cell-type specific (38; 39). To confirm our findings reported in the genetically modified mice, we intend to apply recently developed Epac1 and Epac2 inhibitors to WT mice in our acute cigarette smoke exposure model (40-42). The mode of action and specificity of these Epac1 and Epac2 inhibitors, however, warrant further studies (42). Specific activators for Epac1 and Epac2 are still lacking. Additionally, chronic cigarette exposure of the genetically modified mice will increase our knowledge on the impact of Epac1 and Epac2 on chronic inflammation as observed in COPD.

Aberrant epithelial repair of damage caused by cigarette smoke is also regarded as a pathophysiological feature of COPD (43; 44). Cigarette smoke-induced inflammation, together with oxygen radicals present in cigarette smoke may disturb epithelial repair and barrier function (45). With respect to such barrier function it is of interest that the A-kinase anchoring protein (AKAP) family member AKAP9 enhanced the endothelial barrier function in concert with Epac1 (11; 46; 47). Members of the AKAP family compartmentalize cellular cAMP upon generation of multiprotein complexes consisting of either the β_2-adrenergic receptor, PDE4, PKA or Epac or a combination of these proteins (11; 48). In **chapter 6**, we demonstrated that CSE reduces the barrier function in human bronchial epithelial cells as well as the membrane expression of E-cadherin and AKAP9. Next to the expression of PKA, Epac1 and Epac2, CSE did also not alter the expression of AKAP5 and AKAP12, which are known to interact both with the β_2-

adrenergic receptor (49), showing a specific role for AKAP9 in CSE-induced effects on the barrier in this thesis. Silencing of AKAP9 reduced the functional epithelial barrier and prevented the ability of st-Ht31, an inhibitor of AKAP-PKA interactions, to restore membrane localization of E-cadherin (**chapter 6**). Our data indicated that AKAP proteins, most likely AKAP9, maintain the bronchial epithelial barrier function and may be important in the pathophysiology of COPD. In **chapter 7**, we found that the mRNA expression of PKA-RI was reduced in Epac2-/- mice. As a distinct subset of AKAP members is known to interact with PKA-RI (50), it is reasonable to assume that the observed reduced PKA-RI expression most likely causes alterations in the functioning of PKA-RI bearing AKAP complexes resulting in changes in biological functions and disturbed physiology. These alterations in compartmentalization of cAMP may play a role in COPD characteristics like inflammation and remodeling.

Altogether, Epac1 and Epac2 seem to be involved in cigarette smoke induced inflammation, although with a clear distinction regarding their relative contribution (Figure 1 and 2). Epac1 is related to anti-inflammatory effects both in CSE-exposed human airway smooth muscle cells and in bronchial smooth muscle of COPD patients. In contrast, our studies performed in Epac2-/- demonstrated that Epac2 acts primarily pro-inflammatory. The pro-inflammatory role of cAMP-regulated Epac2 seems to be contradicting in the context of the anti-inflammatory effects of cAMP-elevating drugs. It is important to emphasize, however, that though both β_2-agonists and PDE4 inhibitors elevate cAMP, they modulate distinct cellular functions and in different cell types. It has been reported that β_2-agonists effectively reduce airflow obstruction by inducing bronchodilation (51; 52) and cytokine release *in vitro* (53-55), while evidence for anti-inflammatory properties of β_2-agonists *in vivo* is still lacking (6; 7; 51; 56). On the other hand, PDE4 inhibitors such as rolipram only marginally reduce airflow obstruction, but reduce airway inflammation and thereby possibly the exacerbation frequency (57-62). Compartmentalization of cAMP driven by AKAP family members, may be responsible for the distinct biological effects of cAMP (63). In this thesis, we have reported on the existence of AKAP-based multiprotein complexes which seem to determine bronchial epithelial barrier functioning. As AKAP complexes have been shown to interact with both Epac1 and Epac2 (9; 11), future studies should define

to which extent AKAPs determine pro- and/or anti-inflammatory properties of Epac1 and Epac2. Overall, the findings presented in this thesis indicate that Epac1 and Epac2 have different roles in cigarette smoke induced inflammation. Focus of future research should be on the development of specific activators of Epac1 and/or inhibitors of Epac2 to alter chronic inflammation in COPD and on identifying the interaction between Epac and AKAP as a possible cause of the differences observed between Epac1 and Epac2.

Epac1 and Epac2: Remodeling

Tissue remodeling is another feature of COPD and covers different characteristics of the disease such as mucus hypersecretion, airway fibrosis and emphysema (64). The role of cigarette smoke in remodeling processes has been well established and an increased mucus secretion, onset of airway wall extracellular matrix (ECM) protein deposition and onset of emphysema by cigarette smoke has been observed (45; 65). The majority of ECM proteins, including collagens and fibronectin, are produced by fibroblasts, but can also be produced by other structural lung cells like smooth muscle cells and epithelial cells (66; 67). Alterations in the tightly controlled balance of production and degradation of ECM proteins causes structural changes in the lung such as emphysema, characterized by excessive degradation of parenchymal ECM (68), and (small) airway fibrosis characterized by excessive deposition of ECM proteins (69; 70). Matrix metalloproteases (MMPs) and tissue inhibitors of metalloproteinase (TIMPs) regulate the balance between production and degradation of ECM proteins (71). In COPD patients, both MMP9 and its main inhibitor TIMP1 are elevated (72; 73), making the final outcome with regard to ECM production and/or degradation more difficult to predict in COPD.

Interestingly, it has been reported that cAMP-elevating drugs reduced synthesis of collagen I in human lung fibroblasts (74; 75). In addition, it has been reported that both PKA and Epac inhibited the production of the ECM proteins collagen I and III (76). Binding of Epac1 to the activated TGF-β1 type I receptor subsequently decreased the phosphorylation of Smad2 and Smad2-dependent transcription (77) raising the possibility that Epac1 inhibited collagen production via TGF-β1 (78). The PDE inhibitor cilostazol, adenosine and the cAMP analog 8-Br-cAMP have been shown to reduce MMP9 and TIMP1 gene expression and activity in different

cell types (79-82). Although a role for cAMP in the regulation of remodeling has been shown, the exact role of the cAMP effectors Epac and PKA on the different aspects of remodeling is not completely known and was further investigated in this thesis. In **chapter 5**, we demonstrated that CSE exposure of the human bronchial epithelial cell line 16HBE14o- leads to increase of MMP9 mRNA and thereby of the MMP9/TIMP1 ratio. Induction of MMP9 mRNA was reduced by specific PKA activation. Pro-MMP9 levels induced by CSE were reduced by the β_2-agonist fenoterol, an effect specifically mimicked by pharmacological activation of PKA (**chapter 5**). So, inhibition of PKA may enhance the MMP9/TIMP1 ratio and thereby reduce emphysema. In contrast, activation of PKA may reduce emphysema progression by a decrease of the MMP9/TIMP1 ratio.

As Epac did not seem to be involved in the regulation of MMP, we tried to identify the role of Epac1 and Epac2 in other remodeling processes in the lung by exposure of Epac1-/- and Epac2-/- mice to cigarette smoke. In **chapter 7**, we reported that Epac1-/- mice expressed higher levels of the pro-fibrotic cytokine TGF-β1 (mRNA), collagen I (mRNA and protein) and fibronectin (mRNA and protein). Based on the findings demonstrated in this thesis, we propose that particularly Epac1, but not Epac2, acts anti-fibrotic (**chapter 7**). Mucus hypersecretion represents another factor due to remodeling effects in the airways (83-85). Interestingly, Epac1-/- and Epac2-/- were characterized by a constitutively higher expression of MUC5AC mRNA at basal level. We observed that goblet cells tended to be increased in Epac2-/- and PLCε-/- mice, whereas primarily Epac1-/- mice tended to stain positive for the inducer of goblet cell differentiation SPDEF (**chapter 7**).

Overall, we identified distinct roles of Epac1 and Epac2 in remodeling processes as seen in COPD patients (Figures 1 and 2) (**chapter 5, 6, 7**). This suggest that Epac1 alone is involved in dampening the production of the pro-fibrotic cytokine TGF-β1 as well as the ECM proteins collagen I and fibronectin. In contrast, both Epac1 and Epac2 seem to control the level of MUC5AC. Based on the findings represented in this thesis, Epac1 and Epac2 exert distinct, locally defined roles in remodeling processes of the lung and selective activators or inhibitors for either Epac subtype should be developed. Similar as for inflammation, compartmentalization of Epac

in AKAP-bearing protein complexes will define a potential role of AKAP-dependent compartmentalization of cAMP in remodeling processes such as balancing between MMP/TIMP or mucus hypersecretion.

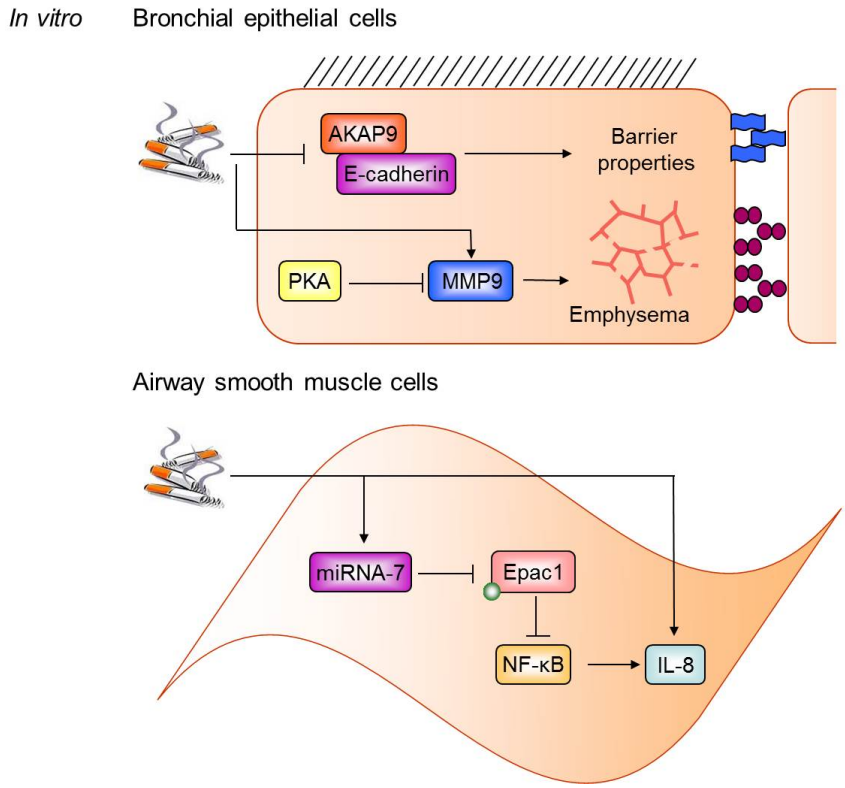


Figure 1: Epac: Implications for structural lung cell functioning. In human bronchial epithelial cells, cigarette smoke extract reduces the expression of both AKAP9 and E-cadherin, and thereby disrupts the epithelial barrier. Next, PKA reduces (pro)-MMP9 expression and activation; modulation of pro-MMP9 level by PKA most likely leads to a reduction in the progression of emphysema. In airway smooth muscle cells, cigarette smoke extract enhances miRNA-7 levels which inhibits the expression of Epac1. On the other hand, primarily Epac1 inhibit NF-κB and thereby cigarette smoke extract-induced IL-8 release. Please note that we here only illustrate the main results of our current studies. For further details, see text.

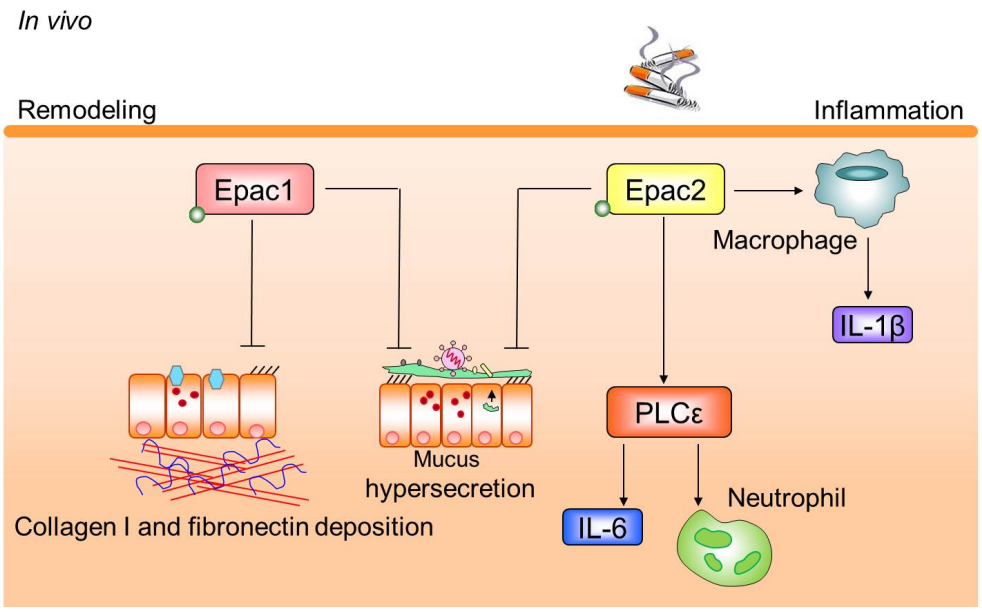


Figure 2: Epac1 and Epac2: Implications in inflammation and remodeling *in vivo*. In an acute mouse model of cigarette smoke exposure, Epac1 bears the capacity to reduce the deposition of collagen I and fibronectin in lung tissue. Both Epac1 and Epac2 regulate mucus hypersecretion in mice. Epac2 contributes to cigarette smoke induced-increase in macrophages and IL-1β release. Possibly via PLCε, Epac2 also enhances neutrophils and IL-6 release. Please note that we here only illustrate the main results of our current studies. For further details, see text.

Clinical implication and future perspectives

In the GLUCOLD study (Groningen Leiden Universities Corticosteroids in Obstructive Lung Disease), a recent 30 month randomized, placebo-controlled trial the effect of the corticosteroid fluticasone and the β_2-agonist salmeterol on airway inflammation and on the progressive decline in FEV_1 has been investigated in COPD patients (62). In this study it was demonstrated that combination of salmeterol with fluticasone did not further improve anti-inflammatory effects compared to fluticasone alone but did improve the level of FEV_1 without further changing the FEV_1 decline (62). In two placebo-controlled, double-blind, multicentre trials (M2-124 and M2-125), the PDE4 inhibitor roflumilast increased FEV_1 and reduced the frequency of excacerbations (58). As with

glucocorticosteroids, the beneficial effects of roflumilast were restricted to a specific subset of COPD patients, implicating that COPD disease management requires further improvement. In human lung fibroblasts, combination of the β_2-agonist indacaterol with roflumilast inhibited the release of several pro-inflammatory and pro-fibrotic mediators including fibronectin (86). Concerning mucus hypersecretion, the β_2-agonist salmeterol improves mucocilialy transport, possibly by increased presence of ciliated epithelium or improvement of ciliary beat frequency (87).

These studies implicate an important role for cAMP in COPD related features. In this thesis the focus was on the cAMP effectors, especially Epac, and we defined distinct roles of Epac1 and Epac2 in inflammatory and remodeling processes in experimental models of COPD. We showed that Epac1 exerted anti-inflammatory properties, effects that seem specific for airway smooth muscle and being sensitive to cigarette smoke (**chapter 3**). In an acute model of cigarette smoke exposure in mice, we unravelled pro-inflammatory actions of Epac2. In addition, we showed that Epac1 alone is able to reduce the remodeling parameters TGF-β1, collagen I and fibronectin, whereas for the reduction of MUC5AC a concerted action with Epac2 seemed to be required (**chapter 7**). Based on these novel findings, development of selective Epac1 and Epac2 activators and/or inhibitors could be of additive value to alleviate symptoms of COPD including airway obstruction and remodeling. Targeting of these selective Epac activators or inhibitors to specific areas in the lung would be a solution to the opposite effects observed between Epac1 and Epac2 in different lung compartments. Research into compartmentalization of cAMP by distinct subsets of AKAPs should be another subject of future research. In **chapter 6** we identified a role for AKAP9 in the regulation of the epithelial barrier function. Future studies should focus on a potential direct interaction of members of the AKAP family with Epac1 or Epac2 to define a potential role of AKAP-Epac complexes in the regulation of COPD characteristics, not only in relation to the bronchial epithelium but also including inflammation, emphysema and small airway fibrosis.
Overall, future research should focus on the distinct functional properties of Epac1 and Epac2 in the context of the pathophysiology of (obstructive) lung diseases including COPD. Such studies should include attempts to further define

the impact of AKAP-based multiprotein complexes to determine the functional impact of compartmentalized cAMP signals on inflammation and remodeling in COPD.

Main conclusions

Overall, the studies presented in this thesis show that:

- CSE-induced IL-8 release from airway smooth muscle cells is inhibited by activation of either PKA or Epac (**Chapter 3**).
- Epac and PKA reduces CSE-induced IL-8 release from human airway smooth muscle cells via inhibition of NF-κB and ERK1/2, respectively (**Chapter 3**).
- Epac1, but not Epac 2, protein expression is decreased in COPD patients, a process presumably caused by cigarette smoke (**Chapter 3**).
- *In vivo*, Epac1 inhibits cigarette smoke-induced remodeling processes (**Chapter 7**).
- *In vivo* Epac2, presumably via PLCε, acts pro-inflammatory in cigarette smoke-induced inflammation (**Chapter 7**).
- An interaction between Epac1 and miRNA-7, known to be induced in COPD, has been identified in human airway smooth muscle cells, which could explain CSE-induced downregulation of Epac1 expression in these cells (**Chapter 4**).
- CSE-induced MMP9 mRNA expression and activity in the human bronchial epithelial cell line 16HBE14o- is reduced by activation of PKA, but not of Epac (**Chapter 5**).
- In the human bronchial epithelial cell line 16HBE14o- the epithelial barrier function is diminished by cigarette smoke by specifically reducing E-cadherin levels (**Chapter 6**).
- AKAP9 directly interacts with E-cadherin to maintain barrier properties in the human bronchial epithelial cell line 16HBE14o- (**Chapter 6**).

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