GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

The aim of this thesis was to study the effects of CS on mitochondrial function as a potential pathophysiological mechanism in smoking induced disease (in particular chronic obstructive pulmonary disease (COPD)) and to gain insight in the mechanisms involved in airway epithelial dysfunction by smoking.

Cigarette smoke disturbs mitochondria and airway epithelial cells
Smoking cigarettes remains the major risk factor for the development of COPD. There is little knowledge of the pathophysiology of COPD. In this thesis we were able to introduce a new pathophysiological concept in the development of COPD and other cigarette smoke-induced diseases.

The lungs extract oxygen which is essential for mitochondria to generate ATP, necessary for life, by oxidative phosphorylation. The respiratory tract which is equipped with airway epithelial cells and epithelial lining fluid (ELF) is exposed to a higher level of oxygen tension than that of most tissues within the body (12; 17). Leakage of electrons during the oxidative phosphorylation and higher oxygen tissue levels, are favorable conditions to generate reactive oxygen species (ROS). Because oxygen is a strong oxidizer, electrons can be easily accepted by this molecule, converting it into $O_2^{\cdot -}$, a very potent free radical. Nevertheless, during normal respiration, airway epithelial cells contain enough antioxidants to protect the cells from oxidative injury. However, the oxidant burden in the lungs is enhanced in smokers. ROS either inhaled by cigarette smoke (CS) or released by the activated neutrophiles, alveolar macrophages and eosinophiles are not capable of diffusing through the plasma membranes of the epithelial barrier of the lung (1; 4; 11). These extra-cellular ROS will act on the external environment by depletion of extracellular reduced thiols and by direct attack on vulnerable lipids and proteins of the cellular surface (19; 24). Other substances, like lipophilic compounds easily pass the membrane of cells, disturb mitochondrial function and enhance ROS generation. The mitochondrial electron transfer chain (ETC) is essential in this ROS generation. Furthermore, lipophilic compounds inhaled by smoking, contribute to an imbalance between ROS and antioxidant defenses (3). In this thesis we showed that GSH in epithelial cells, which plays a key role in the maintenance of the cellular redox balance, is irreversibly lost when exposed to gaseous-phase CS. Identification by mass spectrometry shows that the decrease of GSH is attributable to the formation of glutathione-aldehydes derivatives. Persistent smokers may in that case inhale more ROS than can be scavenged by the residual anti-oxidants, resulting in increased vulnerability for oxidative stress. This makes the re-synthesis of GSH essential for cellular survival and protection of the lung.

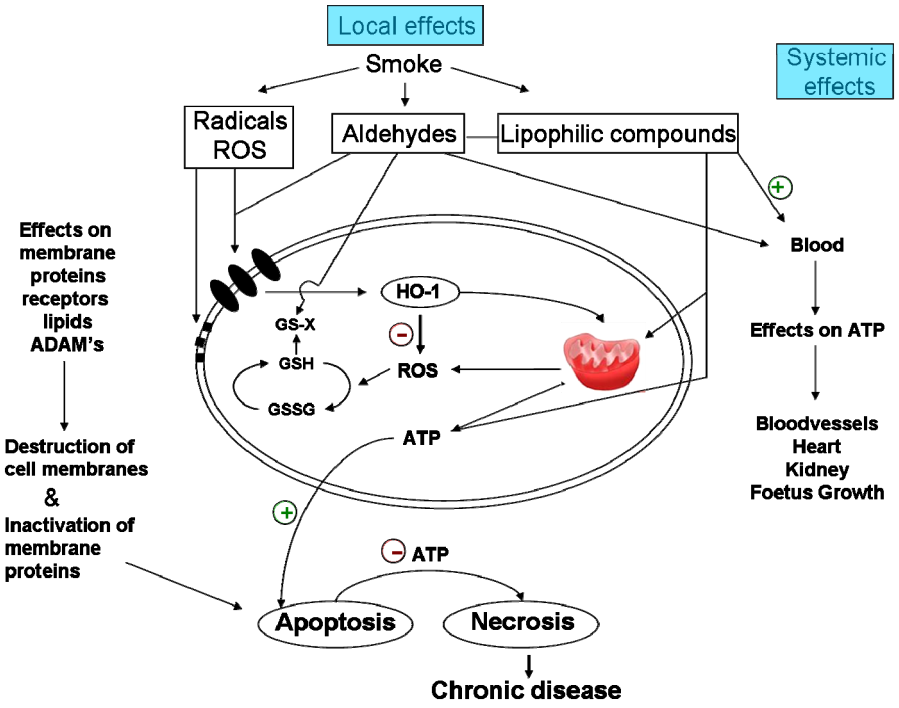

Figure 1. Effects of different compounds inside smoke on lung tissue cells

Aldehydes like acrolein and crotonaldehyde are one of the most prominent lipophilic compounds in gaseous-phase CS. Interestingly, it has recently been shown that acrolein acts as a mitochondrial toxin, with has comparable effects on mitochondrial function as we have observed in chapter 1 (22). Our *in vitro* experiments with airway epithelial cells and isolated mitochondria show that CS extract (CSE) is able to block complex I and II of the ETC. As a consequence of that, the consumption of oxygen and production of ATP is diminished. Removal of the lipophilic compounds from the CSE by hexane extraction significantly attenuate the effects on mitochondria, by restoring the mitochondrial membrane potential ($\Delta\psi_m$) and intracellular ATP synthesis. This may indicate that lipophilic compounds (aldehydes, nicotine, polycyclic aromatics, phenols etc.) inside CSE easily enters the cells and disturb mitochondrial function (9). Mitochondria are the main producers of cellular energy, but are also considered a key regulatory center of apoptosis. ATP depletion can result in necrosis or apoptosis. Apoptosis is an energy dependent process and, therefore, if the energy depletion is above a critical level necrosis will ensue (8; 10). A switch to necrotic cell death and release of their cellular contents, will result in an inflammatory response in the environment of these dying cells. Necrotic cell death may play an important role in the development of lung and airway inflammation and modulation of this pathway might reveal new

treatment modalities. The effects of smoke at the lung tissue cells are summarized in figure 1.

In this thesis we describe that heme oxygenase-1 (HO-1) may have a potential important protective role in CS-induced inflammation and cell death. HO-1 expression is induced by pro-inflammatory stimulants such as cytokines, heavy metals or CS. HO-1 is the rate-limiting enzyme in heme degradation, catalyzing the cleavage of the heme ring to form ferrous iron, carbon monoxide (CO), biliverdin and bilirubin, all of which have antioxidant and anti-inflammatory activities (Fig. 2) (16; 20).

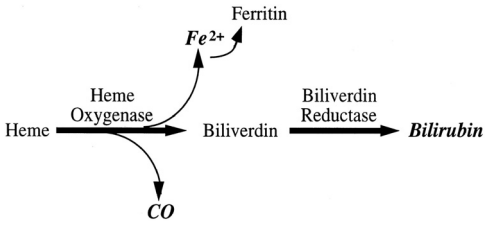

Figure 2. Enzymatic cleavage of heme by heme oxygenase-1.

We demonstrate that active HO-1 was present in higher concentrations in mitochondria than in the cytosol after stimulation with CSE. Overexpression of HO-1 in airway epithelial cells clearly preserves mitochondrial ATP production and prevent cell death, in the presence of CSE. HO-derived end products such as CO, biliverdin and bilirubin may contribute to this metabolic protection. Further studies are needed to elucidate the precise contribution of these end products inside the matrix of mitochondria.

FUTURE PERSPECTIVES

CS is the major risk factor for the development of COPD but also extends to systemic disease manifestations. In these patients, increased levels of oxidative stress parameters were found. We describe that lipophilic compounds inside the smoke of cigarettes induce a profound generation of ROS in alveolar epithelial cells with mitochondria, but not in alveolar epithelial cells that are devoid of mitochondria. We obtain similar results with CS devoid of ROS and water-soluble compounds, but still containing the lipophilic fraction. Our suggestion is that the lipophilic compounds are the major effectors of oxidative stress and systemic toxic effects after smoking cigarettes (see figure 1; right side). Based on our proposals two studies were awarded by grants provided by the Graduate School for Drug Exploration (GUIDE) for two Post-graduate students as a follow up of the study presented in this thesis.

First study: About local effects

To extend our results of chapter 2, we will try to identify which lipophilic compounds present in CS are the main compounds that disturb the mitochondrial function and thereby induce the intracellular ROS production. Furthermore we will investigate the effects of both gaseous-phase ROS and intracellular generation of ROS on release of inflammatory mediators by airway epithelial cells *in vitro*. To expand the *in vitro* results and to study the hypothesized acute affects of CS-induced intracellular generated ROS on airway inflammation, oxidant and anti-oxidant responses *in vivo* in mice and humans will be studied.

In chapter 1 we demonstrate that lung epithelial cells exposed to CS-extract (CSE) decrease there mitochondrial functions [4]. To investigate if also the structure of the mitochondria changed after CS exposure we recently exposed Beas-2b bronchial epithelial cells to CSE and observed a marked mitochondrial autophagy (Fig. 3A,B).

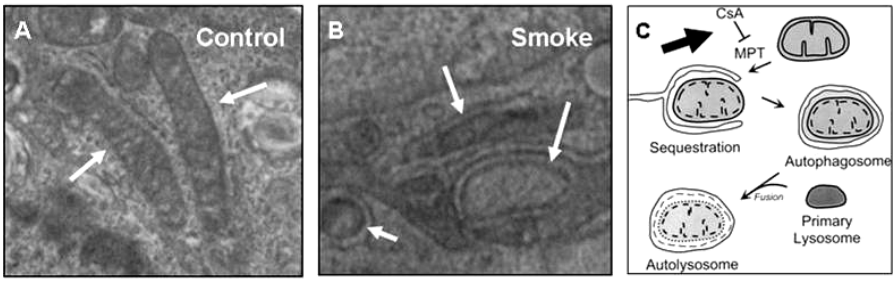

Figure 3. Beas-2b bronchial epithelial cells under normal culture circumstances showing normal mitochondria (A) and exposed to cigarette smoke extract showing marked mitochondrial autophagy (B) on electron microscopy (25.000x magnification, white arrows: mitochondria). (C): Cartoon showing the process of mitochondrial autophagy that can be inhibited by cyclosporine-A (CsA, black arrow).

Mitochondrial autophagy ('mitochondrial apoptosis') precedes cell death, possibly as a consequence of mitochondrial Ca^{2+} overload and increased oxidative stress, resulting in opening of the mitochondrial permeability transition pore (MPTP) and disruption of the mitochondrial matrix (2; 6). Furthermore, preliminary data with isolated mitochondria show that Ca^{2+} leads to mitochondrial swelling. Mitochondria exposed to increased levels of CSE and Ca^{2+} had left-shifted curves indicating that CSE strengthen mitochondrial swelling (Fig. 4). This process leads to the release of cytochrome c and other apoptotic factors such as apoptosis-inducing factor. Besides the well-known anti-inflammatory effects of cyclosporine-A (CsA), therapeutic intervention with CsA prevents opening of the MPTP (see chapter 6) thereby protecting mitochondria from autophagy (Fig. 3C). Therefore, we will investigate whether treatment with CsA under CS-induced toxic circumstances may provide protection against CS-injury (7).

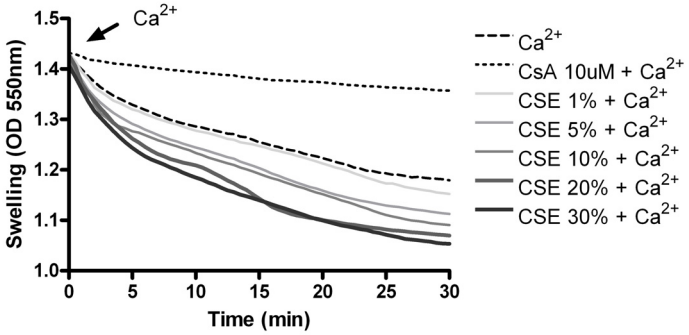

Figure 4. Mitochondrial permeability transition pore opening in isolated mitochondria.

Second study: About systemic effects

Smoking is a risk factor for development of COPD, cardiovascular disease and cancer. Evidence is now accumulating that smoking is also an important risk factor for development and progression of renal disease (13). In a cross-sectional study in the general population, it has been demonstrated that smoking is associated with microalbuminuria and low glomerular filtration rate in non-diabetic subjects (18). Similar associations in cross-sectional studies in patients with diabetes suggest that smoking increases susceptibility for development of diabetic nephropathy. Prospective studies in patients with diabetes have also documented a more rapid decline of renal function in association with smoking (14), even despite angiotensin-converting enzyme inhibition (5). Smoking has also been documented to be associated with a more rapid decline of renal function in patients with lupus nephritis, polycystic kidney disease, and other primary renal diseases (15; 21; 23).

To explore the effects of smoking, and to gain insight in the mechanisms involved in the induction of renal damage by smoking we will investigate in cultured tubular epithelial cells and endothelial cells the effects of the different lipophilic compounds of CS for their potential profibrotic and proinflammatory effects. Furthermore we will perform studies with Rho null cells from tubular epithelial cells and endothelial cells that are devoid of mitochondria. This will allow us to investigate whether different compounds of CS may induce ROS from other cell components than mitochondria. Animal studies will be performed to address the question whether the association between CS and development and progression of proteinuric nephropathies is indeed a causal relationship.

Smoking cessation is important for all smokers. It prevents development of smoke-related diseases. We will try to motivate currently smoking patients with either diabetic nephropathy and/or a status after renal transplantation to be admitted to the outpatient stop smoking clinic, were we will follow them through their follow-up. In the outpatient clinic, patients are supported in gradually decreasing uptake of nicotine supplementation and buproprion. Finally, they will be free of support. Follow-up of these patients, including repeated collection of 24h urine samples will allow us to investigate effects of smoking, nicotine, stopping of smoking, and (in

some cases) restart of smoking on creatinine clearance, albuminuria, markers of oxidative stress (e.g. F2-isoprostanes), and markers of tubulointerstitial involvement (e.g. β2-microglobulin, KIM-1, MCP-1, neopterin, and collagen fragments). Furthermore we will investigate if assessment of 24h urinary cotinine excretion in renal transplant recipients is valuable for detection of underreporting of current smoking. We will also assess whether there is a dose-effect relationship between smoking and future occurrence of graft loss.

Further remarks on systemic effects of smoking

It is known that smoking has deleterious effects on many organs increasing the risk for heart and brain diseases and blood vessel abnormalities. It maybe suggested that lipophilic compounds found in smoke are responsible for these adverse effects. Vulnerability of patients may vary according to the balance between destructive actions of these lipophilic compounds on intracellular ROS production and the anti-oxidative capacities of different tissues under attack. Further research on these anti-oxidative capacities of the different tissues and the genetic make-up of individuals are worthwhile to be studied for all manifestations of disease that are associated with smoking.

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