CHAPTER 7

Summary, general discussion,
conclusions and future perspectives

Summary

Chronic Obstructive Pulmonary Disease (COPD), a chronic respiratory disease, is one of the leading causes of death today, with a worldwide increase in incidence. It is currently the fourth leading cause of death (97, 98). The majority of COPD patients (excluding alpha1AT deficiency) are characterized by a chronic inflammatory response in the lungs in response to noxious particles and gasses, including cigarette smoke, leading to accelerated lung function decline. Epithelial cells are the first line of defense against inhaled insults such as cigarette smoke. In this thesis we investigated how epithelial cells respond to oxidative stress, specifically with respect to mitochondrial function, and whether mitochondrial dysfunction contributes to the pathogenesis of COPD (5, 6, 15, 72, 80). Because only 10-15% of the smokers eventually develop COPD, we propose that a genetic susceptibility contributes to the onset and progression of the disease (21), leading to overproduction or poor neutralization of ROS. This is supported through GWAS and specific gene association studies (12, 29, 64, 65, 73, 74) as well as familial aggregation studies (52, 61, 75), which may contribute to the onset or progression of the disease. In contrast to asthma, where inhaled glucocorticoids (IGC) are able to suppress inflammatory responses, glucocorticoid (GC) treatment is less beneficial in COPD patients. Reduced sensitivity to IGCs has been clinically associated with neutrophilic airway inflammation. Although neutrophils are considered relatively unresponsive to glucocorticoids, the production of pro-inflammatory cytokines/chemokines involved in neutrophilic infiltration, e.g. CXCL8, by airway epithelium is normally suppressed by corticosteroids (24). However, recent studies have shown that macrophages (37) and bronchial epithelial cells (30) of COPD patients are less responsive to GC, which may thus lead to the development of GC-insensitive airway inflammation. Here, oxidative stress has been implicated as mediator of the observed GC unresponsiveness (30, 44, 69). GCs normally exert a broad spectrum of anti-inflammatory effects after binding to their GC receptor (GRα). After ligand-binding, translocation of the receptor to the nucleus suppresses pro-inflammatory gene transcription through the recruitment of histone deacetylases (HDACs), in particular HDAC2. HDAC2 is able to induce deacetylation of histones containing inflammatory genes, thereby restricting access of the transcriptional machinery and inhibiting transcription (4). It has been shown in COPD macrophages that HDAC2 expression and activity is reduced, and therefore unsuccessful in suppressing the inflammatory response (18, 36, 37). Furthermore, overexpression of HDAC2 in macrophages could overcome GC insensitivity (38). Increased expression of the dominant negative form of the receptor, GRβ, which is seen in steroid resistant neutrophils, may also contribute to GC insensitivity in other cells (81). Furthermore, GCs can directly influence gene transcription and are able to bind to glucocorticoid response elements (GREs) that are present in the promoter of several anti-inflammatory genes (17). Here, posttranslational modification of the GR, by phosphorylation, nitration, acetylation or ubiquitination may

influence the receptors function and induce GC insensitivity. Cigarette smoke has been shown to induce oxidative stress-dependent activation of p38 MAPK pathway, resulting in GR phosphorylation and inhibiting its translocations to the nucleus (50). Oxidative radicals can either derive from cigarette smoke directly or be generated intracellularly mainly by mitochondria (43). The long term-effects of smoking on mitochondrial function have never been thoroughly investigated in COPD, although some studies that indicate mitochondrial function changes caused by acute cigarette smoke exposure, resulting in OXPHOS and apoptotic abnormalities (77, 84, 85). This thesis aimed to assess the effects of long-term cigarette smoke-induced oxidative stress on GC responsiveness as well as on mitochondrial structure and function in bronchial epithelial cells and the relation between these processes in COPD. Furthermore, we used a novel technique, lipidomics, to monitor cigarette smoke-induced and/or disease related-changes in sputum from COPD patients as well as in long-term cigarette smoke exposed bronchial epithelial cells.

To address our hypothesis that cigarette smoke-induced oxidative stress in lung epithelial cells results in mitochondrial defects, in ***chapter 2*** we studied the effect of long-term cigarette smoke exposure on mitochondrial function and morphology in human bronchial epithelial cells. We were especially interested in the long-term effects of CSE, because COPD usually develops after smoking for 20 years or longer. Surprisingly, most in vitro studies rely on relative short cigarette smoke exposure (ranging from 15 min to 48 hours) to understand the acute effects of cigarette smoke on cells that are thought to play key roles in COPD disease. We know from previous studies that long-term cigarette smoke exposure induces changes in the airway epithelial layer, with increased mucus cell hyperplasia and decreased numbers of cilia and ciliated cells (57, 76). In addition to these changes, we hypothesized that long-term cigarette smoke exposure induces mitochondrial dysfunction in airway epithelial cells. In long-term CSE-exposed human bronchial epithelial BEAS-2B cells, we observed increased expression of oxidative stress marker MnSOD and of the inflammatory cytokines CXCL8, IL-1β and IL-6 at mRNA and protein level, which was accompanied by a damaged mitochondrial phenotype including increased fragmentation and branching, and reduced numbers of cristae. All these changes, except for fragmentation, remained present after CSE depletion, suggesting that these are persistent upon smoking cessation. This is in line with the increased mRNA expression levels of OPA1, a critical regulator of cristae formation and fission/fusion, observed in these long-term CSE-exposed cells. We compared the findings in long-term cigarette smoke-exposed BEAS-2B cells with mitochondrial abnormalities in bronchial epithelial cells derived from ex-smoking COPD patients. Here we observed striking similarities, including cristae depletion and increased mitochondrial branching and fragmentation when compared to cells from non-smoking individuals. Also, in bronchial epithelial cells from current smoking COPD patients, we observed higher mRNA expression levels of mitochondrial damage markers PPARGC1α and PINK1 than in cells from

nonsmoking controls, with an intermediate level of these markers in cells from smoking controls. Both markers are involved in mitochondrial biogenesis and clearance, suggesting an increased mitochondrial turnover due to damage in epithelial cells from COPD patients. Together, our data from this study show that long-term exposure to CSE leads to structural and functional changes in mitochondria that persist upon smoking cessation which could all contribute to (the onset of) COPD disease by inducing chronic inflammation and hamper tissue repair.

In ***chapter 3***, we investigated the mechanism of cigarette smoke-induced GC unresponsiveness in airway epithelial as well as inflammatory cells. We hypothesized that exposure to cigarette smoke induces GC unresponsiveness by inducing oxidative stress, especially in innate immune cells (e.g. epithelial cells and macrophages) of COPD patients. Previous studies already linked an increased burden of oxidative stress to GC responsiveness in COPD patients (15, 30, 44, 69). We recently observed that oxidative stress reduces GC responsiveness in bronchial epithelial cells and that epithelial cells from COPD patients display reduced GC sensitivity compared to those from non-smokers, with an intermediate effect on control smokers (30). In chapter 3, we confirm that the treatment of epithelial cells with cigarette smoke extract induces oxidative stress, and that this leads to reduced GC responsiveness in bronchial epithelial cells. We next assessed whether GSK3β, a multi-functional redox-sensitive serine/threonine kinase, may be involved in oxidative stress-induced GC unresponsiveness in COPD. Importantly, we found that bronchial epithelial cells and monocytes in lung tissue from smokers and COPD patients display higher levels of phosphorylated GSK3β than in non-smoking controls, a finding that was recapitulated in isolated epithelial cells. We observed that (CSE-induced) oxidative stress is capable of inactivating GSK3β by phosphorylation at serine-9, and that CSE also reduces GC responsiveness in bronchial epithelial cells. We also observed that the pharmacological inhibition of GSK3β leads to reduced GC responsiveness, with a loss of suppressive effects on pro-inflammatory cytokine production. Thus, the increased levels of phopsho-GSK3β in epithelial cells and monocytes may be related to the reduced responsiveness that has been observed in COPD. With respect to the involved mechanism, several studies proposed that oxidative stress-induced histone deacetylase 2 (HDAC2) inactivation in oxidative stress is involved in GC unresponsiveness (4, 5, 17, 53, 93). Although loss of HDAC2 activity appeared, at least in part, responsible for the loss of GC responsiveness upon GSK3β inactivation in monocytes, our data showed that a phospho-p65-dependent mechanism is more likely involved in bronchial epithelial cells (Chapter 2 Supplement). Thus, although the functional output of GSK3β inactivation was similar in inflammatory and structural cells, the downstream mechanisms involved were actually cell-specific.

GSK3β is involved in the regulation of several mitochondrial processes (13, 51, 59, 79) and in chapter 2 we have shown that cigarette smoking affects mitochondrial function and morphology in lung epithelial cells. This raised the question whether mitochondrial dysfunction in these cells contributes to reduced GC responsiveness as well as chronic airway inflammation and impaired lung tissue repair. In ***chapter 4*** of this thesis, we studied the effect of mitochondrial dysfunction on pro-inflammatory responses, barrier function, wound healing and GC responsiveness in lung epithelial A549 cells depleted of mitochondrial DNA (Rho-0). We have shown that the depletion of mtDNA induces an increase in the baseline production of the pro-inflammatory cytokine IL-6 and chemokines CXCL8 (IL-8), CCL20, CCL3 (MIP1α) and CCL4 (MIP1β). When assessing the suppressive effect of the inhaled glucocorticoid budesonide on the pro-inflammatory response, we observed that the A549-Rho0 cells, depleted of mtDNA, are insensitive to budesonide, in contrast to A549 wild-type cells. In addition, we observed that GCs were unable to significantly improve the barrier function of A549-Rho0 cells, in contrast to A549 wild type cells, while the wound healing response of A549-Rho0 cells was also impaired. Increased lactate levels in A549-Rho0 cells indicated activity of a compensatory secondary metabolism, glycolysis. PI3K, a mediator of the glycolysis, has been linked to GC unresponsiveness in COPD (35, 48, 49). Therefore, we studied the effect of a pharmacological PI3K/Akt inhibitor and observed that this induced a significant decrease in the release of CXCL8. Moreover, in the presence of the Akt inhibitor, the unresponsiveness of A549-Rho0 to budesonide was completely reversed, the suppressive effect on CXCL8 almost returning to the effect in wild type cells. This suggests a role for increased PI3K/Akt signaling in GC unresponsiveness in cells harboring a mitochondrial dysfunction. Together, our data from this chapter demonstrate that mitochondrial dysfunction in epithelial cells may have important consequences for COPD, leading to increased pro-inflammatory activity that cannot be reduced by glucocorticoids and impaired restoration of barrier function upon injury. Thus, the reduced mitochondrial function as observed in epithelial cells from COPD patients (chapter 2) could contribute to the reduced GC responsiveness previously observed in these cells (30).

In ***chapter 5***, we used a novel methodology, lipidomics, to evaluate whether lipid levels in induced sputum of COPD patients may serve as specific markers for COPD. This was especially of interest as changes in lipid metabolism may reflect changes in mitochondrial function. This because the mitochondrial matrix is responsible for fatty acid degradation, a process in which fatty acids are broken down, resulting in release of energy. Induction of sputum by inhaling hypertonic saline has shown to be a useful tool to investigate obstructive airway diseases. Also, in elderly patients with COPD it is considered to be a safe tool for assessing airway inflammation (7, 23), although a disadvantage of induced sputum is that the cellular origin of the lipid compounds cannot be determined. We were able to identify

more than 1,500 lipid compounds in induced sputum of the participating individuals. Levels of sphingolipids, a major class of lipids, were significantly higher in smokers with COPD than in smokers without COPD. Of these, we have identified more than 200 known single lipid compounds: 168 sphingolipids and 36 phosphatidylethanolamine lipids. In addition, 5 tobacco-related compounds were significantly higher in smokers with COPD compared to smokers without COPD. This latter observation may reflect the differences in pack-years between these groups. In comparison to the never-smoking controls, only 20 (glyco)sphingolipids and 6 tobacco-related compounds were higher expressed in smokers without COPD. After two-months of smoking cessation, we observed a reduction in expression of only 26 sphingolipids in smokers with and without COPD, while the other 175 were still significantly enhanced compared to non-smokers, indicating persistent changes in lipidome profile. In line with these results, several histopathological studies suggest that airway inflammation persists in ex-smoking individuals, although other studies indicate that smoking cessation improves respiratory symptoms and bronchial hyper-responsiveness (90, 91). We cannot exclude that longer periods of smoking cessation will eventually lead to lower expression of additional individual lipid compounds in induced sputum, which requires further investigation. Additionally, we found only one lipid component to be reduced in induced sputum of smokers and COPD patients compared to non-smokers, which was identified as neuraminic acid, a terminal monosaccharides modification of most glycoconjugates and glycosphingolipids. After smoking cessation, neuraminic acid levels were restored, further indicating that its increase was due to cigarette smoking and not a disease-related effect. To conclude, expression of lipids from the sphingolipid pathway is higher in smokers with COPD compared with smokers without COPD.

Many different lung cell types including epithelial cells, macrophages and neutrophils, may be responsible for the observed changes in lipid compounds, either upon active secretion or release of their lipid (membrane) compounds into the lung sputum upon necrotic cell death. The release of lipids may contribute to airway inflammation (86, 92). Free fatty acids can activate TLR-4, thereby inducing similar inflammatory responses as lipopolysaccharides (47). In line with this, we found a correlation with inflammation in sputum and lung function decline for the 13 lipids with the highest increase in smokers with and compared to those without COPD. Furthermore, lipids released from the sphingolipid pathway, such as ceramides, are major players in the induction of apoptosis and cellular senescence, and both cellular responses are increased in the lungs of COPD patients (28, 86). Additionally, cigarette smoke-induced changes in lipid accumulation and/or composition have been reported to be harmful to the cell as they compromise integrity of cell membranes and organelle membranes, including those from mitochondria (47, 92). Thus, the observed changes in lipid profile of smoking COPD patients may be related to the abnormalities in mitochondrial structure in epithelial cells from COPD patients. There are already initial

studies that use lipidomics and airway epithelial cells for determining airway disease (95).
As a follow-up on chapter 5, in ***chapter 6*** we studied whether epithelial cells may be responsible for the observed changes in lipid profile in sputum of smokers, by assessing how long-term cigarette smoke exposure affects the intracellular lipidome expression in human bronchial epithelial cell line BEAS-2B. In this study we showed that lipids from the sphingolipid pathway are altered by long-term CSE exposure in epithelial cells, which is in line with our findings in chapter 5, indicating that the sphingolipids in sputum from smoking individuals may be derived from airway epithelial cells. In further line with our data from chapter 5, we observed significant alterations in 9 sphingomyelins of which 2 components were up-regulated and 7 were down-regulated in long-term CSE-exposed BEAS-2B cells. Additionally, we found significant changes in 5 ceramides, of which 2 were up-regulated and 3 were down-regulated, and we observed and a reduction in 3 neuraminic acid containing lipid components belonging to the class of glycosphinogolipids. Thus, cigarette smoke exposure strongly reduces neuraminic acid containing lipid components both in vivo in sputum and in epithelial cell in vitro. N-acetylneuraminic acid is one of the most important members of this sugar family and is involved in a variety of biological interactions including cell adhesion and migration (42). Moreover, it is an important addition to mucins and has shown to be an antioxidant scavenger (34). Therefore, we expect that alterations in its levels may have implications in cell infiltration and aberrant cellular repair responses of COPD patients. Nine other glycosphinogolipids belonging to the same lipid class showed strong up-regulation upon CSE exposure, especially lipids belonging to N-acetylhexosamine-trihexosyl-ceramides. In line with the observed upregulation of sphingomyelins, sphingomyelinase activity, as determined by the ratio of ceramides and sphingomyelin, showed increased activity in epithelial cells upon long-term exposure to CSE. Furthermore, we observed that CSE exposure lowered the expression of the lipid converting enzymes SGMS2, FASN and SMPD1, which could be responsible for the observed CSE-induced changes in lipid profile in bronchial epithelial cells. We anticipate that especially the CSE-induced changes in sphingolipid compounds may have important consequences, as they regulate various cellular processes like apoptosis, regeneration and senescence (26, 28, 92), all of which are of relevance to COPD.

General discussion

Together, our study shows that cigarette smoke-induced oxidative stress induces mitochondrial function and morphology changes and that epithelial cells from COPD patients display mitochondrial abnormalities. We hypothesized that oxidative stress results in stronger mitochondrial damage in epithelial cells from COPD patients than control smokers as a consequence of impaired antioxidant responses (15, 22). Normally, cigarette smoking induces an upregulation of antioxidant genes. Increased expression of 16/44 antioxidant-related genes has been observed in airway epithelium from smokers compared to non-smokers (27), while levels of the ROS scavenger Vitamin E were reduced with disease severity in lung tissue of COPD patients (1). Mitochondrial damage may be related to increased pro-inflammatory activity and reduced GC responsiveness in COPD, as depletion of mitochondrial DNA in lung epithelial cells resulted in loss of suppressive effects of glucocorticoids on epithelial pro-inflammatory cytokine production. In addition, mitochondrial dysfunction led to increased glycolysis, which has been associated with increased PI3K activity. PI3K has been implicated in oxidative-stress induced GC unresponsiveness (48, 49). Indeed, we observed that inhibition of PI3K/Akt activity restored the responsiveness of pro-inflammatory epithelial responses to GCs in mtDNA-depleted cells. Of note, PI3K can induce phosphorylation of GSK3β at Ser-9 (94), which results in a reduction of GSK3β activity. We further demonstrated that this inhibitory phosphorylation of GSK3β mediates GC insensitivity in bronchial epithelial cells, and that levels of phosphorylated GSK3β are increased in bronchial epithelial cells of COPD patients. Of interest, it is well known that inhibition of GSK3β activity stimulates glycolytic processes (87), supporting the notion that it may play a role in mitochondrial function. Mitochondria may also be important as sensors of cellular stress by quantitating the local accumulation of specific lipids and glycolipids and initiate stress-induced apoptosis (83). Sphingolipids have been implicated in apoptotic and autophagy processes (46). Accumulation of the sphingolipid ceramide can affect mitochondrial functions either direct or indirectly (82, 83). In early stage apoptosis, a ganglioside (GD3) is actively synthesized and relocates to the mitochondrial membrane initiating apoptosis by opening the transition pore complex (83). Thus, it is tempting to speculate that the cigarette smoke-induced changes in lipid compounds may contribute to the observed mitochondrial abnormalities in COPD and thus the pathogenesis of the disease. We observed higher levels of lipids from the sphingolipid pathway in smokers with COPD compared with smokers without COPD. Specifically, ceramides has been shown to induce alveolar epithelial apoptosis and alveolar enlargement in a mouse model (63). Furthermore, Bowler and co-workers recently reported an association between sphingomyelins and emphysema and between glycosphingolipids and COPD exacerbations (8). Together, we propose that cigarette smoke-induced oxidative stress and changes in lipidome lead to mitochondrial damage and a switch to glycolysis,

which is accompanied by the activation of PI3K/Akt, with subsequent activation of pro-inflammatory pathways and inactivation of GSK3β, leading to reduced responsiveness to glucocorticoids. In turn, we show that a proper mitochondrial function is important for restoration of the epithelial barrier upon injury, and may thus play a role in the abnormal tissue repair, another important pathological feature of COPD. Excessive ROS production by damaged mitochondria may increase pro-inflammatory signaling (e.g. the PI3K/Akt pathway) and further damage mtDNA components, leading to further impairment of mitochondrial function and enhancement of pro-inflammatory responses (chapter 2, (33)). Mitochondrial impairment in epithelial cells from COPD patients is supported by our finding that the expression of mitochondrial biogenesis gene expression marker PPARGC1α was increased in epithelial cells from COPD patients compared to control smokers. This indicates a necessity for generating new mitochondria in epithelial cells from COPD patients. In contrast to epithelial cells, it was found lowered in skeletal muscle from COPD patients (70). Another mitochondrial damage marker, PINK1, was also significantly increased in epithelial cells from COPD patients compared to non-smokers, although not when compared to control smokers. This is in line with a recent study showing elevated levels of PINK1 in epithelium of COPD patients. Here, authors also showed that PINK1 knock-out mice are protected from cigarette smoke-induced COPD manifestations including airspace enlargement (54).

We have not studied effects of cigarette smoke on cell death in epithelial cells from COPD patients this thesis, but increased epithelial apoptosis has also been observed in COPD (11). GSK3β is able to mediate apoptosis through p53 and BAX activation, triggering the caspase cascade and induce programmed cell death (20, 32, 39, 59). p53 can also influence cell metabolism at specific points, notably through the up-regulation of glutamate synthesis and inhibition of fatty acid synthesis and glycolysis (41, 55, 68). GSK3β, together with the Akt-dependent kinase tau protein kinase 1, is able to interact with pyruvate dehydrogenase (PDH), thereby reducing levels of acetyl-CoA and blocking the TCA cycle, thus impacting on mitochondrial respiration (25, 40). Additionally, GSK3β can negatively regulate several aspects of insulin signaling and thus limit the uptake of glucose (71). Inactivation of GSK3β promotes glucose uptake and together with reduced levels of PDH will generate a metabolic shift towards glycolysis (20, 31). We speculate that especially the effects of GSK3β on the apoptotic response might force the cell to go into necrosis, which may subsequently result in the release various cellular components, including membrane lipids and damage associated danger signals (66) into the environment, contributing to the chronic inflammatory response.
An overview of these processes is illustrated in *figure 1*. Taken together, we expect that more knowledge about mitochondrial function, GSK3β regulation and lipid release will greatly contribute to improve our insight in the inflammatory processes and cellular regeneration

of COPD disease and might improve the method of treatment and the outcome of this general permanent and progressive disease. This may provide new insights in the role of mitochondria in the development and treatment of **C**hronic **O**bstructive **P**ulmonary **D**isease.

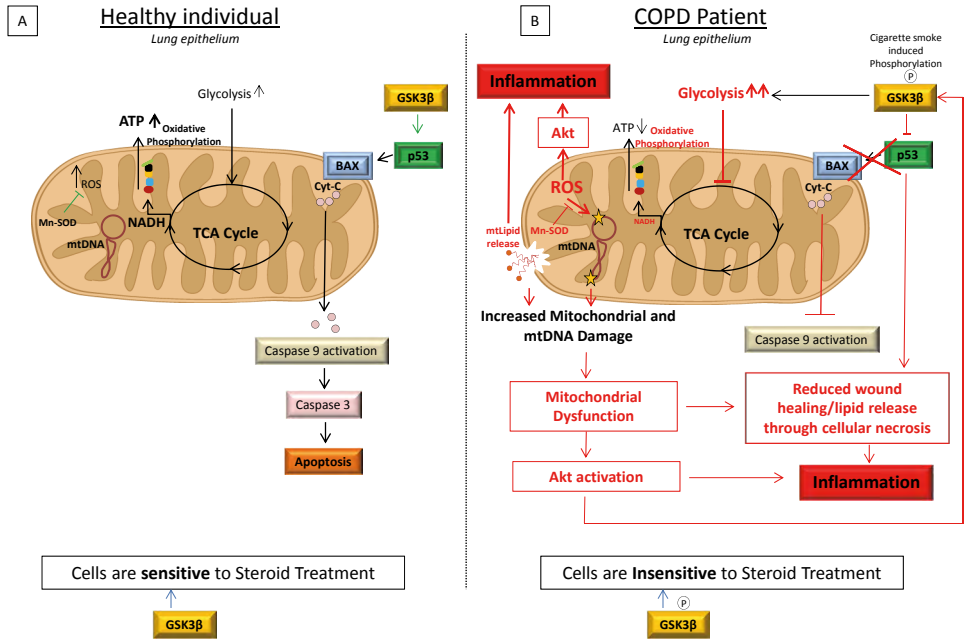

Figure 1. Model of molecular mitochondrial processes in A) lung epithelial cells from healthy individual and B) lung epithelial cells from COPD patient. A) In epithelial cells from healthy individuals, the majority of ATP is produced by oxidative phosphorylation and an adequate anti-oxidant response (e.g. Vitamin E, Catalase, MnSOD) will neutralize the produced reactive oxygen species. A balanced active and inactive form of GSK3β will ensure limited glycolysis, steroid sensitivity and a proper apoptotic response. B) Hypothetical model of the effects of mitochondrial damage in epithelial cells of COPD patients. Increased ROS production and/or an inadequate anti-oxidant response can trigger an inflammatory response and further mitochondrial damage. Mitochondrial dysfunction can lead to cellular decay, amplify the pro-inflammatory response, and lead to impaired wound healing upon injury. Inactivation of GSK3β reduces steroid responsiveness, and stimulates the glycolysis and can block cells from going into mitochondrial induced apoptosis, potentially inducing necrosis, leading to the release of lipid components.

Future perspectives

For a COPD patient, the moments that "take your breath away" are worst in life. COPD is a slow progressive disease and especially the end stage is characterized by very severe airflow limitation and exacerbations that are sometimes life-threatening. A severely impaired quality of life is also common at this stage. Smoking cessation and improvement of physical health until now are considered the only effective interventions inhibiting the further progression of COPD; in particular the further decline in lung function, however, inflicted lung damage cannot be restored. Current treatment of COPD is based on suppressing the inflammatory response in the lung using corticosteroids but this appears to exert little benefit because of an impaired response mechanism. Current research aims to improve the sensitivity of corticosteroids studying the involvement of reactive oxygen species and HDAC2 activity. Studies have shown that oxidative stress induces steroid insensitivity mediated by the activation of Akt and the inhibition of HDAC2 activity (4, 17, 36, 38, 48, 53). Our study supplements these results as we have shown that Akt is indeed involved in GC unresponsiveness in mtDNA-depleted cells, however, we found only partial HDAC2 involvement in steroid insensitivity in human monocytes and no involvement of HDAC2 in bronchial epithelial cells. We did identify a novel player regulating GC response, GSK3β, in both cell types. Pharmacological inhibition of GSK3β led to complete steroid insensitivity in cultured human monocytes and bronchial epithelial cells. Future studies should focus on preventing inactivation/phosphorylation of GSK3β when using corticosteroids to treat COPD patients. Additionally, it would be of interest to study the possible link and role of GSK3β and mitochondrial function. It might be interesting to study the effect of anti-oxidants and/or an Akt inhibitor on the inflammatory response in combination with GC treatment. (Mitochondrial targeted) antioxidants may also prove beneficial in protecting the mitochondria against excessive ROS production. Ahmad *et al*. have shown that mitochondrial targeted antioxidants greatly improved impaired mitophagy, a phenotype observed in mouse lungs with emphysema as well as in lungs of chronic smokers and patients with COPD (2). Additionally, protection of the mitochondria and stimulating the formation of new mitochondria might be a novel therapeutic strategy for COPD. In this respect, substances like L-carnitine, lipoic acid, and coenzyme Q10 are known to protect mitochondria, while pyrroloquinoline quinone (PQQ) has been shown to stimulate mitochondrial biogenesis by increasing the expression of PPARGC1α (14, 78). We observed that mRNA expression of PPARGC1α is increased in epithelial cells from COPD patients potentially as consequence of an unsuccessful attempt to upregulate mitochondrial biogenesis. Reducing the formation of ROS may also prove beneficial in preventing GSK3β phosphorylation and thus protect mitochondria and promoting the sensitivity to GCs.

In the future, it will further be of interest to explore abnormalities in autophagy/mitophagy processes in lung epithelial cells of COPD patients. In chapter 2, we imaged epithelial cells of COPD patients and found structural and functional abnormalities in the mitochondria. Normally, when autophagy/mitophagy is hampered, defective mitochondria accumulate. The persistent presence of the defective mitochondria in epithelial cells from COPD patients raised the question whether clearance by mitophagy could be disturbed. A recent study showed that mitophagy-dependent necroptosis contributes to the progression of COPD (54). Also, it has been shown that when autophagy is hampered, this leads to increased release of the pro-inflammatory cytokine IL-1β upon mtDNA exposure. Enhanced levels of IL-1β have been observed in COPD and disturbed autophagy may thus have important consequences for the inflammatory response in COPD (3, 9, 56). mtDNA is also able to induce various inflammatory cytokines next to IL-1β including CLCX8, IL-6 and TNF-α (10, 45, 60, 62, 96). These cytokines can also be released by exposure through cigarette smoke directly or indirectly due to the induction of necrotic cell death leading to the release of DAMPs in the environment. It would be interesting to study whether pro-inflammatory responses of epithelial cells are reduced upon cigarette smoke or DAMP exposure when the autophagy/mitophagy process is blocked.

In addition, restoring the neuraminic/sialic acid concentration in respiratory mucus might be a promising therapeutic strategy. The airway epithelium is covered by a renewable layer of gelatinous mucus. Mucus lining the respiratory tract is thought to act as scavenger of free radicals, consisting mostly of water, salt, lipids, ascorbic acid, reduced glutathione (GSH) and mucin (19, 58, 67). Mucins are large neuraminic acid or sialic acid containing glycoproteins present at the interface between epithelium and their extracellular environment. A recent study indicated that sialic acid is the key component in hydroxyl oxidant savaging capacity of mucins (58). Also, sialic acid has been proposed to inhibit viral entry by mimicking sialylated receptors on the cell surface, thus a reduction of sialic acid in the mucus lining could increase the susceptibility for viral infections (16). This may lead to more frequent COPD exacerbations, which are often associated with viral or bacterial infections (88, 89). Neuraminic/Sialic acid containing components were the only components lowered in our lipidomic study (chapter 5). Lowered levels were found in smokers as well as COPD patients, indicating that this was caused by cigarette smoking. This is in line with our prolonged CSE exposed BEAS-2B cell study where neuraminic/sialic acid components were also lowered (chapter 6). This cigarette smoke-induced loss of neuraminic/sialic acid may lead to increased epithelial damage due to impaired scavenging of oxidants. Restoring neuraminic/sialic acid concentration in the airways might protect the respiratory epithelium from further decay.

Future research may also focus on the use of lipidomics in recognizing mitochondrial dysfunction in cells or biopsies. We used lipidomics successfully to identify specific lipid components in induced sputum of COPD patients and in lung epithelial cells exposed

to cigarette smoke. A cell line harboring a mitochondrial dysfunction (Rho-0 or cybrids, a cytoplasmic hybrid formed by the fusion of a whole cell lacking mtDNA with a cytoplast) could serve as a model to generate a specific lipid spectrum specifically for mitochondrial impairment. Mitochondrial fractions can also be isolated from healthy- and affected cells to investigate which lipidomic profile is specific for healthy and dysfunctional mitochondria. These lipidomics spectra can be compared to individual patient samples/spectra for diagnostics use of COPD disease. This would especially be of interest for a disease like COPD because it reduces the invasiveness of current procedures.

Concluding remarks

Taken together, our findings suggest an important role for mitochondrial functionality in COPD disease. Mitochondrial function is affected by cigarette smoke-induced oxidative stress, especially in epithelial cells from COPD patients, leading to increased inflammatory signaling, reduced wound healing capabilities and reduced GC responsiveness. These abnormalities observed in mitochondrial function in epithelial cells from COPD patients are accompanied by increased phosphorylation/inactivation of GSK3β. Our data show that both deficiencies lead to GC responsiveness. Glucocorticoids have broad anti-inflammatory effects, but exert little benefit in COPD patients. Understanding the mechanisms of this unresponsiveness may lead to important novel avenues in the treatment of COPD disease. Furthermore, we show that the novel technique lipidomics may be suitable for diagnostic use in induced sputum of COPD patients. This thesis may thus open avenues for further research on the cellular and molecular mechanism underlying the pathophysiology of COPD, and provide novel insights for future therapeutic strategies.

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