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Cigarette smoke-induced mitochondrial dysfunction and oxidative stress in

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SUMMARY AND MAIN CONCLUSIONS

In this thesis we studied the effects of cigarette smoke (CS) on mitochondrial function and oxidative stress in epithelial cells and discussed the potential of these phenomena in the pathogenesis of chronic obstructive pulmonary diseases (COPD). In the first three chapters we demonstrated that CS disturbs mitochondrial function, increase reactive oxygen species (ROS) generation and alters the regulation of cell death. In chapter 4 and 5 we investigated the effects of gaseous-phase CS and H_2O_2 on the antioxidant glutathione and thiol proteins regulating redox state, morphology and cell death pathways in airway epithelial cells. In chapter 6 we studied the negative side effects of cyclosporine A (CsA), an immunosuppressive drug and potent inducer of mitochondrial permeability transition pore (MPTP) closure, on mitochondrial membrane potential ($\Delta\psi_m$), oxidative stress and calcium homeostasis in relation to chronic renal allograft dysfunction.

In **chapter 1** of this thesis we investigated the effects of CS extract (CSE) on mitochondrial function and mode of cell death in human lung epithelial cells. Therefore, at first we isolated mitochondria and examined the effects of CSE on complex I and II activity of the electron transfer chain (ETC), $\Delta\psi_m$, proton motive force, oxygen consumption and examined the effects of CSE on intracellular energy production and cell death in lung epithelial cells. We demonstrated that CSE acts as a blocking agent of complex I and II of the ETC. Inhibition of the two entry points of the ETC caused a decrease in $\Delta\psi_m$ and proton motive force. As a result of that, oxygen consumption and production of ATP were diminished. Furthermore, we demonstrated that CSE inhibited caspase-3 and -7 activities in early apoptotic cells and switched epithelial cell apoptosis into necrosis. We concluded that compounds in CSE act as blocking agents of the mitochondrial ETC and that the loss of intracellular ATP generation switches early apoptotic epithelial cells into necrosis.

In COPD patients markers of increased oxidative stress are present. ROS in the gaseous-phase of CS are thought to constitute the main component of the oxidative stress present in COPD. In **chapter 2** we described that ROS present in the gaseous-phase of CS are not able to diffuse through the membranes of the epithelial cells of the lungs. Despite this fact, evidence of systemic oxidative stress parameters such as higher malondialdehyde (MDA) levels, lower serum vitamin C and reduced glutathione (GSH) levels have been found in smokers and patients with COPD. We hypothesized that instead of ROS, lipophilic compounds present in CS enter the cell and induce an intracellular burst of ROS by disturbing mitochondrial function. Therefore, we investigated the acute effects of CSE and lipophilic free CSE on mitochondrial function. We also used A549 alveolar epithelial cells and A549-ρ0 cells that lack a functional ETC and examined if CSE was able to induce an intracellular burst of ROS production. Furthermore, we used an ancient method, the Narghile or so called waterpipe, to quench ROS from the gaseous-phase of CS to investigate the effects of intracellular generated ROS on thiol redox status in lung epithelial cells. We demonstrated that CSE deteriorates the intracellular levels of ATP, most probably trough inhibition of the ETC (**chapter 1**). Removal of lipophilic compounds from CSE significantly restored the intracellular levels of

ATP. Exposure of CSE to A549 and A549-ρ0 cells showed an increase in ROS generation in A549 cells whereas A549-ρ0 cells did not. We furthermore showed that ROS in the gaseous-phase of CS directly induces thiol oxidation in A549 cells whereas water-filtered CS did not. We concluded that lipophilic compounds present in CS disturbed mitochondrial function leading to increased generation of intracellular ROS. A functional mitochondrial ETC was essential in this ROS generation. Furthermore, ROS inside the gaseous-phase of CS and generated by the mitochondria themselves were both responsible for the oxidation of free thiol groups.

In **chapter 3** we described the significance of heme oxygenase-1 (HO-1), a stress protein, which contributes to the protection of the airway epithelium against toxic compounds present in CS. HO-1 catalysis the rate-limiting step in the oxidative degradation of heme, confers protection against exogenous stresses and inhibits apoptotic cell death. In ex-smoking patients with COPD compared with ex-smoking healthy control subjects, lower expression of HO-1 were found. In this study we hypothesized that HO-1 exerts a potential regulatory and protective function in COPD by preserving mitochondrial function, and by inhibiting cell death associated with CS exposure. Therefore at first we characterized the localization of HO-1 in the epithelial cells. In mice we examined the mRNA expression of HO-1 in the lung after exposure of CS. Thereafter we examined the functional activity of HO-1 in whole cells and mitochondrial extracts after treatments with hemin (substrate of HO-1) and CSE. To investigate the protective role of HO-1 in CSE-induced cell death process, we used Beas-2b bronchial epithelial cells overexpressing HO-1 and control Beas-2b cells. We demonstrated that HO-1 levels were significantly increased in cytosolic and mitochondrial fractions of alveolar A549 cells and bronchial Beas-2b cells. The mitochondrial localization of HO-1 was confirmed using immunogold-electron and confocal microscopy. HO-1 activity increased dramatically in mitochondrial fraction and whole cell extract after exposure of hemin and CSE. HO-1 mRNA expression levels were elevated in the lungs of mice exposed to CS. Furthermore, over expression of HO-1 levels inhibited CSE-induced cell death and preserved cellular ATP levels. We concluded that functional compartmentalization of HO-1 in the mitochondria of airway epithelial cells preserved mitochondrial ATP production and prevented cell death, in the presence of CSE.

In patients with COPD, increased levels of oxidative stress parameters have been documented suggesting that antioxidants may be insufficient to prevent oxidative damage from CS. To ensure an appropriate defense against lung injury, the respiratory tract is equipped with the epithelial lining fluid (ELF) and the airway epithelium, which both contain large amounts of the reduced GSH. GSH plays a key role in the cellular redox balance and is thought to be one of the most important antioxidant defenses in the airways. CS is known to deplete total glutathione (GSH + GSSG) in the airways. In **chapter 4** we investigated if compounds of the gaseous-phase of CS react irreversibly with the reduced form of GSH to form GSH derivatives that cannot be reduced, thereby causing this depletion. Therefore at first we tested whether CS compounds were able to modify the free thiol (-SH) groups of

GSH in solution compared to hydrogen peroxide (H_2O_2). Thereafter we investigated if gaseous-phase CS irreversibly modified GSH in A549 cells and primary bronchial epithelial cells. Enzymatic assays combined with mass spectrometry were used to quantify the amount of total glutathione and identify the GSH modification. We demonstrated in a solution of GSH and in airway epithelial cells that gaseous-phase CS irreversibly reduces the amount of total glutathione whereas H_2O_2 did not. Mass spectrometry showed that GSH was modified to glutathione-aldehyde derivatives. Identification by MS2 showed that GSH was bound to acrolein and crotonaldehyde and another, yet to be identified structure. We concluded that CS does not oxidize reduced GSH to oxidized GSSG but, rather, reacts to nonreducible glutathione-aldehyde derivatives, thereby depleting the total available GSH pool. Under these circumstances, a chronic lack of protection against oxidative stress might be induced.

The airways of smokers are constantly exposed to high levels of ROS. These reactive compounds may directly participate in specific tissue injury and cell death, which is followed by recovery and repair by the proliferation of the remaining cells. In **chapter 5** we investigated the response and recovery of A549 cells after various concentrations of H_2O_2. We hypothesized that resistance and recovery would be dependent on the concentration of the oxidative agent, duration of exposure and on the quiescent or proliferating state of the cells. Therefore at first we studied morphological changes in quiescent or proliferating A549 cells exposed to different concentration of H_2O_2 and incubation time. Because free thiol groups play an important role in the defense against oxidative stress, the effect of H_2O_2 on the cellular level of free thiol groups were measured relative to the protein content. Thereafter, cell numbers, cell viability and recovery were tested. We demonstrated that proliferating A549 cells recovered a 1-h challenge with up to 1 mM H_2O_2 whereas quiescent A549 cells did not. Proliferating A549 cells did not sustain a more prolonged challenge (6 or 24 h) with 0.5 mM or 1.0 mM H_2O_2. The severe conditions resulted in loss of cells by detachment from the plate surface, reduced numbers of viable cells primarily due to necrosis and a strong reduction of the intracellular free thiol content. Furthermore, a relation was found between cell morphology, free thiol content and the number of necrotic cells for proliferating A549 cells. This correlation was less strong for quiescent A549 cells. We concluded that quiescent cells were more sensitive to oxidative stress than proliferating cells. Intracellular free thiol levels apparently played a decisive role in cell survival, preferentially protecting proliferating cells.

Immunosuppressive therapy with cyclosporine A (CsA) to prevent rejection of transplanted solid organs is associated with undesired effects that promote deterioration of transplant function and accelerated atherogenesis. It has been suggested that ROS plays an important underlying role. CsA is also a potent inhibitor of the mitochondrial permeability transition pore (MPTP). In **chapter 6** we investigated whether closure of the MPTP by CsA resulted in a concomitant increase in $\Delta\psi_m$ and increased production of mitochondrial ROS. We used fluorescent probes to asses $\Delta\psi_m$, ROS and $[Ca^{2+}]$ in human kidney cells (HK-2) and a line of human small cell lung carcinoma (GLC4), because these do not express CsA-sensitive P-

glycoprotein. Transfected GLC4 cells were used expressing P-glycoprotein as control for GLC4 cells. NIM811 and PSC833 were applied as selective MPTP and P-glycoprotein blockers. We also isolated mitochondria from fresh pig livers and studied the effects of CsA on mitochondrial function. We demonstrated that CsA and PSC833 induced a more than two-fold increase in $\Delta\psi_m$ in HK-2 cells, whereas NIM811 had no effect. None of the three cyclosporine analogs induced an increase in $\Delta\psi_m$ in GLC4 cells. The MPTP blockers CsA and NIM811, but also the non-MPTP blocker PSC833, induced comparable degrees of increased ROS production and cytosolic [Ca^{2+}]. Furthermore, blockade of the MPTP in isolated mitochondria by CsA affected neither $\Delta\psi_m$, ATP synthesis, nor respiration rate. During state III respiration and in the presence of Ca^{2+} mitochondrial ROS generation was increased. Addition of CsA resulted in significant attenuation of generation of mitochondrial ROS. We concluded that CsA and its analogs induce ROS generation and cytosolic Ca^{2+}. However, neither mitochondria, nor involvement of P-glycoprotein nor inhibition of calcineurin play a role in CsA-induced oxidative stress and disturbed Ca^{2+} homeostasis. One must be very cautious when using fluorescent probes in P-glycoprotein-expressing cells when effects of substances with P-glycoprotein-blocking properties, such as CsA, are investigated, because this may result in false-positive signals.

MAIN CONCLUSIONS OF THE STUDIES IN THIS THESIS

About the effects of CSE on isolated mitochondria and intact cells

1 CSE acts as a blocking agent of the mitochondrial ETC

2 CSE-induced depletion of cellular ATP switches lung epithelial cell apoptosis into necrosis

3 Overexpression of HO-1 levels in airway epithelial cells inhibited CSE-induced cell death and preserved cellular ATP levels

4 CSE induces the activity of HO-1 protein

About the effects of gaseous-phase CS on intact cells

5 ROS inside the gaseous-phase of CS and generated by the mitochondria themselves are able to change the thiol redox state of the cell

6 CS induces mRNA expression of HO-1 in the lungs of mice

7 Lipophilic compounds in CS disturb mitochondrial function and induce mitochondrial ROS generation

8 Gaseous-phase CS is able to deplete free thiol groups

9 Unsaturated aldehydes generated during the combustion of tobacco irreversibly modify GSH into GSH-aldehyde compounds

About the effects of oxidative stress on intact cells

10 Quiescent alveolar A549 cells are more sensitive to oxidative stress than proliferating A549 cells

11 Intracellular free thiol levels apparently play a decisive role in cell survival after oxidative stress challenges

About the effects of CsA on isolated mitochondria and intact cells

12 The MPTP blockers CsA and NIM811, but also the non-MPTP blocker PSC833 induce increased production of ROS and cytosolic Ca^{2+} in GLC4 cells

13 Neither mitochondria, involvement of P-glycoprotein or inhibition of calcineurin play a role in CsA-induced oxidative stress and disturbed Ca^{2+} homeostasis

14 Ca^{2+} induced generation of mitochondrial ROS can be prevented by CsA

15 One must be cautious when using fluorescent probes in P-glycoprotein-expressing cells when substances with P-glycoprotein-blocking properties, such as CsA, are investigated, because this may result in false-positive signals