Compartmentalized cAMP Signaling in COPD

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General Introduction

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Chronic obstructive pulmonary disease (COPD)

Chronic obstructive pulmonary disease (COPD) is one of the major health problems to induce morbidity and mortality. Based on the estimates from the World Health Organization, 65 million people have moderate to severe COPD all over the world. It is predicted that COPD will become the third leading cause of death (~ 8.3 million) and the fifth leading cause of disability by 2030 (Barnes, 2000; Laudette et al., 2018). According to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines, COPD is characterized by progressive and not fully reversible airflow limitation. Obstruction of small airways, emphysema, enlargement of air spaces and destruction of lung parenchyma, loss of lung elasticity, closure of small airways, fibrosis, inflammation, mucus hypersecretion, and pulmonary hypertension are the key features of COPD lung tissue (Barnes, 2000; Giembycz and Maurice, 2014; Vogelmeier et al., 2017). Currently, none of the existing medications used to treat COPD have been shown to improve the long-term decline of lung function. Therefore, novel medications are urgently needed for COPD prevention and treatment.

Exposure to cigarette smoke (CS) is considered to be the primary cause of COPD. Therefore, the most effective way to prevent the development of COPD is smoke cessation (Bergeron and Boulet, 2006; Tønnesen, 2013). Additionally, other factors including exposure to indoor pollution from biomass fuels and outdoor air pollution including occupational dusts particularly in developing countries and genetics may also contribute to pathogenesis of COPD (Boswell-Smith and Spina, 2007; Vogelmeier et al., 2017; Wang et al., 2018).

Pathogenesis and pathophysiology of COPD

COPD is characterized by chronic inflammation and airway obstruction, which is not fully reversible (Vogelmeier et al., 2017). The inflammation in COPD most likely occurs in peripheral airways (bronchioles) and lung parenchyma (Barnes, 2014). It has been shown that patients with severe COPD have infiltration of macrophages and CD8+ T cells and an increased number of neutrophils in bronchial-biopsy (Di Stefano et al., 1998; O'Shaughnessy et al., 1997). A dramatic increase of macrophages and neutrophils has been observed in bronchoalveolar lavage fluid and induced sputum (Keatings et al., 1996; Pesci et al., 1998). Moreover, multiple inflammatory mediators, including lipids, chemokines, cytokines and growth factors also play a crucial role during COPD development (Barnes, 2014; Lamela and Vega, 2009). Chronic inflammation is able to induce structural alterations and mucus hypersecretion, thereby further causing narrowing of small airways and decline in lung function. Epithelial cells and macrophages secrete transforming growth factor-β (TGF-β), which triggers fibroblast proliferation and thus contributes to tissue remodeling (Barnes, 2014). The inflammatory cytokines, proteases and growth factors produced by airway smooth muscle cells are associated with remodeling process and induce phenotypic changes of smooth muscle from contractile to proliferative phenotype (Aghasafari et al., 2018; Chung, 2005).

As cigarette smoke can induce oxidative stress, there is accumulating evidence that oxidative stress is involved in COPD (Kirkham and Barnes, 2013; Wang et al., 2018). Except for cigarette smoke, exogenous sources of reactive oxygen species (ROS) such as air pollutants, or endogenously released ROS from leukocytes and macrophages can also induce oxidative stress and break the balance between oxidant and antioxidant (Kirkham and Barnes, 2013). The activated immune cells, for instance neutrophils and macrophages, are able to release ROS during the inflammatory process(Meijer et al., 2013). Endogenously released ROS reacts with lipid, protein, DNA, RNA and mitochondrial DNA, thereby, leading to epithelium injury and contributing to COPD development (Boukhenouna et al., 2018).

Fibrosis, which is a key feature in chronic pulmonary diseases, plays a vital role in COPD pathogenesis. It has been shown that epithelial-to-mesenchymal transition (EMT), which was triggered either by environmental stresses such as oxidative stress (Rhyu et al., 2005) or by extracellular mediators such as TGF-β1 (Hackett et al., 2009), contributed to pulmonary fibrosis (Jolly et al., 2018; Rout-Pitt et al., 2018; Sakuma, 2017). EMT was first identified in the 1980s by Greenburg and Hay (Greenburg and Hay, 1986). EMT is a process in which epithelial cells gradually lose epithelial proteins including E-cadherin, ZO-1, which are responsible for cell-cell contact, and undergo transition to a more mesenchymal phenotype as they gain mesenchymal markers such as N-cadherin, vimentin and fibronectin (Nieto, 2011; Zuo et al., 2019b). Transcription factors involved during EMT process are Snail, Slug, Zeb and Twist (Baulida, 2017; Yang et al., 2013). In 2010, Sohal and colleagues demonstrated for the first time that the fibroblast protein marker S100A4 was significantly increased in cells within reticular basement membrane clefts of smokers and COPD patients compared with never-smoking control subjects by immunohistochemistry, indicating an active EMT process in the large airway of CODP patients which was highly correlated with cigarette smoke exposure (Sohal et al., 2010). However, the role of cAMP scaffold in TGF-β1/cigarette smoke-induced EMT is still unclear.

Cyclic AMP targeted therapies in COPD

According to GOLD guidelines, the aim of therapy in COPD is to relieve symptoms, to reduce the frequency and severity of exacerbations and to improve health status and exercise performance (Vogelmeier et al., 2017). Unfortunately, no existing COPD medication has been conclusively shown to modify the long-term clinical outcomes. Cyclic AMP (cAMP) has been proven to be a promising target in COPD treatment due to its excellent performance on bronchodilation and anti-inflammation which is mediated by cAMP/ cAMP-dependent protein kinase A (PKA) and exchange proteins activated by cAMP (Epacs) (Dekkers et al., 2013; Oldenburger et al., 2012b; Roscioni et al., 2011b, 2011a; Schmidt et al., 2013). Nowadays, medicines used for COPD treatment relies mainly on bronchodilator therapy (β_2-agonists, anticholinergics and theophylline), and on PDE4 inhibitors used in concert with either corticosteroid or bronchodilator treatment especially in COPD patients with a high risk of

exacerbations (Giembycz and Maurice, 2014; Maji et al., 2018; Vogelmeier et al., 2017; Wang et al., 2018).

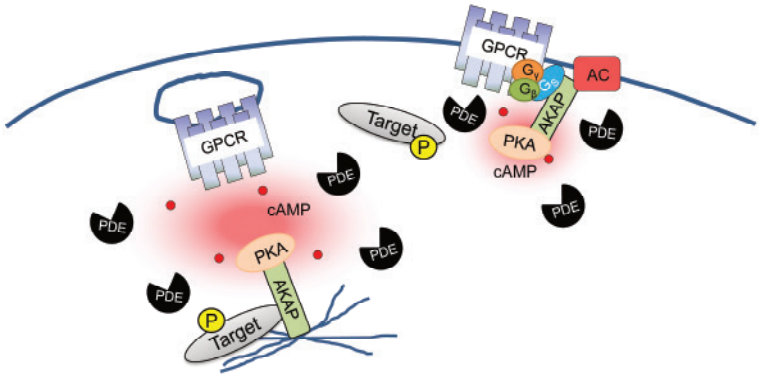

Figure 1. Compartmentalised cAMP signaling. Two distinct cAMP pools are shown in the schematic. One cAMP pool is generated by an AC anchored at the plasma membrane and activated by a GPCR exposed to the extracellular stimulus; the other one is generated by an internalized GPCR in the cytoplasm. PDEs, as key actors in limiting the spread of cyclic nucleotides, are responsible for cAMP hydrolysis and hence compartmentalize the cyclic nucleotide signal. The AKAP family, which binds to the regulatory subunits of PKA and targets PKA to discreet sites/macromolecular complexes, is also indicated in the schematic. GPCR, G-protein coupled receptor; AC, adenylyl cyclase;PDE, phosphodiesterase; PKA, cAMP-dependent protein kinase; AKAPs, A-kinase anchoring proteins;

cAMP compartmentalization

The first evidence for a compartmentalized cAMP signaling has been provided in the heart more than 40 years ago. Hayes et al. and Buxton et al. demonstrated differences in heart contractility when comparing hearts perfused with different agonists to activate the cAMP cascade. The force of heart contraction was enhanced with β_1-adrenoceptor agonist isoproterenol, whereas there was no change in heart contractility when activating prostaglandin E1 receptor with PGE1, even though cAMP was elevated and soluble PKA activity was also increased in both cases (Buxton and Brunton, 1983; Hayes et al., 1979). These findings provided functional evidence for the selectivity of cAMP action, indicating a compartmentalized cAMP signaling.

As one of the most important second messengers, cAMP localizes in well-organized intracellular signaling microdomains. As shown in **Fig 1**, cAMP is synthesized from adenosine triphosphate, following activation of adenylyl cyclases (ACs) (Omori and Kotera, 2007). Subsequently, cAMP binds to specific intracellular effector proteins, such as cyclic nucleotide-gated ion channels, PKA and Epacs (Oldenburger et al., 2012a; Omori and Kotera, 2007). In addition, PDEs, as key actors in limiting the spread of cyclic nucleotides, are responsible for cAMP and cGMP hydrolysis and

hence compartmentalize the cyclic nucleotide signal. The superfamily of PDEs is composed of 11 families with a distinct substrate specificity, molecular structure and subcellular localization (Omori and Kotera, 2007; Zuo et al., 2019a). Each PDE family has at least one (e.g. *Pde5a*) and often multiple coding genes, resulting in the mammalian PDE superfamily being composed of more than 21 genes (Omori and Kotera, 2007; Page and Spina, 2012). Moreover, most PDE encoding genes have distinct promoters, and multiple transcriptional products which are generated by alternative splicing, resulting in nearly 100 different PDE messenger RNAs (Conti and Beavo, 2007; Otero et al., 2014). In addition, the communication between β_2-adrenoreceptor, cAMP effectors, PDEs and other downstream targets are coordinated by A-kinase anchoring proteins (AKAPs) (shown in **Fig 1**) (Beene and Scott, 2007; Carnegie et al., 2009; Han et al., 2015; Poppinga et al., 2014). Members of the AKAPs family bind to the regulatory subunits of PKA and target PKA to discreet sites/macromolecular complexes, thereby playing a central role in the regulation of cAMP compartmentalization (Beene and Scott, 2007).

Scope of the thesis

The main objective of this thesis is to explore the role of compartmentalized cAMP signaling in the pathogenesis of COPD, focusing on PDE subfamilies and AKAPs. Using *in vitro*, *ex vivo* and *in vivo* approaches, we investigate the potential therapeutic targets in cAMP signaling pathways and link it with the current therapies available on the market.

In **chapter 2**, we provide an overview of Epac function and cAMP scaffolds in the heart and the lung. We highlight recent studies in heart and lung pertaining to cAMP compartmentalization, which provides more insights in understanding the role of cAMP scaffolds in different organs.

In **chapter 3**, we review the regulation of several PDEs (PDE3, PDE4, PDE5, PDE7 and PDE8) and demonstrate the roles of their selective inhibitors in chronic pulmonary diseases (COPD and asthma). In addition, the combination of different PDE inhibitors is also described, thereby providing a more comprehensive overview of the up-to-date research findings.

In **chapter 4**, we describe a new method to monitor cAMP dynamics in the airway by combining Förster resonance energy transfer (FRET) and precision cut lung slices (PCLS). Using this novel setup, the effect of cigarette smoke on cAMP hydrolyzing enzymes, PDE3 and PDE4, is studied. We show that cigarette smoke upregulates the activity and expression of both PDE3 and PDE4, which in turn, induces changes of intracellular cAMP dynamics. Moreover, these findings from PCLS are further confirmed using human bronchial epithelial cells and airway smooth muscle cells transfected with the cAMP biosensor adenovirus, indicating the different strategies in epithelial cells and airway smooth muscle cells with cAMP hydrolysis. In addition, functional changes of PDE3 and PDE4 after cigarette smoke exposure are examined by airway contractility and ciliary beating frequency (CBF) test. This study provides

strong evidence of the underlying changes induced by cigarette smoke regarding PDE3 and PDE4, providing increased impetus towards the development of improved dual PDE3/4 inhibitors for clinical use in smoke-related airway diseases.

In **chapter 5**, we discuss the scientific and therapeutic value of a recently published research paper in American Journal of Respiratory Cell and Molecular Biology "PDE8 is expressed in human airway smooth muscle and selectively regulates cAMP signaling by β_2-AR-AC6". PDE8, an IBMX insensitive PDE, can be inhibited by PF-04957325, which is a highly potent and selective PDE8 inhibitor developed by Pfizer. The role of PDE8 was demonstrated by Johnstone *et al*. for the first time in human airway smooth muscle cells. We highlight the findings on the transcript, protein and functional presence of PDE8 and β_2-AR-AC6-PDE8 signalosome which is expressed in caveolae.

In **chapter 6**, we review the recent knowledge about the role of cAMP scaffolds and oxidative stress in EMT process. How cAMP scaffolds (PDEs and AKAPs) and their distinguished signalosomes in different subcellular compartments may contribute to COPD is described here.

In **chapter 7**, we investigate the role of cAMP compartments during TGF-β1/ cigarette smoke induced EMT by modulating intracellular AKAPs. The contribution of PKA-AKAP complexes to EMT process is studied using the peptide st-Ht31, which inhibits the interaction between RII subunits of PKA and AKAP. Among more than 50 members, specific attention focuses on Ezrin, AKAP95 and Yotiao. The role of Ezrin, AKAP95 and Yotiao on EMT process is studied by small interfering RNA.

In **chapter 8**, the nasal and bronchial brushes and lung tissues from never-smokers, ex-smokers and current smokers are used to study the effect of cigarette smoke on PDE3 and PDE4 protein expression. In addition, human mRNA is isolated from bronchial and nasal brushings of never-smokers and healthy smokers.

In **chapter 9**, we provide a summary of our work, give future perspective of cAMP studies, and also describe the challenges.

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