

Small Airways in Asthma: Distal is Different

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Commentary

Goblet cell metaplasia (GCM) and mucus production are typical features of bronchial asthma, the most common chronic airway disease worldwide. In lung biopsies from patients with fatal asthma mucus plugging in small distal airways was causally linked with the death of the patient. Moreover, excess mucus production in the airways leads to endoplasmic reticulum stress (ER), activation of unfolded protein response (UPR) and cell death. Thus, it is of special importance to decipher the regulation of mucus production along the airway tree. We previously showed that distal airways significantly differ from proximal airways in their ability to respond to IL-13 stimulation. This study aimed at investigating if factors of ER stress or UPR potentially contribute to an impaired ability to produce mucus in distal airways. Proximal and distal airways of female C57BL/6 mice were microdissected and gene expression was determined by real-time RT-PCR. ER stress proteins BiP, CHOP and Foxp1 showed no different expression in airway sections. However, expression of IRE1 β and Agr2 was significantly lower in distal compared to proximal airways. This further supports our hypothesis of distal airways being protected against excess mucus production in order to prevent life-threatening mucus plugging.

Bronchial asthma is clinically characterized by a combination of symptoms including productive cough, rhonchus, shortness of breath, chest tightness, and a variable degree of broncho-obstruction [1]. This complex disease phenotype arises on the basis of a chronic inflammatory response in the airways that permanently causes tissue destruction and corresponding repair processes. This ultimately leads to airway remodeling, mucus hyperproduction, and the development of airway hyperresponsiveness (AHR). Whether these pathological features are also present in small airways has been a matter of debate for quite a long time. Distal airways are defined by their diameter of about less than 2 mm and located after the eighth branching generation. Due to their peripheral localization and small size the pathophysiological role of distal airways could not be similarly investigated like the proximal sections of the airway tree. Thus, the contribution of distal airways to the formation of asthma symptoms has long been neglected for a long time and they have even been described as the “quiet zone of the lung” [2].

However, a number of studies indicate that distal airways indeed play a critical, pathophysiological role in asthma and have marked impact on lung function in patients. This view is especially supported by autopsy findings from fatal asthmatics. These observations of course do not reflect the pathological situation of stable chronic asthma; however, they provide precious information about the disease in its

most extreme form. Hence, proximal as well as distal airways of fatal asthmatics reveal marked inflammation as indicated by infiltration of eosinophils and lymphocytes [3-6]. Furthermore, airway remodeling as evident by increased layer thickness of the adventitia, submucosal, and smooth muscle area is significantly higher in distal airways of these patients compared to healthy controls or patients suffering from chronic obstructive pulmonary disease (COPD) [7]. The most striking finding that underpins their contribution to fatal asthma is vast plugging of distal airways by mucoinflammatory exsudates that inhibits ventilation of the attached alveolar structures and thus can be described as a major contributing cause of death in asthma [8].

Transbronchial biopsies and surgically resected lungs from asthmatic patients further provided insight into the pathology of distal airways in stable moderate-to-severe asthma. These examinations found even higher numbers of activated eosinophils, lymphocytes, and chymase positive mast cells in distal as compared to proximal airways [9,10] that positively correlated with a decline in lung function [11,12]. In line with that further studies reported an increased expression of T helper (TH) 2 type cytokines like Interleukin (IL)-4 and 5 and chemokines like eotaxin and monocyte chemoattractant protein (MCP)-4 in distal airways [13,14]. Whether these findings indeed describe a stronger inflammatory response in distal airways or are eventually the result of an insufficient drug delivery to these airway sections remains elusive. However, they demonstrate that distal airways are by far not a “quiet zone” in asthma. This is especially true for their impact on lung physiology. In healthy individuals small airways only contribute about 10% to the total resistance of the entire airway system. In contrast, they contribute up to 60% to total airway resistance in asthma patients as demonstrated by direct bronchoscopic measurement of intrabronchial pressure [15]. This observation can directly be linked to asthma symptoms including airflow limitation, symptoms of dyspnea, cough, and wheezing that are all functional consequences of an increased airway resistance. And this is in turn caused by a combination of airway inflammation, broncho-constriction, and non-contractile mechanisms. The latter include changes of the airway structure (e.g. fibrosis and hyperplasia of smooth muscle cells and goblet cells), mucus hypersecretion, inflammation-related edema, and fibrin accumulation. Due to their structure, size and location distal airways are much more vulnerable to these factors than proximal airways. Hence, following Poiseuille’s law reduction of the luminal diameter of an airway leads to a disproportional increase of its resistance. Since the diameter of distal airways is per definition comparatively small and its resistance consequently high, any further reduction of the diameter carries the risk of premature closure. Furthermore, compared to proximal airways that contain cartilaginous elements the wall structure of small airways is much more fragile and is differentially subjected to

other mechanical influences. Thus, their opening depends among others on airway liquid surface tension and elastic recoil of the attached alveolar structures. Loss of these factors due to mechanical disruption of the mentioned structures or the diluting effect of plasma extravasation have been shown to increase airway resistance and air trapping in severe asthmatics [16]. The combination of any of these non-contractile factors with smooth muscle contraction, which is also markedly increased in distal airways of asthmatic patients as assessed by histamine provocation [17] could amplify airway resistance and consequently premature airway closure [18-20]. This can ultimately lead to the dramatic situation depicted by fatal asthma.

Altered and increased mucus production in distal airways seems to play a pivotal role in the formation of this life-threatening condition of asthma. While clinical studies demonstrated that patients dying from this disease displayed small airway plugging by mucoinflammatory exudates, a conglomerate of mucus, fibrin, plasma exudates, and inflammatory cells, a quite recent study mechanistically demonstrated the impact of mucus and its well-known component Muc5AC on lung function in mice. In different mouse models of experimental allergic asthma animals lacking this mucin did not only reveal diminished mucus hyperproduction and goblet cell hyperplasia, but surprisingly did not display an exaggerated airway response towards the methacholine (MCh) inhalation [21]. Whether this observation could really be traced back to an involvement of Muc5Ac in the development of AHR as suggested by the authors has not been proven. However, the most striking finding of this study was that wildtype animals undergoing provocation with the highest concentration of the secretagogue MCh displayed marked occlusion of distal airways by mucus plugs. This resembled the pathology observed in fatal asthmatics. In contrast, mice deficient for Muc5AC revealed impressively less mucus occlusion of distal airways suggesting mucus hypersecretion as a major factor in the pathogenesis of fatal asthma. Since only a small proportion of asthmatic patients indeed get in danger to develop fatal asthma, one could raise the question whether fatal asthma could be the dramatic result of a fundamentally deregulated mucus secretion in distal airways.

Mucus in general is a gel-like, viscous secretion composed of various macromolecules, inorganic salts and water. It can be found throughout the body on mucous membranes for example in the gastrointestinal, urogenital, visual, auditory, and respiratory systems. In humans the mucus of the respiratory tract is produced by goblet cells of the airway epithelium and submucosal glands. There, it functions as first line of defense against airway infection and plays a major role in mucociliary clearance of the airways. Pathogens, particles and other chemicals are caught up in a mucus layer covering beating cilia, which constantly sweep the mucus from distal to proximal airways to finally force it out of the lung [22-24]. This mucus layer is composed of a low-viscosity and therefore low resistance liquid layer up to the height of the cilia supporting the cilia beating and an overlying high-viscosity gel layer [23,25,26]. Healthy airway mucus consists of 98% water, 0.7% mucins, salts and other macromolecules, for example with anti-microbial activity [27]. The physical, viscoelastic property of mucus is determined by the quality and quantity of the mucins.

Mucins are high molecular weight glycoproteins with variable numbers of serine, threonine and proline rich repeats. Serine and threonine are sites of O-linked glycosylation for the peptide backbone [28,29]. From the 22 known human mucin genes 16 mucins genes are expressed in the lung, of which Muc1, Muc4, Muc5AC, Muc5B, and

Muc16 reveal the highest expression profiles [30-34]. There are two different types of mucins, the membrane-tethered mucins like Muc1, Muc4, and Muc16, which contribute to the periciliary layer, and the secreted gel-forming mucins like Muc5AC and Muc5B, which form the upper gel-layer and determine the mucus viscosity [28,35-37]. Whereas Muc5B is produced by both, goblet cells and the submucosal glands, Muc5AC is mainly produced by goblet cells and is widely used as marker for goblet cell metaplasia [38-41]. Furthermore, increased Muc5AC expression is induced during airway inflammation, whereas Muc5B expression is constitutive and remains unaltered [42,43].

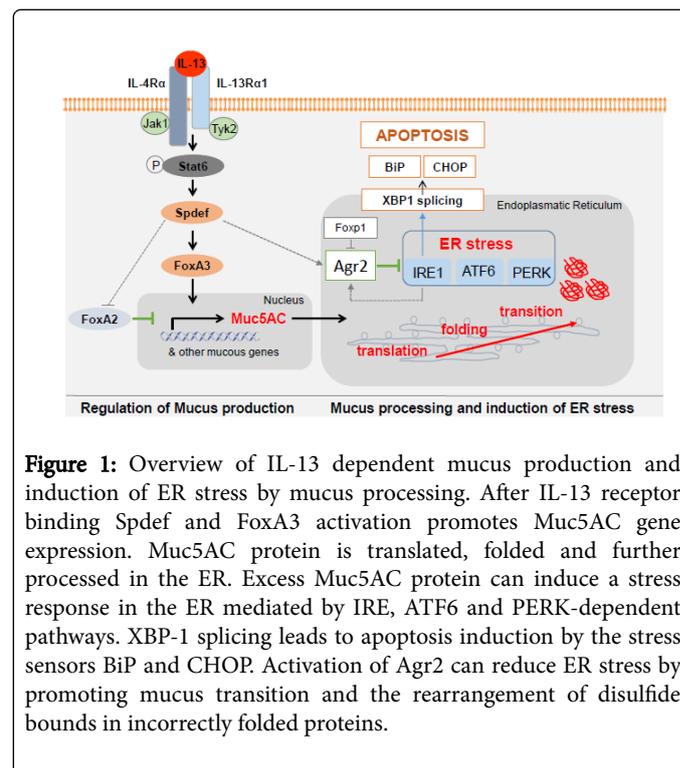


Figure 1: Overview of IL-13 dependent mucus production and induction of ER stress by mucus processing. After IL-13 receptor binding Spdef and FoxA3 activation promotes Muc5AC gene expression. Muc5AC protein is translated, folded and further processed in the ER. Excess Muc5AC protein can induce a stress response in the ER mediated by IRE, ATF6 and PERK-dependent pathways. XBP-1 splicing leads to apoptosis induction by the stress sensors BiP and CHOP. Activation of Agr2 can reduce ER stress by promoting mucus transition and the rearrangement of disulfide bounds in incorrectly folded proteins.

property for ciliary clearance mucins absorb more than 100-fold their mass of water after secretion due to the high water binding capacity of their polysaccharides [48,49]. One of the most potent inducers of Muc5Ac and thus of GCM and mucus hypersecretion is IL-13. The IL-13 dependent regulation of mucus production and subsequent mucus processing is depicted in Figure 1. This cytokine mainly produced by TH2 cells is capable of inducing all hallmarks of experimental asthma in mice also including AHR and allergic airway inflammation. In our previous work we were therefore interested in the IL-13-dependent regulation of mucus production in different sections of the airway tree.

In a mouse model of OVA-induced experimental asthma, we made the apparently contradictory observation that goblet cells and mucus production were nearly absent in distal airways although Club cells were present and inflammatory cell infiltration and the expression of the TH2 cytokine IL-13 in distal airways was as high as in proximal airways [50]. We further found that epithelial cells of distal airways express significantly less IL-13 receptor alpha 1 (IL-13R α 1), a strictly regulated receptor chain that heterodimerizes with IL4R α to form the IL-13 receptor. Consequently, SAM-pointed domain ETS-like factor (Spdef) and Forkhead box A3 (FoxA3), key factors involved in GCM and mucus production, were also less expressed in distal airways contributing to the diminished GCM and mucus production. Based on these data we suggested that epithelial cells of distal airways could be less sensitive towards IL-13 signaling and consequently for the induction of GCM and mucus production, so that these cells are less capable to produce excessive amounts of mucus. Such insensitivity towards IL-13 could therefore represent a mechanism protecting distal airways from mucus occlusion and airway trapping. We further wondered if this would be the only mechanism to prevent such a physiologically dangerous outcome in distal airways.

It has previously been demonstrated that excessive production of mucins leads to stress responses in the ER, the so called unfolded protein response (UPR) [28,51], and that this is necessary to maintain proper folding of mucins and therefore the secretory capacity of goblet cells (Figure 1) [52-54]. Excess ER stress can further increase inflammation by activation of nuclear factor kappa B (NF κ B) and CAAT-enhancer-binding protein homologous protein (CHOP) and can even induce apoptosis. In order to prevent cell death, UPR pathways are activated. In eukaryotic cells three UPR pathways exist: (1) inositol-requiring enzyme 1 (IRE1), (2) activating transcription factor 6 (ATF6), and (3) PKR-like ER kinase/pancreatic eIF2 α kinase (PERK) [55,56]. Upon accumulation of unfolded proteins or induction of ER stress, GRP78, glucose regulated protein 78 (BiP) acts as a chaperone and UPR becomes activated by the release of sensor proteins [57]. Therefore, the induction of UPR is essential for the maintenance of cellular health and mucus production. As already mentioned IRE1 proteins are important ER stress sensors. A comprehensive study by Martino et al. described IRE1 β to be also involved in GCM and mucus production. Accordingly, IRE1 β , expressed in mouse and human airway epithelial cells, mediates mucin production and is up-regulated in airways of asthmatics. The application of OVA-induced experimental asthma in IRE1 β knock-out mice resulted in attenuation of mucus production independently from the inflammatory response, suggesting an important role of IRE1 β in mucus production. Increased IRE-1 β expression mediates IL-13 dependent splicing of the ER stress associated XBP-1 gene linking mucus production and ER stress. By this, IRE1 β also regulates anterior gradient protein homolog 2 (Agr2), which belongs to the protein disulfide isomerase (PDI) family and resides to the ER [51,58]. PDI

proteins support the transition of proteins to the ER by the rearrangement of disulfide bonds in incorrectly folded substrate proteins [59]. Agr2 was initially described to play a role in the production of the intestinal mucus protein Muc2 [60], but also as a chaperone required for mucin packaging [61]. Beside its role in the intestine, Agr2 is also expressed in mucus-producing goblet cells and localizes with Muc5AC during airway GCM in a mouse model of experimental allergic asthma. Thus, Agr2 deficient mice display impaired export of mucins from the ER and no activation of UPR [61-63]. These studies support the role of Agr2 in reducing ER stress during the production of large amounts of mucins. Consistent with its role in mucus processing the expression of Agr2 in airway epithelial cells is regulated *via* the IL-13-dependent STAT-6 pathway and Spdef [61,63]. Recently, Foxp1 was identified as another transcriptional regulator of Agr2. Expression of Foxp1 in the murine lung acts as suppressor of GCM by repression of Agr2 [64,65]. Overwhelming ER stress caused by inappropriate regulation of mucus processing eventually leads to the induction of the stress proteins BiP and CAAT-enhancer-binding protein homologous protein (CHOP) [54].

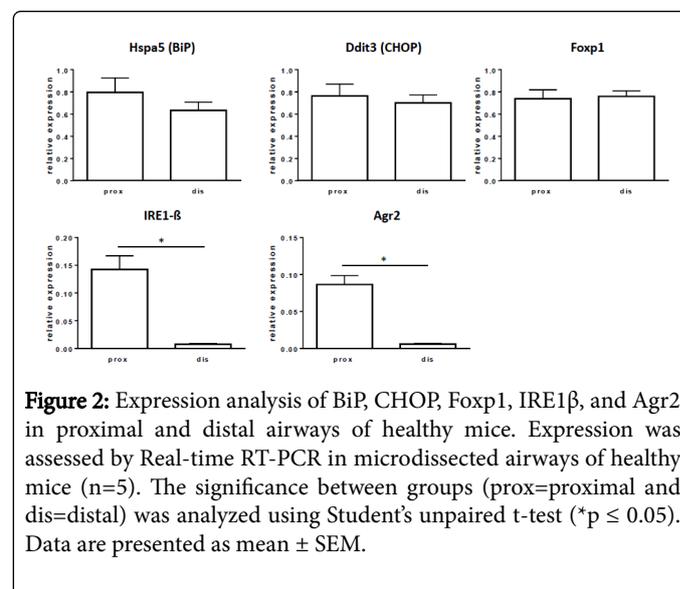


Figure 2: Expression analysis of BiP, CHOP, Foxp1, IRE1 β , and Agr2 in proximal and distal airways of healthy mice. Expression was assessed by Real-time RT-PCR in microdissected airways of healthy mice (n=5). The significance between groups (prox=proximal and dis=distal) was analyzed using Student's unpaired t-test (*p \leq 0.05). Data are presented as mean \pm SEM.

Here we aimed to further extend our work on the role of distal airways in asthma and hypothesized that diminished mucus production in distal airways is not only caused by attenuated IL-13R α 1 expression and signaling but also by an inappropriate capability to process excessive amounts of mucins. Therefore, we performed real-time RT-PCR analysis on microdissected proximal and distal airways from healthy mice. We assessed mRNA levels of IRE1 β , Agr2, Foxp1, BiP and CHOP in proximal and distal airways of healthy mice (Figure 2). No difference in Foxp1, BiP, and CHOP mRNA levels between proximal and distal airways are observable implicating the absence of ER stress as expected under steady-state conditions. However, IRE1 β and Agr2 show significantly lower mRNA levels in distal airways compared to proximal airways. One could speculate that the reduced expression of these members of the ER stress protection system could be associated with a reduced capacity to handle large amounts of misfolded proteins including mucins. On the one hand this could be interpreted as a limited ability of distal airway cells to produce mucus. On the other hand it could just be an economical adaption of these cells to their diminished readiness to develop into goblet cells due to a special degree of insensitivity towards IL-13. From another point of

view one could even speculate that this could further be a mechanism contributing to the protection of distal airways from mucus plugging. Consequently, epithelial cells from distal airways could rather undergo apoptosis instead of producing large amounts of mucus that harbors the risk of airway occlusion.

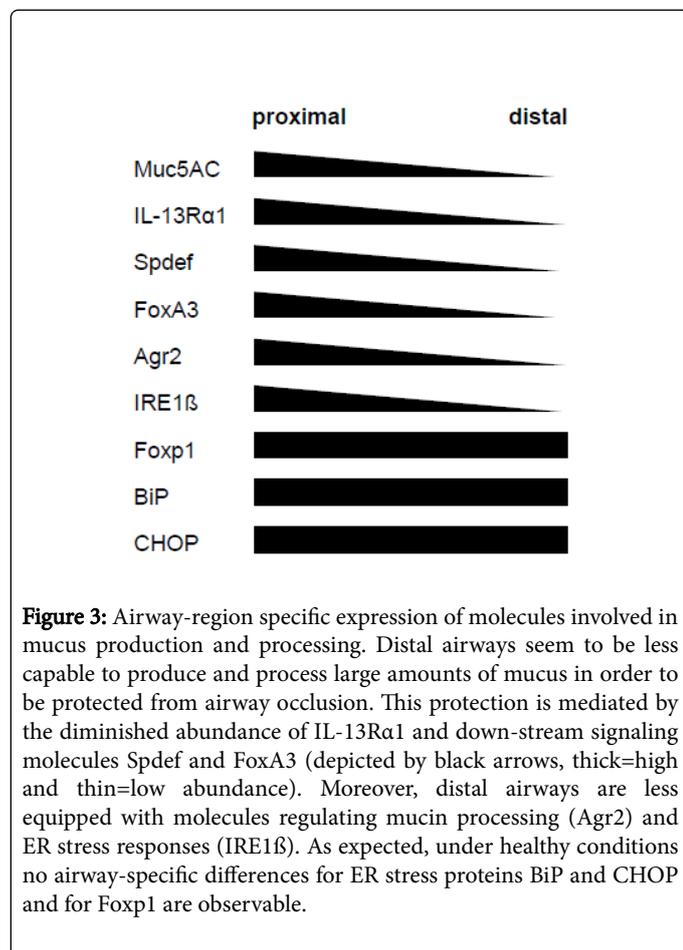


Figure 3: Airway-region specific expression of molecules involved in mucus production and processing. Distal airways seem to be less capable to produce and process large amounts of mucus in order to be protected from airway occlusion. This protection is mediated by the diminished abundance of IL-13Rα1 and down-stream signaling molecules Spdef and FoxA3 (depicted by black arrows, thick=high and thin=low abundance). Moreover, distal airways are less equipped with molecules regulating mucin processing (Agr2) and ER stress responses (IRE1β). As expected, under healthy conditions no airway-specific differences for ER stress proteins BiP and CHOP and for Foxp1 are observable.

In conclusion, we provided further evidence that epithelial cells of distal airways are indeed different from those of proximal airways: These cells are not only comparatively insensitive towards induction of mucus hypersecretion by IL-13 and but also reveal a diminished UPR indicating a reduced capacity to manage excessive production of proteins and mucus (Figure 3). Thus, both observations could somehow represent mechanisms protecting distal airways from mucus hyperproduction and consequently from occlusion. In turn, impairment of these mechanisms by inherent genetic or exogenous factors (e.g. airway infections) in patients with chronic inflammatory airway diseases could therefore increase the risk of life-threatening events like fatal asthma.

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