Time course of cigarette smoke-induced changes of systemic inflammation and muscle structure

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Abstract

It has become more evident, that long term cigarette smoking (LTCS) has an important extrapulmonary toxicity. The aim of the study was to investigate the time dependent effects of cigarette smoke exposure on exercise capacity, markers of systemic inflammation and skeletal muscle structure.

C57BL/6J-mice were either exposed to mainstream cigarette smoke for 6 hours/day, 5 days/week (Smoke Exposed, SE group) or assigned to the control, unexposed group (Con group). SE Group mice were exposed for 8, 16, 24 and 32 weeks to smoke and unexposed Con mice were used as age matched controls. Exercise capacity was investigated by spiroergometry. Systemic inflammatory status was analyzed by flow cytometry and multiplexed fluorescent immunoassay. For analysis of muscle tissue histological techniques and microarray analysis were used.

Mice of the SE group exhibited a lower increase of body mass and a decrease of $\text{VO}_{2\text{max}}$ (p<0.05). An increase of lymphocyte CD62, ICAM and VCAM expression was found in SE mice (p<0.05). A biphasic trend of protein up- and down-regulation was observed in markers of systemic inflammation, tissue deterioration and allergic reactions such as CRP, eotaxin, haptoglobin, M-CSF-1, MIP-1 γ. Thereby, the expression of several chemotactic proteins in plasma correlated with their expression in muscle. A time dependent decrease of muscle mass, oxidative type-I fibers and muscle cross-sectional area was found (p<0.05). Microarray analysis revealed a SE–induced up-regulation of several pathways of metabolic processes and tissue degradation.

Taken together it was found that the loss of exercise capacity and systemic inflammation are early events of SE, which might induce muscular atrophy and loss of oxidative muscle capacity.
Introduction

Tobacco smoking is one of the most potent and prevalent addictive habits. It is known that smoking affects multiple organ systems resulting in numerous so-called tobacco-related diseases like chronic obstructive pulmonary disease (COPD). While the effects of cigarette smoke on the respiratory tract are well established, it is becoming more evident that smoking has an important extrapulmonary toxicity. In this regard, numerous studies have demonstrated several extrapulmonary effects of smoking including systemic inflammation and muscle dysfunction. These extrapulmonary effects are particularly pronounced for long-time cigarette smoking (LTCS) which leads to a high prevalence of COPD (15, 16, 64). The so-called systemic effects of LTCS and COPD have been recognized as being also clinically relevant. In fact, it is likely that important clinical outcomes, including mortality or health status, eventually result from the interplay between the intrapulmonary and extrapulmonary effects of LTCS (44).

A common systemic effect of LTCS is a decreased exercise capacity. This functional decline was thought to be primarily caused by airway obstruction resulting in increased work of breathing during activity. However, over longer periods there are several other systemic abnormalities which might contribute to functional declines in LTCS or COPD patients (49). Central extrapulmonary effects of smoking are changes in the cellular and humoral inflammatory status (21). Regarding circulating inflammatory cells it was shown that the hematopoietic system is stimulated resulting in an increased release of leukocytes and platelets into the circulation (30). In addition to neutrophils and macrophages it was also demonstrated that T cells, which include both CD4+ and CD8+ cells, were activated and were present in the alveolar walls in emphysema during COPD development (42). The systemic inflammatory status in smokers and COPD patients is also characterized by an increased level of several inflammatory cytokines such as tumor necrosis factor (TNF)-α and C-reactive protein (CRP) (60). However, inflammation affects several other tissues including skeletal muscle (1, 4). Primarily two types of muscle abnormality were observed in muscles of patients with moderate and severe COPD (6). On the one hand, atrophy occurs in the anaerobic type-IIx fibres, leading to a loss of muscle strength. On the other hand COPD patients suffer from the depletion of aerobic type-I fibres and a reduction in mitochondria and oxidative enzymes within both type-I and -IIa fibres, resulting in decreased muscle endurance (20, 23, 27). The mechanisms of muscle wasting in COPD are intensively discussed. It is assumed that both a reduction of activities as well as the inflammatory status induces skeletal muscle abnormalities (27, 28, 60). In addition, a large number of oxidants contained in cigarette smoke may also exert direct deleterious effects on skeletal muscles (6).

Recently, Seimetz et al. (53) established a mouse model of smoke exposure demonstrating that 8 months of smoke exposure induced pulmonary hypertension and emphysema and
right heart hypertrophy. However, functional capacity, systemic inflammation and effects of muscle tissue were not elucidated.

The main objective of this study was to investigate the time dependent effects of cigarette smoke exposure on exercise capacity, markers of systemic inflammation and skeletal muscle tissue in a mouse model. Therefore, mice were exposed to cigarette smoke using a whole body cigarette smoke exposure system. We hypothesized that cigarette smoke exposure reduces maximum oxygen consumption, increases markers of systemic inflammation and affects skeletal muscle composition in favor of loss of oxidative fibers and atrophy.
Methods

Experimental animals and smoke exposure

Animals

Adult male C57BL/6J mice, 20–22 g, were obtained from Charles River Laboratories, Sulzfeld, Germany. Animals were housed under controlled conditions with a 12-hour light/dark cycle and food and water supply ad libitum. Animals were randomly allocated to tobacco-smoke exposed (SE group) and unexposed groups (Con) of 40 mice each. All experiments were approved by the governmental ethics committee for animal welfare (Regierungspräsidium Giessen, Germany).

Experimental design and tobacco-smoke exposure

Mice of the SE groups were exposed to mainstream smoke of 3R4F cigarettes (Lexington, KY, USA) at 140 mg particulate matter/m³ for 6 hours/day, 5 days/week, while mice of the Con were kept under control conditions (no smoke exposure). Both groups (SE and Con) were subdivided into groups of 10 which were exposed for 8, 16, 24 and 32 weeks to smoke (SE group) or used as age matched controls (Con group). Cigarette smoke was generated with a customized smoke generator (Burkhart, Wedel, Germany). Mainstream smoke was mixed with fresh air and delivered via tube into a chamber where the mice were sitting in cages in respective groups. There was a constant flow-in and –out (around 10 l/min) and the particle concentration was routinely measured several times per run to ensure constant smoke level. In addition, humidity, temperature, and O₂ level in the smoke chamber were continuously measured during the run. To get the desired smoke concentration the particle concentration was routinely measured several times during each run. Therefore, the smoke passes a filter pad for 3 minutes which is weighted before and after. Using the weight difference and flow rate [liter/min], the particle concentration/m³ was calculated.

Determination of exercise capacity

Exercise capacity was determined as previously described (Krüger et al. 2009). Briefly, by using a treadmill spiroergometry (custom made), maximal oxygen uptake (VO₂max) and maximal running speed (Vmax) of mice were determined. The treadmill was placed in a metabolic chamber where air was led through at a rate of 0.5 l/min. Samples of 200 ml/min of gas were extracted to the paramagnetic oxygen analyzer (type 1155, Servomex) and the carbon dioxide analyzer (Lair 12, M&C Instrument). All animals were acclimated to the treadmill before VO₂max and Vmax were tested during a continuous, progressive test on the treadmill ergometer until exhaustion. After 10 min of acclimatization in the treadmill chamber the test uptake started at 0.15 m/s, increasing every 3 min by 0.05 m/s. Exhaustion was
determined when oxygen consumption reached a plateau (leveling off) and mice repeatedly
stopped running.

Cell isolation procedure and analysis of inflammatory surface markers

At least 4 days after the last exercise bout, mice were anaesthetized by isoflurane and
sacrificed by cervical dyslocation. Blood was collected by cardiac puncture. In order to
investigate systemic inflammatory status, lymphocytes were isolated by density gradient
centrifugation. Briefly, 100 µl of whole blood anticoagulated with EDTA was layered upon a 1
ml of Percoll (Nycomed, Oslo, Norway) and centrifuged at 400g for 20 min at room
temperature. After centrifugation the lymphocyte band was removed and cells were
repeatedly washed. Cell suspensions were incubated with monoclonal antibodies anti-CD3,
anti-CD4, anti-CD8 (FITC-conjugated, ImmunoTools, Friesoythe, Germany), anti-CD62 anti-
CD54/ICAM, anti-CD106/VCAM (PE conjugated, Beckman Coulter, Krefeld, Germany) and
analyzed by Flow cytometry (EPICS XL Flow Cytometer, Beckman Coulter, Krefeld,
Germany).

Cytokine analysis

Serum was tested for apolipoprotein A-I (Apo A-I), CD40 (CD40), CD40 Ligand (CD40-L),
CRP, endothelin-1 (ET-1), eotaxin, epidermal growth factor mouse (EGF Mouse), factor VII,
fibrinogen, fibroblast growth factor 9 (FGF-9), fibroblast growth factor basic (FGF-basic),
granulocyte chemotactic protein-2 mouse (GCP-2 Mouse), granulocyte-macrophage colony-
stimulating factor (GM-CSF), growth-regulated alpha protein (KC/GRO), haptoglobin,
immunoglobulin A (IgA), interferon γ (IFN-γ), interferon γ-induced protein 10 (IP-10),
interleukin-1 alpha (IL-1 alpha), interleukin-1 β (IL-1 β), interleukin-10 (IL-10), interleukin-11
(IL-11), interleukin-12 subunit p70 (IL-12p70), interleukin-17A (IL-17A), interleukin-18
(IL-18), interleukin-2 (IL-2), interleukin-3 (IL-3), interleukin-4 (IL-4), interleukin-5 (IL-
5), interleukin-6 (IL-6), interleukin-7 (IL-7), leukemia inhibitory factor (LIF), lymphotactin,
macrophage colony-stimulating factor-1 (M-CSF-1), macrophage inflammatory protein-1
alpha (MIP-1 α), macrophage inflammatory protein-1 β (MIP-1 β), macrophage inflammatory
protein-1 γ (MIP-1 γ), macrophage inflammatory protein-2 (MIP-2), macrophage inflammatory
protein-3 β (MIP-3 β), macrophage-derived chemokine (MDC), matrix metalloproteinase-9
(MMP-9), monocyte chemotactic protein 1 (MCP-1), monocyte chemotactic protein 3 (MCP-
3), monocyte chemotactic protein-5 (MCP-5), myeloperoxidase (MPO), myoglobin,
oncostatin-M (OSM), serum amyloid p-component (SAP), serum glutamic oxaloacetic
transaminase (SGOT), stem cell factor (SCF), T-cell-specific protein, RANTES,
thrombopoietin (TPO), tissue factor (TF), tissue inhibitor of metalloproteinases 1 mouse
(TIMP-1 Mouse), TNF-α, vascular cell adhesion molecule-1 (VCAM-1), vascular endothelial
growth factor A (VEGF-A) and von Willebrand factor (vWF) by a multiplexed fluorescent bead-based immunoassay (Luminex, Myriad RBM, Austin, Texas). Briefly, this method operates with capture-antibodies which are attached to fluorescent microspheres and then measured by flow cytometry. All assays have been validated in a multiplex based upon the principles of immunoassay and tested for the parameters of least detectable dose (LDD), precision, cross-reactivity, linearity, spike recovery, correlation to an alternative method, dynamic range, and matrix interferences.

Muscle weight, fiber typing and determination of muscle fiber cross-sectional area

Freshly prepared muscles (m. rectus femoris, m. soleus and m. gastrocnemius) were weighed and frozen quickly. Serial cross sections of these muscles (20 μm thick) were cut on a cryostat microtome at -25°C. Muscle cross sections were mounted on cover slips and stained for myosin ATPase (mATPase) with acid pre-incubation using a modified method according to Hämäläinen and Pette (29). Fiber type delineation are based on methods exploiting their differential pH-dependent mATPase stability. Briefly, sections were pre-incubated for 5 min in sodium acetate (54.3 mM) – sodium barbital (32.6 mM) solution adjusted with HCl to pH 4.6. After washing, the sections were incubated for 30 min at 37°C in substrate solution (2.7 mM ATP, 100 mM glycine, 54 mM CaCl₂, 100 mM NaCl, pH adjusted to 9.6). After incubations in 11 mM CaCl₂ and 2% CoCl₂, a black insoluble product was developed in 1% ammonium sulfide for 50 s. As a result type 1 fibers stained darkly, while type II fibers were stained light grey. After washing with distilled water, the sections were analyzed by light microscopy (Leica DMI 6000B, Leica Microsystems, Wetzlar Germany). In order to calculate percentages of the type I and type II fibers and measure muscle fiber cross-sectional area, Leica Application Suite software and Leica QWin software were used (Leica Microsystems, Wetzlar Germany).

Microarray analysis

A total of 20 RNA samples from 20 different mice (n=5/time point) were subjected to 10 dual-color hybridizations. Purified total RNA (1 µg, each) was amplified and Cy-labeled using the dual-color LIRAK kit (Agilent) following the kit instructions. The samples were labeled with either Cy3 or Cy5 to match a balanced dye-swap design for each group. Cy3- and Cy5-labeled RNAs were hybridized overnight to 4x44K 60mer oligonucleotide spotted microarray slides (Mouse Whole Genome 4x44K; Agilent Technologies, design ID 014868). Hybridization and subsequent washing and drying of the slides were performed following the Agilent hybridization protocol.

The dried slides were scanned using the GenePix 4100A scanner (Axon Instruments, Downingtown, PA). Image analysis was performed with GenePix Pro 5.1 software, and
calculated values for all spots were saved as GenePix results files. Stored data were evaluated using the R software (48) and the limma package (54) from BioConductor (24). Log mean spot signals were taken for further analysis. Signals of replicate spots (same probes) within arrays were averaged. The M/A data were calculated from spots with intensities greater 50. M/A data were LOESS normalized (54,55,56) before averaging over arrays. Genes were ranked for differential expression using a moderated t-statistic (53). Pathway analyses were done using gene set tests on the ranks of the t-values with mixed alternative (54) Gene sets were defined from the KEGG database (ftp://ftp.genome.jp/pub/kegg/genomes) from March 2011 and GeneOntology database from September 2012 (19).

Statistical analysis

Data are means ± SEM, unless indicated otherwise in the figure legends. Differences between the points of time were performed by analysis of variance followed by Bonferroni’s Multiple Comparison Test. For comparison of two groups, a Student’s t test was performed. Pearson’s correlation analysis was used to identify any significant relationship. We considered p values below 0.05 as statistically significant for all analyses.
Results

Body mass and exercise capacity

Both groups, Con and SE mice, started with a similar average body mass and showed increase of body mass with time. However, mice of the SE group exhibited a statistically significant lower body mass compared to the corresponding controls at all time points (p<0.05, Table 1).

We further tested the exercise capacity of the mice after 8, 16, 24 and 32 weeks. As depicted in Figure 1, smoke-exposed mice showed a significant reduced VO$_{2\text{max}}$ compared to the age-matched controls at the 16, 24 and 32 week time points. A remarkable attenuation of gain in Vmax compared with respective controls was observed at 24 weeks (p<0.05, Figure 1).

Inflammatory markers on lymphocytes

In order to evaluate inflammatory changes on cellular level, surface markers on lymphocytes were analyzed. A statistically significant increase of CD62 expression was found after 24 and 32 weeks in mice of the SE group compared to age matched controls (p<0.05, Figure 2A). Similarly, an increase of ICAM expression and VCAM expression was found in SE mice. Thereby, ICAM expression increased early on CD4 and CD8 cells (16 weeks), while an increase of VCAM was found at 24 weeks of SE (p<0.05, Figure 2B and C). On CD8 cells, a late increase of both markers was found (week 32) (p<0.05, Figure 2D and E).

Inflammatory serum markers

A panel of 59 antigens was analyzed using Rodent MAP to evaluate time course of systemic inflammation. Basically a biphasic trend of protein up- and down-regulation could be observed. For instance, markers of systemic inflammation, tissue deterioration and allergic reactions like CRP, CD40 ligand, eotaxin, factor VII, fibrinogen, haptoglobin, IL-4, lymphotactin, M-CSF-1, MIP-1 γ, MDC, MCP-1, MCP-3, MCP-5, MPO, SAP, SGOT, TPO and TIMP-1 Mouse, were already upregulated after smoke exposure of 8 weeks, whereas IgA, VCAM-1 and vWF were downregulated at the same time point. Most of the cytokines named above showed a biphasic increase during smoke exposure. The early up-regulation (after 8 weeks) was followed by passing normalization (16 weeks) and again by increases after 24 weeks of smoke exposure. The upregulated cytokines frequently remained at a higher level as well after 32 weeks of smoke exposure in comparison with the control group (Figure 3).
While muscle weight of the Con group increased slightly over time, a smaller increase or decrease was found after 24 and 32 weeks in weight of m. rectus femoris, m. soleus, and m. gastrocnemius of the SE group (p<0.05, Table 2). In addition to muscle weight, changes in muscle fiber type and muscle fiber cross-sectional area were analyzed selectively for type I and type II fibers. Here a time dependent decrease of oxidative type-I fibers in m. rectus femoris, m. soleus, and m. gastrocnemius was found (p<0.05, Figure 4A). In parallel, SE mice showed a decrease of muscle fiber cross-sectional area in both fiber types (type I and type II fibers) (p<0.05, Figure 4B). Type II fiber area decreased early in the rectus femoris muscle (16 weeks). In contrast, a statistically significant decrease of area of muscle fibers in type I fibers was not observed until week 32 (p<0.05, Figure 4B).

Microarray analysis of muscle tissue

Microarray analysis revealed effects of smoke exposure on several muscular signaling pathways. The pathways were selected by controlling of the false-discovery rate to 5%. While only the PPAR pathway was clearly affected at 8 weeks, we could identify effects on several pathways at 16 weeks of SE. In particular, the identified pathways were related to diseases (Parkinson’s disease, Alzheimer’s disease, Huntington disease), general metabolic processes (oxidative phosphorylation, nitrogen metabolism, fatty acid metabolism, citrate cycle) as well as to the protein/amine acid metabolism and degradation (alanine, aspartate and glutamate metabolism, β-alanine metabolism, glutathione metabolism, valine, leucine and isoleucine degradation, cysteine and methionine metabolism, arginine and proline metabolism). Furthermore, the pH regulative proximal tubule bicarbonate reclamation pathway was regulated and PPAR signaling pathway was still upregulated. While no pathway regulation could be identified at 24 weeks, at 32 weeks the only altered pathway was olfactory transduction (Table 3).

Analysis of single genes revealed a down-regulation of several genes known to be related to increasing percentage of type I fibers. This down-regulation was observed at 16 and 24 weeks of smoke exposure (e.g. Pdk4: -- 1.00 (-1.1, 0.53) (24 weeks), Ppargc1b: - 0.87 (-1.33, -0.41) (16 weeks). We further found an up-regulation of several genes which are involved in muscle degradation (e.g. FOXO1: 0.83 (0.43, 1.11(24 weeks) FBXO32: 0.63 (0.18, 1.06 (16 weeks) while several genes which are involved in muscle growth were down-regulated (e.g. LGFBP5: −0.54 (0.08, 1.01) (32 weeks). Other genes which were found to be regulated are involved in protein degradation (e.g. Uchl1: 0.65 (-0.03, 1.34) (12 weeks)), in cell death (e.g. Fas: 0.40 (0.18 , 0.61 (12 weeks)), cell cycle (e.g. Cdk5r1: -0.31 (-0.55, -0.08) (12 weeks), oxidative stress (e.g. Gst3: 0.42 (-0.94, 0.10), and anaerobic metabolism (e.g.
Ldhb: 1.19 (0.01,2.36) (12 weeks) (values are given as mean log₂ fold-changes with 95% confidence intervals in brackets).

Correlations between the time course of muscle gene expression and plasma protein expression

We further analyzed the correlation between the expression of selected genes in muscle and cytokines in plasma at all time points. Here it was found that the expression of several inflammation related genes, like CCL-2, CCL-7, CCL-9 or CSF-1, which exhibit chemotactic activities on leukocytes, correlated with the level of respective plasma cytokines over time (p<0.05) (Table 4).
Discussion

In the present study, the time course of changes in functional capacity, systemic inflammation and muscle structure was investigated in a mouse model of smoke exposure. In response to SE, mice showed an early decline of exercise capacity accompanied by an up-regulation of several adhesion molecules on lymphocytes and inflammatory serum markers. Remarkably, inflammatory markers in blood showed a mixture of acute and chronic inflammatory changes during smoke exposure. In skeletal muscle pathways of protein degradation were regulated early, while a corresponding time dependent decrease of muscle weight, muscle cross-sectional area, and type I fibers occurred. It was also found that the expression of several inflammatory cytokines in serum correlated with their upregulation in muscle suggesting a spillover of inflammatory mediators.

A progressive decline of VO\textsubscript{2}max in both LTCS as well as COPD patients was previously demonstrated (59). Interestingly we found that endurance capacity declined early after smoke exposure (8 weeks). This immediate decrease of VO\textsubscript{2}max might be the result of CO saturation of the blood which decreases the oxygen transport capacity (34). Seimetz et al. (53) demonstrated in the same mouse model that functional and structural pulmonary vascular remodeling is an early event after smoke exposure, most probably resulting in a reduced oxygen delivery. The later progressive decline of VO\textsubscript{2}max might be the result of additional mechanisms because the VO\textsubscript{2}max depends on both central as well as peripheral factors which comprise lung diffusion, stroke volume, blood volume, capillary density of the skeletal muscle and muscle oxygen extraction (7). Therefore, it has been speculated that also the loss of type I oxidative muscle fibers supported the decline of VO\textsubscript{2}max. However, several other factors including pulmonary hypertension and changes in cardiac output are known to accelerate the stepwise decline of exercise capacity in COPD patients (30).

In order to investigate the time course of systemic inflammation, expression of adhesion molecules on lymphocytes was analyzed. It was previously demonstrated that ICAM-1, VCAM-1 and CD62L were associated with air pollution, the occurrence of asthma, and COPD (2). However, in these studies, molecules were found increased in several forms such as soluble in serum (2), expressed in pulmonary fibroblasts (65), and expressed on the endothelium (31). We have demonstrated that these molecules also showed increased expression on the lymphocyte cell surface in response to smoke exposure, indicating an increased activation state of these cells. Upregulation of these surface proteins might induce lymphocyte recruitment through the bronchial circulation. It is likely that the biphasic regulation of some of these markers reflect a status of acute and chronic inflammation (14). The early changes of ICAM-1 expression might be associated with the upregulation of inflammatory serum markers. In this regard, Huang and colleagues (31) demonstrated
correlations between CRP levels and sICAM-1, suggesting a relationship between systemic inflammation and adhesion molecule expression. However, the reasons for a biphasic increase can only be speculated. D'Hulst et al. (15) demonstrated that activated T cells showed a biphasic infiltration pattern in the lungs of smoke-exposed mice. Therefore, it is assumed that ICAM-1 might mediate migration into lung tissue. Possibly, a first increase of cellular infiltration is followed by an anti-inflammatory counter-regulation, which might account for the decreased expression observed during the time course. Furthermore, it can be speculated that an interim decrease of inflammation might be the result of a temporary habituation to the smoke exposure.

Similar to lymphocyte adhesion molecule expression, early and late up-regulation of markers of systemic inflammation and tissue deterioration were found in plasma. Increased levels of CRP, IL-1 β and CD40 ligand indicate early systemic inflammatory conditions during SE. These findings were supported by previous studies which demonstrated that even short term smoking increased systemic inflammation (61). Many chemokines like MCP-1, MCP-3, MCSF, and eotaxin, which are known to attract macrophages/monocytes or T cells, were found to be upregulated early after smoke exposure. It has been speculated that these cells were activated early in order to leave the circulation (12). In this regard, D'Hulst et al. (15) demonstrated an early increase of total number of neutrophils and macrophages in the bronchoalveolar lavage of mice during SE.

Increased levels of factor VII and fibrinogen indicated that hemostatic function is altered due to smoke exposure, which is supported by previous data (59). However, it is a matter for discussion if changes in hemostasis area direct result of smoking, caused by smoking-induced vascular damage, or are associated with inflammation. In this regard, Hunter et al. (31) showed that increased synthesis of fibrinogen is related to expression of CRP. It is assumed that inflammation-induced high fibrinogen levels may promote the cardiovascular risk of smokers through arterial wall infiltration and effects on blood viscosity (11). The increase of cytokines like TIMP-1or MMP might be a result of tissue deterioration. An increase of these markers was previously observed in sputum of COPD patients and might result from a breakdown of the extracellular matrix of airways or other tissue (42).

Regarding muscle tissue, a time-dependent atrophy of muscles and specifically of type I fibers was observed. The atrophy of both fiber types promotes both a functional loss of both strength as well as endurance capacity (50,60). A relationship between muscle atrophy and systemic inflammation was proved by several studies demonstrating that inflammation triggers catabolic processes resulting in skeletal muscle wasting (13,33,37,60). Thereby, different cytokine-mediated pathways are known to negatively impact muscle protein metabolism. On the one hand, inflammatory conditions increase the demand for amino acids to synthesize acute phase proteins which leads to reduced muscle protein stores (3,5). On
the other hand, cytokines like TNF-α or IL-1 are known to activate the adenosine triphosphate (ATP)-ubiquitin-dependent proteolytic systems resulting in degradation of muscle proteins and inhibition of muscle repair system (40,45,63). In order to find associations between selected inflammatory serum proteins and related genes in muscle correlations analyses was performed. Here it was found that the expression of several chemoattractants in serum and their respective genes in muscle correlated indicating a potential spillover of inflammation from plasma to muscle. However, current data are descriptive and it is not possible to show such a causal relationship.

Reduction of fiber area was accompanied by a reduction in the proportion of type I fibers. It was previously demonstrated that the loss of muscle quality is primarily reflected by loss of oxidative phenotype: a slow-to-fast or I-to-II fiber type shift is a consistent finding in lower-limb muscles of patients with COPD. Thereby, fiber type transitions seem to play an important role (28,38,39,40). Whether muscle dysfunction is a direct effect of smoking, inactivity or systemic inflammation remains to be determined. Gosker et al. (29) speculated that the reduction of type I fibers might be the result of hypoxemic stress in smokers. However, the molecular and cellular mechanisms underlying the deleterious effects of hypoxemia on skeletal muscle still need to be elucidated.

Analyzing muscular signaling pathways revealed that after 8 weeks of SE only the PPAR pathway was found to be upregulated, while after 16 weeks various pathways of diseases, metabolism and tissue degradation were regulated. An effect of LTCS on PPAR pathway was previously reported in muscles of COPD patients, in conditions of hypoxia (46), and in patients with systemic inflammation (9). In addition, reduced PPAR-a and PPAR-d activity was also demonstrated in other disease states with muscle abnormalities similar to COPD. Accordingly, PPAR-a and PPAR-d mRNA concentrations were reduced in quadriceps muscle of patients with spinal cord injury who also have an atrophic and anaerobic muscle phenotype compared to healthy controls or endurance athletes (36). Thereby, PPAR-a mRNA concentration positively correlated with the proportion of type-I fibers indicating their role in shifts of muscle fibers. The PPAR-pathway seems to be generally disturbed in diseases which are characterized by a reduced muscle oxidative capacity (23,50). In addition to the PPAR pathway, also some disease-related pathways like the Huntington disease and Parkinson's disease pathways were found to be significantly regulated. It is suggested that the regulation of the Parkinson's disease pathway is linked to apoptosis and protein degradation (25). The Huntington disease pathway includes genes encoding expression of PGC-1γ, which is known to affect muscle fiber switch and protein breakdown (12,13).

Further indications of an increased breakdown of myofibrillar proteins come from the activation of pathways which are mainly linked to protein degradation like the valine, leucine and isoleucine degradation pathway (16). Protein degradation might secondarily affect
changes of fiber composition because it was shown that components of the degradation machinery are present in higher quantities in oxidative fibers compared to low oxidative fibers (58). Perturbations in amino acid metabolism were previously demonstrated in COPD patients (17). It was found that COPD patients displaying cachexia can be distinguished from patients not displaying cachexia by increased serum levels of aspartate and arginine levels. Therefore, it is suggested that the up-regulation of some aminoacid metabolism pathways might be the result of tissue degradation. An upregulation of arginine–proline metabolism can be classified as a general feature of cachectic patients because it was observed in COPD subjects as well as in cachectic cancer patients (17,44). Recently, decreased glutamate and reduced glutathione levels were reported in the quadriceps femoris of patients with COPD. Thereby, glutamate is an important precursor for the first and rate-limiting step in the synthesis of reduced glutathione, one of the most important antioxidants in muscle (5). Reduced muscle glutathione levels often result in an antioxidant/oxidant imbalance, increasing the susceptibility of the muscle to oxidative stress and cell injury (18). However, current concepts assume that oxidative stress plays a relevant role in the generation of skeletal muscle abnormalities in COPD (5).

In summary, the present data give an overview about the time course of some important extrapulmonary effects of LTCS and COPD pathogenesis. A decrease of endurance capacity and systemic inflammation seem to be early events of smoke exposure which progress during the status of chronic inflammation. In parallel, several muscular signaling pathways are regulated which might induce protein degradation resulting in atrophy and loss of oxidative fibers. Of course, since this study is primarily descriptive, the causal relationships between the different changes must be further explored.

References


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Figure legends
Fig. 1: Exercise capacity VO\textsubscript{2max} and V\textsubscript{max} of SE and Con mice after 8, 16, 24 and 32 weeks. * means p<0.05 compared to Con at the same time point.

Fig. 2: Changes of cellular level surface markers on different lymphocyte populations at 8, 16, 24 and 32 weeks of smoke exposure. * means p<0.05 compared to Con at the same time point.

Fig. 3: Heat map of inflammatory serum markers 59 antigens in the time course of smoke exposure. The colors represent mean log-ratios between the SE and the Con group at each time point.

Fig. 4: Changes of muscle fiber type (A) and muscle fiber cross-sectional area (B) of SE and Con mice after 8, 16, 24 and 32 weeks. * means p<0.05 compared to Con at the same time point.
<table>
<thead>
<tr>
<th>Group</th>
<th>8 weeks</th>
<th>16 weeks</th>
<th>24 weeks</th>
<th>32 weeks</th>
</tr>
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<tr>
<td>SE</td>
<td>19.5 ± 0.9</td>
<td>24.3 ± 1.0*</td>
<td>27.8 ± 1.0*</td>
<td>30.1 ± 1.6*</td>
</tr>
<tr>
<td>Con</td>
<td>20.9 ± 1.2</td>
<td>25.7 ± 0.9</td>
<td>29.1 ± 0.9</td>
<td>31.5 ± 1.4</td>
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</tbody>
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Tab. 1. Time course of body weight [g] in smoke exposed mice (SE) compared to age matched control (Con). * means p<0.05 compared to Con same time point.
<table>
<thead>
<tr>
<th>Muscle weights (g)</th>
<th>8 weeks</th>
<th>16 weeks</th>
<th>24 weeks</th>
<th>32 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>m. rectus femoris</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SE</td>
<td>205.0 ± 16.4</td>
<td>207.2 ± 15.4</td>
<td>194.5 ± 17.7*</td>
<td>187.5 ± 16.0*</td>
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<tr>
<td>Con</td>
<td>202.4 ± 17.6</td>
<td>207.6 ± 18.9</td>
<td>215.4 ± 15.5</td>
<td>218.7 ± 17.4</td>
</tr>
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<td>m. soleus</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>SE</td>
<td>9.9 ± 3.4</td>
<td>11.1 ± 2.4</td>
<td>11.5 ± 3.7*</td>
<td>11.9 ± 4.0*</td>
</tr>
<tr>
<td>Con</td>
<td>10.2 ± 1.4</td>
<td>12.3 ± 2.2</td>
<td>13.5 ± 1.6</td>
<td>14.1 ± 2.3</td>
</tr>
<tr>
<td>m. gastrocnemius</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SE</td>
<td>162.9 ± 14.4</td>
<td>176.4 ± 15.4</td>
<td>180.3 ± 13.9*</td>
<td>182.6 ± 14.8*</td>
</tr>
<tr>
<td>Con</td>
<td>165.6 ± 13.4</td>
<td>179.4 ± 12.4</td>
<td>186.6 ± 15.7</td>
<td>191.5 ± 14.0</td>
</tr>
</tbody>
</table>

Tab. 2. Time course of weight change [g] of m. rectus femoris, m. soleus, and m. gastrocnemius in smoke exposed mice (SE) compared to age matched control (Con). * means p<0.05 compared to Con same time point.
<table>
<thead>
<tr>
<th>Time point</th>
<th>Kegg ID</th>
<th>Pathway</th>
<th>Number of genes</th>
<th>-log p</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 weeks</td>
<td>03320</td>
<td>PPAR signaling pathway</td>
<td>74</td>
<td>4.61</td>
</tr>
<tr>
<td>16 weeks</td>
<td>05012</td>
<td>Parkinson's disease</td>
<td>110</td>
<td>11.46</td>
</tr>
<tr>
<td></td>
<td>00190</td>
<td>Oxidative phosphorylation</td>
<td>112</td>
<td>10.49</td>
</tr>
<tr>
<td></td>
<td>05010</td>
<td>Alzheimer's disease</td>
<td>154</td>
<td>8.37</td>
</tr>
<tr>
<td></td>
<td>00020</td>
<td>Citrate cycle (TCA cycle)</td>
<td>29</td>
<td>7.91</td>
</tr>
<tr>
<td></td>
<td>05016</td>
<td>Huntington's disease</td>
<td>163</td>
<td>5.83</td>
</tr>
<tr>
<td></td>
<td>04260</td>
<td>Cardiac muscle contraction</td>
<td>68</td>
<td>4.89</td>
</tr>
<tr>
<td></td>
<td>00910</td>
<td>Nitrogen metabolism</td>
<td>22</td>
<td>4.69</td>
</tr>
<tr>
<td></td>
<td>00710</td>
<td>Fatty acid metabolism</td>
<td>43</td>
<td>4.60</td>
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<tr>
<td></td>
<td>00250</td>
<td>Alanine, aspartate and glutamate metabolism</td>
<td>31</td>
<td>3.89</td>
</tr>
<tr>
<td></td>
<td>00410</td>
<td>beta-Alanine metabolism</td>
<td>22</td>
<td>3.42</td>
</tr>
<tr>
<td></td>
<td>00480</td>
<td>Glutathione metabolism</td>
<td>52</td>
<td>3.28</td>
</tr>
<tr>
<td></td>
<td>04964</td>
<td>Proximal tubule bicarbonate reclamation</td>
<td>17</td>
<td>3.21</td>
</tr>
<tr>
<td></td>
<td>00280</td>
<td>Valine, leucine and isoleucine degradation</td>
<td>45</td>
<td>3.06</td>
</tr>
<tr>
<td></td>
<td>00270</td>
<td>Cysteine and methionine metabolism</td>
<td>34</td>
<td>2.98</td>
</tr>
<tr>
<td></td>
<td>03320</td>
<td>PPAR signaling pathway</td>
<td>74</td>
<td>2.76</td>
</tr>
<tr>
<td></td>
<td>00330</td>
<td>Arginine and proline metabolism</td>
<td>51</td>
<td>2.70</td>
</tr>
<tr>
<td>32 weeks</td>
<td>04740</td>
<td>Olfactory transduction</td>
<td>859</td>
<td>5.37</td>
</tr>
</tbody>
</table>

Tab. 3: Microarray analysis of the effect of smoke exposure on several muscular signaling pathways. The pathways were selected by controlling of the false-discovery rate at 5%.
<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>8 weeks log₂ fold-change</th>
<th>16 weeks log₂ fold-change</th>
<th>24 weeks log₂ fold-change</th>
<th>32 weeks log₂ fold-change</th>
<th>Average log₂ expr. of Gene expression</th>
<th>8 weeks log₂ fold-change</th>
<th>16 weeks log₂ fold-change</th>
<th>24 weeks log₂ fold-change</th>
<th>32 weeks log₂ fold-change</th>
<th>Average log₂ expr. of Plasma protein levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ccl2</td>
<td>-0.24</td>
<td>-0.06</td>
<td>1.21</td>
<td>0.21</td>
<td>7.06</td>
<td>1.09</td>
<td>0.26</td>
<td>1.62</td>
<td>0.78</td>
<td>7.03</td>
</tr>
<tr>
<td>Ccl7</td>
<td>0.00</td>
<td>-0.22</td>
<td>0.83</td>
<td>0.13</td>
<td>6.07</td>
<td>0.87</td>
<td>0.15</td>
<td>1.88</td>
<td>0.64</td>
<td>8.22</td>
</tr>
<tr>
<td>Csf1</td>
<td>0.04</td>
<td>-0.29</td>
<td>0.23</td>
<td>0.02</td>
<td>7.95</td>
<td>0.34</td>
<td>0.09</td>
<td>0.22</td>
<td>0.20</td>
<td>2.23</td>
</tr>
<tr>
<td>Ccl9</td>
<td>0.33</td>
<td>0.70</td>
<td>1.22</td>
<td>0.06</td>
<td>7.80</td>
<td>0.62</td>
<td>0.10</td>
<td>0.23</td>
<td>0.28</td>
<td>3.36</td>
</tr>
<tr>
<td>Il1b</td>
<td>0.29</td>
<td>-0.11</td>
<td>0.09</td>
<td>0.01</td>
<td>5.87</td>
<td>0.11</td>
<td>-0.27</td>
<td>-0.34</td>
<td>0.30</td>
<td>1.95</td>
</tr>
<tr>
<td>Ccl11</td>
<td>0.9</td>
<td>-0.23</td>
<td>0.77</td>
<td>0.19</td>
<td>8.85</td>
<td>0.44</td>
<td>-0.25</td>
<td>0.59</td>
<td>0.82</td>
<td>10.45</td>
</tr>
</tbody>
</table>

Tab. 4: Selected key genes in muscle and cytokines in plasma which are regulated in muscle tissue and plasma (mean log₂ fold-changes after 8, 16, 24 and 32 weeks smoke exposure with 95% confidence intervals in brackets, and log₂ expression as average over all time points).
Fig. 1
Fig. 2

A. CD3+/CD62+ cells [%]

B. CD4+/ICAM+ cells [%]

C. CD4+/VCAM+ cells [%]

D. CD8+/ICAM+ cells [%]

E. CD8+/VCAM+ cells [%]
Fig. 3
Fig. 4

A

M. rectus femoris

M. soleus

M. gastrocnemius

% of total fibers

Typ I (Con)  
Typ II (Con)  
Typ I (SE)  
Typ II (SE)

B

area of fibers [µm²]

weeks

Typ I (Con)  
Typ II (Con)  
Typ I (SE)  
Typ II (SE)

C

32 weeks (CON)  
32 weeks (SE)