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Two weeks smoking cessation reverses cigarette smoke-induced skeletal muscle atrophy and mitochondrial dysfunction in mice

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Abstract

Introduction

Apart from its adverse effects on the respiratory system, cigarette smoking also induces skeletal muscle atrophy and dysfunction. Whether short-term smoking cessation can restore muscle mass and function is unknown. We therefore studied the impact of 1- and 2-weeks smoking cessation on skeletal muscles in a mouse model.

Methods

Male mice were divided into 4 groups: Air-exposed (14 weeks); cigarette smoke (CS)-exposed (14 weeks); CS-exposed (13 weeks) followed by 1-week cessation; CS-exposed (12 weeks) followed by 2 weeks cessation to examine exercise capacity, physical activity levels, body composition, muscle function, capillarization, mitochondrial function and protein expression in the soleus, plantaris and diaphragm muscles.

Results

CS-induced loss of body and muscle mass was significantly improved within 1 week of cessation due to increased lean and fat mass. Mitochondrial respiration and protein levels of the respiratory complexes in the soleus were lower in CS-exposed mice, but similar to control values after 2 weeks of cessation. Exposing isolated soleus muscles to CS extracts reduced mitochondrial respiration that was reversed after removing the extract. While physical activity was reduced in all groups, exercise capacity, limb muscle force, fatigue resistance, fiber size and capillarization and

diaphragm cytoplasmic HIF-1α were unaltered by CS-exposure. However, CS-induced diaphragm atrophy and increased capillary density was not seen after 2 weeks of smoking cessation.

Conclusion

In male mice, two weeks smoking cessation reversed smoking-induced mitochondrial dysfunction, limb muscle mass loss and diaphragm muscle atrophy, highlighting immediate benefits of cessation on skeletal muscles.

Key words: CS-exposure, Smoking cessation, muscle mass, mitochondrial function

Implications

Our study demonstrates that CS-induced skeletal muscle mitochondrial dysfunction and atrophy are significantly improved by 2 weeks cessation in male mice. We show for the first time that smoking cessation as short as 1 to 2 weeks is associated with immediate beneficial effects on skeletal muscle structure and function with the diaphragm being particularly sensitive to CS-exposure and cessation. This could help motivate smokers to quit smoking as early as possible. The knowledge that smoking cessation has potential positive extrapulmonary effects is particularly relevant for patients referred to rehabilitation programs and those admitted to hospitals suffering from acute or chronic muscle deterioration yet struggling with smoking cessation.

Introduction

Cigarette smoke (CS) is a complex mix of toxic substances exerting deleterious effects on different organs. While the adverse effects of CS on the respiratory system are well established, its extrapulmonary toxicity has only been highlighted during the last decades. In particular, the deleterious effects of CS on skeletal muscle function has emerged as an important extrapulmonary consequence of smoking[1,2].

Importantly, the observation that skeletal muscle dysfunction was present in smokers and CS-exposed mice prior to the development of any respiratory symptoms indicates that some compounds in CS could directly affect skeletal muscle function. Young smokers without respiratory symptoms have weaker and less fatigue resistant muscles compared to non-smokers[1,2]. Although part of these effects could have been related to decreased physical activity as reported in many smokers[3,4], CS-exposed animal models confirmed the loss of muscle force and resistance to fatigue prior to the development of any respiratory symptoms[5–8]. These models also revealed that both the respiratory muscles (in particular the diaphragm) and the limb muscles were affected by CS[5–8]. Muscle fiber atrophy with loss of muscle mass due to decreased protein synthesis, increased proteolysis, and enhanced protein oxidation in the muscle as reported in smokers and animal models might have contributed to the CS-induced muscle weakness[6,7,9–11]. In addition,

An impaired oxygen supply and blood flow to the muscle as a consequence of smoking may induce the expression of Hypoxia-inducible factor-1α (HIF-1α) that plays a crucial role in mitochondrial biogenesis, enhanced vasodilatory capacity and capillary proliferation [12]. However, these adaptations may be impaired by noxious substances in cigarette smoke [13]. Skeletal muscles require an adequate supply of blood by the capillary network for the delivery of nutrients, hormones and

oxygen and removal of waste products, heat and metabolites[14]. Various substances in CS may interfere with muscle capillarization and blood supply which may culminate in impaired muscle homeostasis and deleterious structural changes in the long run[7,15,16]. Moreover, reduced oxygen delivery to the mitochondria as a result of CS-induced intermittent tissue hypoxia, or the inability of the mitochondria to use oxygen coupled to the direct effect of CS on the mitochondrial respiratory chain enzyme complexes[17,18] could also contribute to the loss of muscle function seen with CS[6,11,19]. Due to its role in breathing, the diaphragm muscle is constitutively active even when the limb muscles are at rest and requires a constant supply of oxygen and nutrients. This implies that the noxious substances in CS may be constantly transported to the diaphragm making it more susceptible to CS-exposure. In fact, a recent study in mice reported CS-induced diaphragm muscle contractile dysfunction prior to the development of limb muscle alterations and emphysema[6].

Whether skeletal muscle impairment due to CS is reversible has been addressed in long-term smoking cessation studies. For instance, 16 months of smoking cessation was associated with an increase in fat and muscle mass in post-menopausal women[20], something also seen in other studies on smoking cessation[20,21]. Furthermore, the absence of fiber atrophy in muscles of former smokers and non-smokers pairs of monozygotic twins supports the notion that smoking cessation can lead to restoration of muscle mass[3]. Likewise, the observation that both the lean body mass and the force generating capacity of skeletal muscles in former smokers are not different from never smoking controls[22] seems to indicate that normalization of muscle function after cessation of smoking is associated with the recovery of muscle mass. A recent study in mice also confirmed that soleus mass can be normalized after 2 months of smoking cessation but gastrocnemius was still lower [23].

Studies in human lymphocytes from healthy smokers have shown that smoking cessation for as little as 24 hours to 7 days resulted in restored mitochondrial complex III and IV activities and increased maximal mitochondrial respiration[24]. These data suggest that smoking cessation may have immediate beneficial effects on mitochondrial function in blood cells. Whether this also occurs in skeletal muscles has never been explored so far. Therefore, the objective of the study was to comprehensively assess the impact of 1 or 2 weeks smoking cessation on limb and respiratory muscle function, structure, and capillarization in CS-exposed mice. We assessed maximal exercise capacity, physical activity levels, body composition, muscle force and fatigue as well as muscle histology, mitochondrial function and capillarization characteristics. We hypothesized that short-term smoking cessation restores body mass and composition, mitochondrial function and improves limb and respiratory muscle structure and capillarization.

Materials and methods

All experimental procedures were conducted according to the European, national and institutional guidelines for animal welfare and approved by the ethical committee for animal experiments of the University of Leuven, Belgium (authorization number: P010/2014 and P050/2016).

Animals and study design

Eight-week-old male C57Bl/6JolaH mice (n=88) were randomly divided into 4 groups: one exposed to CS for 14 weeks (CS); a group exposed to CS for 13 weeks and 1 week cessation (CS1W); a group exposed to CS for 12 weeks and 2 weeks cessation (CS2W) and a control group (Con). Since the amount of muscle tissue per mouse was not sufficient to perform all analyses in each muscle, the animals were further divided into two series. The first series (n = 52 [13 per group]) was used to determine muscle mass, body mass, mitochondrial function, neutrophilic inflammation, muscle force and fatigue, Dual-energy X-ray Absorptiometry (DEXA) scans, whole

body strength, muscle structure and maximal exercise capacity measurements. The second series (n = 36 [9 mice per group]) were used to assess spontaneous physical activity, body mass, muscle structure and capillarization.

All mice were housed in individually ventilated cages with food and water provided *ad libitum*. Mice were acclimatized during one week to CS by gradually increasing the number of cigarettes per day while the control mice were exposed to room air in soft restrains for the same duration (Figure S1). The mice were nose-only exposed (InExpose System, SCIREQ, Montreal, Canada) to either six 3R4F research cigarettes (Kentucky Tobacco Research and Development Center, University of Kentucky) or room air (Con) in soft restrains twice daily, 5 days a week (Figure S1), as previously described[25]. Total particle density was measured daily (Microdust, Casella CEL, Bedford, UK), and carbon monoxide (CO) levels in CS 3 times per week (EasyLog EL-USB-CO, Lascar electronics, UK), while body mass and food intake were measured weekly.

The average total particulate matter in CS was 188 ± 29 mg/m^3 and CO levels were 871 ± 49 ppm/cigarette. This exposure elevates arterial and venous carboxyhemoglobin levels to 35% immediately after exposure, 6% within 1 hour and back to control levels (1.5%) within 2 hours.

Exercise capacity and physical activity levels

Whole-body function

Maximal exercise Capacity and whole-body strength were measured at baseline (before the mice were exposed to CS or room air in soft restrains) and after 14 weeks. The exercise test consisted in a 5-minute warm up on a treadmill (0% incline, 3 m/min), and the speed was increased by 1 m/min every minute until exhaustion. Maximal exercise capacity was defined as the maximum speed[26]. Latency-to-fall time was measured after placing the mice on a grid that was subsequently inverted and served as a surrogate marker for maximal muscle strength[26].

Spontaneous physical activity was assessed at baseline, at the initiation of smoking, at 11-12 weeks and after smoking cessation, as previously described[27]. Each mouse was first acclimatized to an individual cage for 24 hours and then physical activity was recorded for the next 24 hours using a basic IP camera (Foscam C1; Foscam, Shenzhen, China). After recording, the mouse was returned to its original cage. The videos for the light and dark cycles were combined and analyzed using a customized program (Matlab Release 2017a; The MathWorks, Natick, MA) and data were reported as cumulative distance covered over time.

Measurements at sacrifice

Mice were anesthetized with a mixture of Ketamine (100 mg/kg, Ketalar®, Pfizer, Belgium), Xylazine (10 mg/kg, Rompun®, Bayer, Belgium) and Acepromazine (3 mg/kg, Placivet®, Kela, Belgium) administered intra peritoneally at a dose of 6 µL/g body mass.

Body composition

Fat, lean and bone mass were assessed using a DEXA scanner (Lunar Corp., Madison, WI) as described previously[26].

In situ muscle force and fatigue resistance

The left plantaris with its nerve and blood supply left intact was prepared to elicit contractions via stimulation of the tibial nerve at optimal length, to measure maximal twitch (at 1 Hz) and tetanic (at 200 Hz, train duration: 250 ms) tension normalized to muscle cross-sectional area. Fatigue resistance was assessed during repetitive isometric contractions at 30 Hz, 330ms/s, 4 min preceded by a series of 100 Hz, 100 ms/2 s contractions for 1 min that does induce little or any fatigue at all to activate muscle metabolism and blood flow as described previously[28] . The fatigue index (FI) was calculated as follows: for FI100, force of the last contraction was divided by the highest

contraction in this series while for FI30, force 2 min after the strongest contraction was divided by the strongest contraction in this series.

Lung cell counts

The mice were tracheotomized and the lungs were lavaged 4 times with Dulbecco's phosphate buffered saline (PBS). Total cell counts were done on pooled bronchoalveolar lavage (BAL) fractions using a Bürker hemocytometer with trypan blue. The remaining BAL fluid was centrifuged (1000 g for 10 min at 4°C) and the cell pellets dissolved in PBS were stained with Diff-Quick® (Medical Diagnostics, Düdingen, Germany) to determine differential cell counts on 300 cells per mouse to assess lung inflammation.

Muscle tissue assessments

Muscle mass

The gastrocnemius, plantaris and soleus muscles were removed and weighed before further analysis.

Mitochondrial function

Saponin-permeabilized fibers from the soleus muscle were used to assess mitochondrial respiratory function by high-resolution respirometry (Oxygraph-2k; Oroboros Instruments, Innsbruck, Austria) as described previously[29,34]. In a parallel experiment, mitochondrial function was assessed in freshly extracted soleus muscles from control mice exposed to 5% and 20% cigarette smoke extracts for 30 minutes after which the extracts were removed, and mitochondrial function re-assessed after washing.

Muscle histology

Immunohistochemistry for myosin heavy chain types was done on 10 µm thick serial sections of the plantaris, soleus and diaphragm muscles to determine fiber size and fiber type composition as

described previously[30] (Figure S2B). Serial sections were stained for succinate dehydrogenase (SDH) activity to measure oxidative capacity as described previously[31] (Figure S2E). Serial sections were stained with lectin for capillaries as described previously[32] (Figure S2A). Capillary domains analysis was performed as described previously[33](Figure S2C - D). This analysis provides information on fiber cross-sectional area (FCSA), fiber roundness (calculated as: perimeter^2/[4π·FCSA]), capillary domain area, capillary-to-fiber ratio, local capillary-to-fiber ratio [(LCFR) - the sum of the fractions of the capillary domains overlapping a given fiber], capillary density, capillary fiber density (LCFR/FCSA) and heterogeneity in capillary spacing [logarithmic standard deviation of the domain areas (Log_DSD)].

W*estern immunoblotting*

Forty mg of diaphragm muscle was homogenized in ice cold CER1 lysis buffer containing protease inhibitor followed by protein extraction using the NE-PER nuclear and cytoplasmic extraction kit (Thermo Fisher Scientific; 78833) according to the manufacturer's instructions. Equal amounts of protein from the cytoplasmic fraction (30 µg) were separated under reducing conditions using 8% SDS-PAGE (Biorad mini-PROTEAN) and transferred onto polyvinylidene fluoride membranes (Millipore). The membranes were blocked in 5% non-fat dry milk in TBS-0.1%Tween and then incubated overnight at 4°C with anti-HIF-1α (NB400-479, Novus Biologicals) or anti-β-tubulin (T5201, Sigma) in 1:1,000 dilutions. After incubation with the appropriate HRP-conjugated secondary antibody, specific proteins were detected using an enhanced chemiluminescence system (Sigma). The resulting bands were quantified, and the results expressed as ratios normalized to β-tubulin.

Assessment of subunits of mitochondrial complexes I to IV and ATP synthase by western blotting

Protein levels of key subunits of mitochondrial complexes I-IV and ATP synthase were assessed by western blotting as described before[34]. Briefly, proteins of the soleus muscle were extracted in protein extraction buffer containing 25 mM Tris-HCl, 150 mM NaCl, 1% (v/v) NP-40, 1% sodium deoxycholate and 0.1% SDS. Ten to 15 µg of total (undenatured) protein lysate was loaded on 8-16% gradient Criterion TGX SDS-PAGE gels and proteins were transferred onto nitrocellulose membranes and blocked for one hour with 5% non-fat dried milk powder, and subsequently incubated for 2 hours with an antibody cocktail against complex I subunit (NDUFB8), complex II subunit 30 kDa, complex III subunit core 2, complex IV subunit 1, and ATP synthase α-subunit (MS604, Abcam, Cambridge, UK) at 1:1000 dilution. Membranes were washed with TBS-Tween and incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (DAKO, Heverlee, Belgium; 1:5000).

Statistical analysis

The sample size was calculated based on maximal tetanic force data from previous experiments (25% difference) considering a 10% mortality rate in order to reach a significance level (α) of 0.05 and a power of 0.8. A Shapiro-Wilk test for normality was performed in each group prior to further analysis. Comparison between groups was performed using one-way ANOVA with Tukey's multiple comparison post-hoc test for normally distributed data or Kruskal-Wallis test with Dunn's multiple comparison post-hoc analysis for non-normally distributed data. Capillarization parameters were analyzed using two-way ANOVA with smoking and fiber type as the two variables, followed by Sidak's multiple comparison post-hoc analysis. Mixed model analysis followed by Sidak's post-hoc test was performed when the data had random missing values. Significance level was set at $p < 0.05$. Values are presented as mean ± SD.

Results

Lung assessments

CS-exposure increased total cell count in BAL (P<0.0001; [Figure S3A]), as well as macrophage (P<0.0001;[data not shown]), neutrophil (P<0.0001; [Figure S3B]) and lymphocyte count (P<0.0001;[data not shown]) which were similar to control levels within 2 weeks of smoking cessation.

Body mass, body composition and food intake

Body mass. Body mass declined significantly in all animals at the onset of CS or room air exposure and remained lower in the CS-exposed mice. This was statistically significant from 10 weeks onwards ($p < 0.01$ *vs.* control) [Figure S4A]. Cessation of CS-exposure for 1 week ($p = 0.03$ *vs.* smoking) and 2 weeks ($p < 0.0001$ vs smoking) improved body mass (Figure S4B).

Body composition. Fat mass was 26% lower after CS exposure ($p < 0.001$ *vs.* Con) and remained so after cessation ($p = 0.01$ *vs.* control) [Figure S4C]. Lean mass reduced by 9% in the CS animals and was only 4.6% and 0.5% lower after 1- and 2-weeks smoking cessation as compared to control animals, indicative of rapid improvements (Figure S4D). However, these improvements were not statistically significant.

Food intake. Food intake was reduced briefly in all mice in the first week of CS or room air exposure (Figure S5). There was a significant increase in food intake after 1- and 2-weeks smoking cessation ($p < 0.05$ *vs.* CS).

Physical fitness and Physical activity

Maximal Exercise Capacity, Whole-Body Strength and Spontaneous physical activity

For all these parameters, the baseline is the same and refers to measurements done before the mice were exposed to cigarette smoke or air. Compared to baseline values, maximal running speed was not different in the control group (-5.5%, p=0.18) but decreased significantly by 10% in CS (p=0.02), 9% in CS1W (p=0.05) and 9% in CS2W (p=0.01) groups at sacrifice with no effect smoking cessation. Whole body strength showed a 65-80% decrease in latency-to-fall time in all groups ($p<0.01$ vs baseline) and no significant effect of CS-exposure or cessation. Spontaneous physical activity decreased significantly by 40 – 45% in all groups compared to baseline from the start of the experiments independently of smoking and remained as such during the entire study period (Table S1).

Muscle assessments

Muscle mass

Compared to the Control group, the masses of the gastrocnemius and soleus muscles were 18% and 14% lower respectively in the CS group and similar to control levels in the CS1W and CS2W groups. Though the mass of the plantaris was 16% lower in the CS mice, it only became similar to control levels in the CS2W mice while it was still significantly lower in the CS1W mice compared to controls (Table 1).

In situ contractile properties

Despite the CS-induced decrease in muscle mass, CS-exposure did not significantly alter absolute (N) or specific ($N \cdot cm^{-2}$) tetanic (Figure S6A) and twitch (Figure S6B) plantaris muscle forces. The 100-Hz/100 ms/2s series of contractions induced little if any fatigue, and the percentage decrement in force during this series of contractions did not differ significantly between groups (Figure S6C).

The following more strenuous protocol (30 Hz, 330 ms on 670 ms off for 4 min) induced fatigue that did not differ significantly between groups (Figure S6D).

Muscle histology

Fiber type composition, cross-sectional area (CSA)

In the soleus and plantaris muscles, the fiber CSA (Figure S7A - B) did not differ significantly between groups. There were no significant differences in fiber type composition between groups in any of the muscles (Figure S7C - D). In the diaphragm, the fiber CSA was smaller in the CS than Con mice ($P<0.001$ *vs.* Con) and similar to control levels in CS2W mice (Figure S8A). The percentage of non-contractile material did not differ between groups in any of the muscles.

Capillarization and SDH activity

There were no significant differences in any of the capillary parameters between groups in the soleus and plantaris muscles ($p>0.05$; [data not shown]). By contrast, diaphragm capillary density (Figure S8B) and capillary fiber density of all fiber types (Figure S8C) were significantly increased in CS mice and similar to control levels in CS2W mice. This was not due to angiogenesis, as there were no significant differences between groups in the diaphragm for LCFR (Figure S8D). Two weeks cessation also led to significant improvements in capillary spacing (Figure S8E, $P<0.05$ vs control and smoking). The SDH activity of fibers was not significantly different between groups in any of the muscles. The diaphragm muscle had a higher capillary density than the plantaris and soleus muscles (Figure S8F, $P<0.001$).

Muscle mitochondrial function and protein levels of key markers of mitochondrial complexes

Leak respiration, maximal electron transport capacity and succinate/rotenone (complex II)-linked respiration were lower in CS-exposed animals compared to controls (Figure 1 A-C; $p < 0.01$).

Mitochondrial respiration under all substrate conditions were similar to control levels after 2 weeks of smoking cessation with intermediate values after 1 week of cessation (Figure 1 A – C). Total protein levels of mitochondiral complex I subunit NDUFB8, complex III subunit UQCRC2, and complex IV subunit MTCO1 showed lower values in smoking animals and were similar to controls after smoking cessation (Figure 1D-E). Acute exposure of the soleus to CS extracts also led to reduced respiration that was readily reversed after removal of the extracts (Figure 1F).

HIF-1α protein levels

Though there were no alterations with CS-exposure, smoking cessation for 2 weeks significantly increased cytoplasmic HIF-1α protein content in the diaphragm ($p<0.05$ for CS2W *vs* CS1W; $p=0.052$ for CS2W *vs* CS) [Figure S9 A-B].

Discussion

This study shows for the first time that in male mice, the diaphragm is more susceptible to smoking than the limb muscles, as indicated by a significant smoking induced fiber atrophy in the diaphragm only. However, even the limb muscles showed a lower muscle mass and smoking-induced impairment in maximal mitochondrial respiration. Even though some emphysema could have developed after 3 months of CS exposure, all these detrimental effects of smoking, including lung inflammation, were similar to control levels after 2 weeks of smoking cessation. If the reversibility of many of the smoking-induced defects we see here are as rapid in human smokers, this information may encourage smokers to quit smoking.

In line with previous studies, CS-exposure led to neutrophilic lung inflammation[26,35], reduced body mass[36–38] and decreased gastrocnemius, plantaris and soleus muscle mass[23,26,30,38]. Studies in humans[21,39–41] and mice[23,42,43] have shown that long-term smoking cessation is accompanied by

increased fat and lean body mass[20,21,44,45]. Our study shows a progressive increase in fat and lean mass after 1 to 2 weeks of CS-cessation. Potential contributors to CS-cessation-induced weight gain include amongst others, increased food intake, increased lipoprotein lipase activity and decreased resting metabolic rate[44], but our data show that increased food intake after cessation is the most likely explanation.

Despite the rapid increase in body mass during smoking cessation (7 and 12 % after 1 and 2 weeks respectively), there were no significant changes in the fiber CSA in the plantaris and soleus muscles, nor were there significant changes in force generating capacity. This is in accord with previous studies whereby 12 – 18 weeks of CS exposure in mice did not alter fiber CSA and force in the soleus and extensor digitorum longus (EDL) muscles[26,35]. The fatigue resistance in the plantaris was neither altered by CS-exposure nor smoking cessation, and such absence of changes in fatigue resistance correspond with the absence of significant changes in fiber type composition, similar to the absence of significant changes in *in vitro* contractile properties, fatigue resistance, and fiber type composition in mice exposed to CS for 12 weeks[35]. Others also have reported no significant smoking-induced changes in fiber CSA and fiber type proportions in mice[6,26,30,35] and humans[1,2,46].

Unlike the limb muscles, the diaphragm muscle showed significant reductions in the fiber CSA of all fiber types with CS-exposure. Interestingly, smoking cessation for as short as 2 weeks led to a rapid increase in fiber CSA to control levels. Literature on CS-induced diaphragm muscle fiber atrophy are controversial. Some studies report reduced force and increased atrophic signaling[6] or altered fiber proportions[5] but no changes in fiber CSA, while others report reduced mass[47] or fiber atrophy[48]. These discrepancies may result from differences in dose and duration of CS-exposure or mouse strain used. Whatever the cause of the discrepancies between studies, our study is the

first to demonstrate immediate beneficial effects of smoking cessation on diaphragm muscle structure. Taken together, and in line with previous studies[6], these results show that the diaphragm muscle is more sensitive to CS-induced changes in muscle structure than limb muscles and that it responds rapidly and positively to smoking cessation.

Despite the effects of CS-exposure in the diaphragm, the cytoplasmic protein content HIF-1α was not significantly altered. This could indicate that CS-induced transient intermittent hypoxia did not stimulate HIF-1α signaling or substances in CS may have inhibited its activation. Surprisingly, HIF-1α protein content increased after 2 weeks of cessation. However, the absence of downstream effects such as angiogenesis could show that the accumulated HIF-1α in CS2W mice is not translocated to the nucleus and therefore not activated.

The main determinant of the capillary supply to a fiber is muscle fiber size, while fiber type and oxidative capacity play a minor role at best[49,50]. Our data show that CS-exposure and consequently CS-cessation had no significant effects on the capillarization in the plantaris and soleus muscles. This observation is in discrepancy with a recent study showing capillary regression in the soleus after 8 weeks of CS-exposure[7]. However, the data obtained from the EDL (a predominantly glycolytic muscle similar to the plantaris) in the aforementioned study[7] and another[16] report no effect and thus concur with our study. In the diaphragm, we did see an increase, rather than a decrease in capillary density (CD) and capillary fiber density (CFD) after smoking. While at first glance this may suggest angiogenesis, the absence of an increase in LCFR shows that this is not the case, and the increase in capillary density must be due to the decrease in fiber CSA. Further support comes from the observation that the normal values of capillary density after smoking cessation were associated with an increase in fiber CSA, again without a significant change in the LCFR. We have no explanation for the more homogeneous distribution of capillaries after smoking

cessation than even control muscles, but it would result in improved muscle oxygenation. Perhaps it is a sort of adaptation to an impaired respiratory chain function in a bid to restore homeostasis. Further research is required to confirm these findings.

Studies in skeletal muscles of humans have reported CS-induced impairment of the electron transport system, lower maximal ATP production rate and lower oxygen consumption rates in limb muscle mitochondria[3,18,51]. Studies in mice are contentious, with some[16,52] but not all[6] reporting limb muscle mitochondrial dysfunction after CS-exposure. Here we show that the respiration of the mitochondria was indeed impaired during CS-exposure and restored with smoking cessation. To further investigate the potential direct effects of CS on skeletal muscle mitochondria, we exposed the soleus muscle from control mice to CS extracts. This resulted in reduced respiration that was readily reversed after removal of the extracts. This indicates that substances in CS acutely impair mitochondrial function. Although the smoke extracts contained a large variety of substances that could potentially explain the acute impairment of mitochondrial respiration, it fits the notion that carbon monoxide in cigarette smoke can directly inhibit complex IV activity and mitochondrial respiration[18]. In addition to potential effects of CO, we also observed a significant lower protein expression levels of subunits of mitochondrial complex I, III and IV that were similar to control levels after 1 and 2 weeks of smoking cessation.

CS-exposure or CS-cessation had no effect on maximal exercise capacity, whole body strength and spontaneous physical activity, though the physical activity levels of all mice dropped significantly as from the start of CS/room air exposure (-50%) probably as a result of external stress. This shows that the effects observed in our study were not due to alterations in physical activity (disuse or overuse) and supports the concept that CS has direct effects on skeletal muscles.

These results are in line with a previous study showing no CS-induced alterations in whole body strength and maximal exercise capacity after 24 weeks of CS-exposure[26].

Though we looked very comprehensively at many parameters, our study does not present in depth assessment of specific mechanisms. Also due to the plethora of analyses, the number of mice for some of the analyses may have been rather low but no type II error was detected. In addition, since most of the CS-induced effects on skeletal muscles observed in our study (decreased muscle mass, reduced protein content and function of mitochondrial enzymes and diaphragm atrophy) are in line with previous studies with similar sample sizes, this confirms that the probability of getting false negatives in our study is low. The absence of female mice in this study also limits the scope of interpolation and extrapolation of these data. We present here an extensive assessment of the short-term effects of smoking cessation on skeletal muscles in male mice and whether similar data would be observed in female mice needs to be addressed. Further research is needed to clarify the mechanisms of changes in muscle mass and capillarization, effects of intermittent hypoxia and physical activity alterations.

In conclusion, our study shows that smoking cessation for as short as 2 weeks exerted immediate benefit on the diaphragm and limb muscles with reversal of muscle mass loss, especially lean mass and improvement of mitochondrial function at least in male mice. Obviously, these data could help motivating and stimulating stopping smoking as they highlight a real benefit of short-term smoking cessation on skeletal muscle and body mass.

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Declaration of interests

All authors have no conflicts of interest to disclose

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Figure caption

Figure 1. Mitochondrial function in soleus muscle (n = 19/group): Leak respiration (A), maximal uncoupled respiration (B), succinate/rotenone (complex II)-stimulated respiration (C) and protein levels of key subunits of the mitochondrial complex I to IV (NDUFB8 (CI), complex II subunit, UQCRC2 (CIII), MTCO1 (CIV)), and ATP synthase α-subunit (CV) in Con (open bars), CS (hatched bars), CS1W (brick bars) and CS2W (solid black bars) [D] – [p = 0.07 for the comparison between CS group and Con group for mitochondrial complex IV]. Typical western blot with bands corresponding to the mitochondrial complex protein levels (E) and maximal oxygen consumption after acute exposure of the soleus to 5% and 20% CS extracts (CSE) and after a 30 minute wash out period (F). **P<0.001, *P<0.05 vs control; ##p<0.001, #p<0.05 vs smoking; ns = non-significant. Values are presented as mean and SD.

Table 1: Muscle mass of the right calf complex

	Mass (mg)

Muscle	Con	CS	CS1W	CS2W
Plantaris	20.5 ± 2.9	16.9 ± 1.7**	18.2 ± 2.7*	19.7 ± 2.0#
Soleus	10.8 ± 1.0	9.1 ± 0.7**	10.0 ± 1.3	10.6 ± 1.2##
Gastrocnemius	147 ± 12	126 ± 11**	137 ± 14	146 ± 16##

Con = air-exposed for 14 weeks; CS = CS-exposed for 14 weeks; CS1W = CS-exposed for 13 weeks and stop for 1 week; CS2W = CS-exposed for 12 weeks and stop for 2 weeks (n = 13/group). Values expressed as mean ± SD. ***P<0.001, *P<0.05 vs control; #p<0.05, ##p<0.007 vs smoking.*

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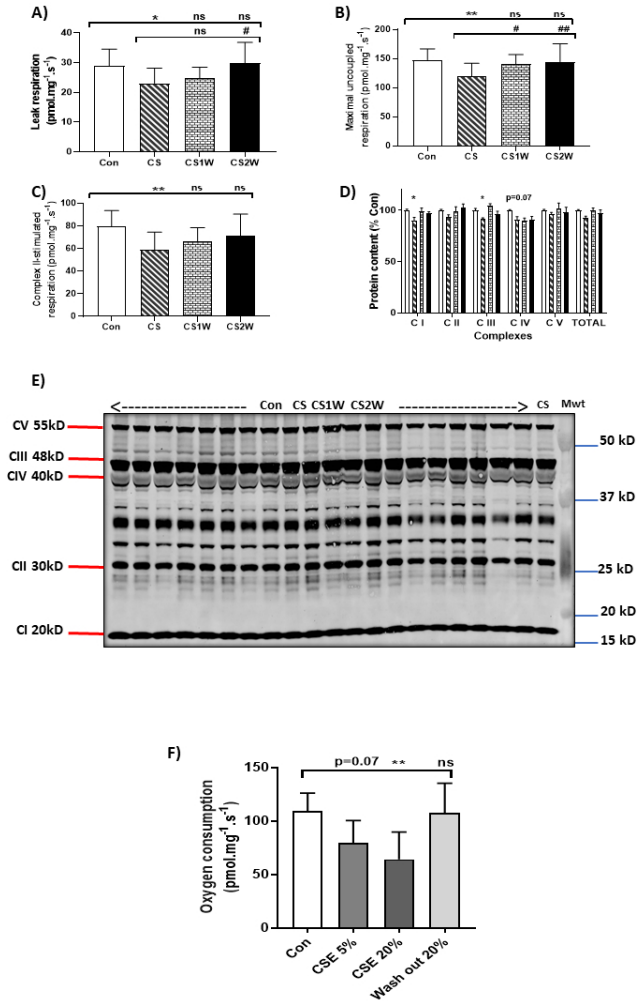

Figure 1. Mitochondrial function in soleus muscle (n = 19/group): Leak respiration (A), maximal uncoupled respiration (B), succinate/rotenone (complex II)-stimulated respiration (C) and protein levels of key subunits of the mitochondrial complex I to IV (NDUFB8 (CI), complex II subunit, UQCRC2 (CIII), MTCO1 (CIV)), and ATP synthase α-subunit (CV) in Con (open bars), CS (hatched bars), CS1W (brick bars) and CS2W (solid black bars) [D] – [p = 0.07 for the comparison between CS group and Con group for mitochondrial complex IV]. Typical western blot with bands corresponding to the mitochondrial complex protein levels (E) and maximal oxygen consumption after acute exposure of the soleus to 5% and 20% CS extracts (CSE) and after a 30 minute wash out period (F). **P<0.001, *P<0.05 vs control; ##p<0.001, #p<0.05 vs smoking; ns = non-significant. Values are presented as mean and SD.

190x338mm (96 x 96 DPI)

Two weeks smoking cessation reverses cigarette smoke-induced skeletal muscle atrophy and mitochondrial dysfunction in mice

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Supplementary material

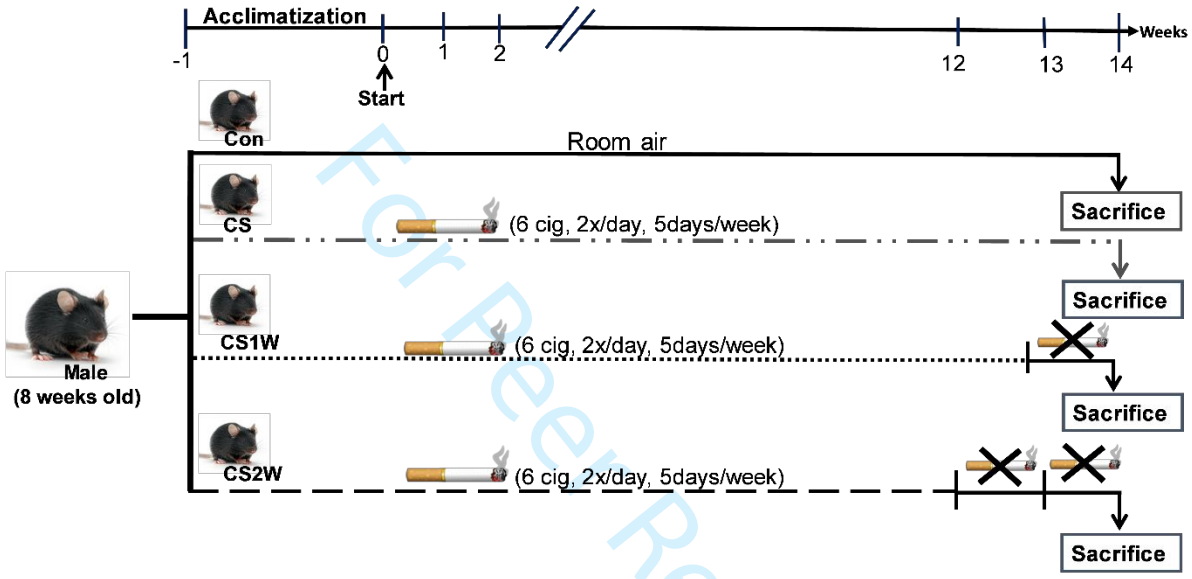

Figure S1. Study design. **Con** = air-exposed for 14 weeks; **CS** = CS-exposed for 14 weeks; **CS1W** = CS-exposed for 13 weeks and stop for 1 week; **CS2W** = CS-exposed for 12 weeks and stop for 2 weeks; Cig = cigarette.

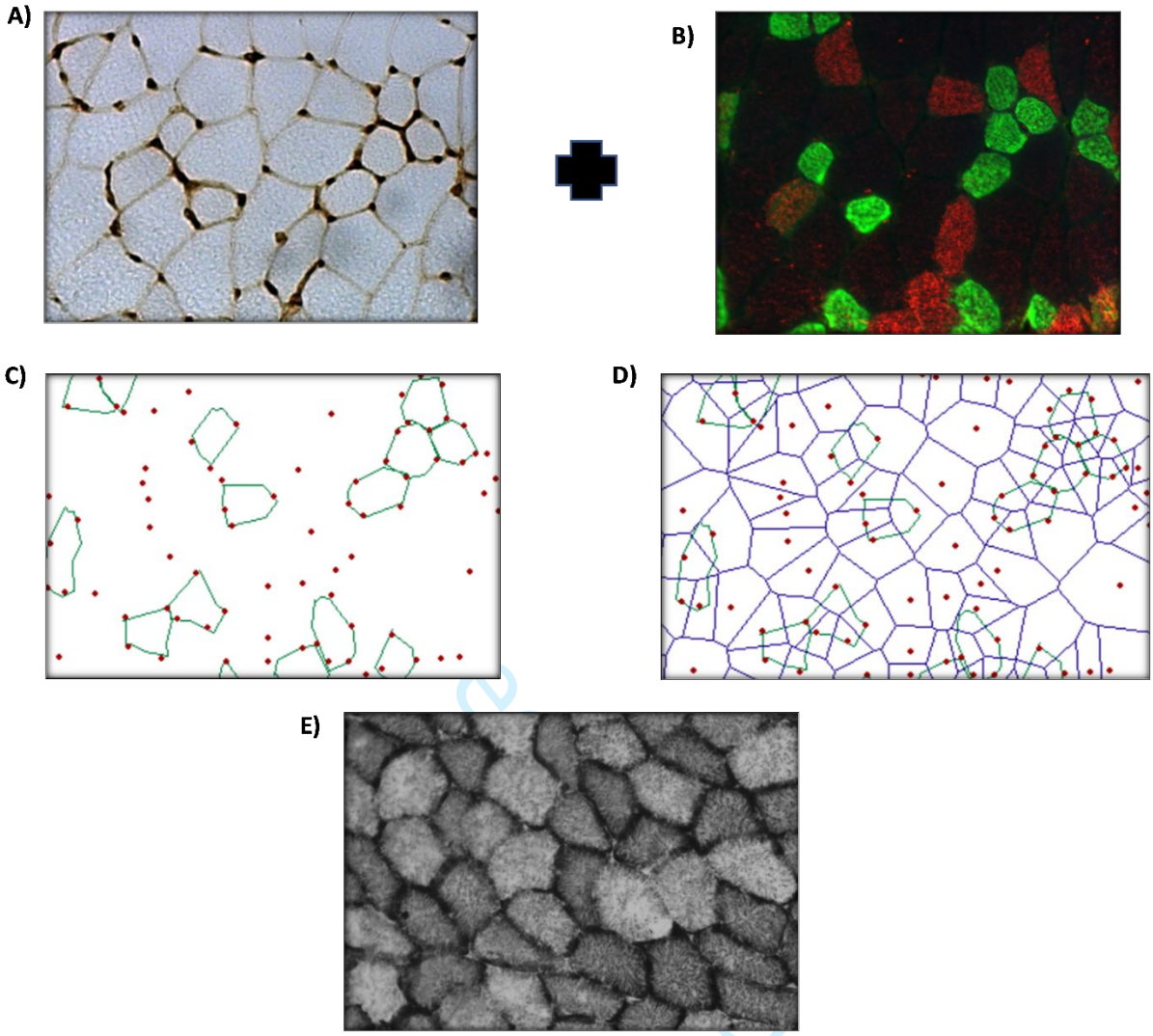

Figure S2. Fiber cross sectional area (FCSA), capillarization and SDH activity measurement. (A) Lectin stained 10 µm section of the plantaris showing capillaries (black dots) around fibers; (B) MHC Stained serial section for identification of fibers and fiber types (Green = IIa, Red = IIx, unstained = IIb). The black plus sign between A and B indicates that both images are used simultaneously to produce C and D in the digitizing tablet. (C) Trace of type IIa fibers (green outlines) with capillaries (red dots) using digitizing tablet which provides FCSA data; (D) The overlap of automatically calculated capillary domains (blue outlines) and type IIa fibers to

determine capillarization parameters. (E) Typical example of 10 µm section of the soleus stained for SDH activity. The darker stained fibers have more SDH activity.

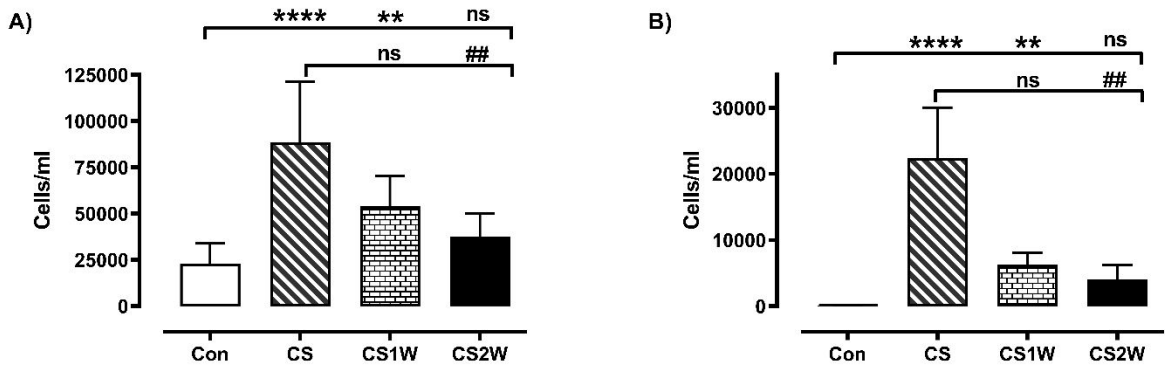

Figure S3. Effects of CS-exposure and CS-cessation on lung inflammation (n = 13 mice per group measured). CS caused neutrophilic lung inflammation which was similar to controls after two weeks of smoking cessation Total cell count [A] and Neutrophil count [B] in BAL. *****P<0.00 01, **P<0.001, *P<0.05 vs control*; ##p<0.001 vs *smoking ns: not significant*. Values are presented as mean and SD.

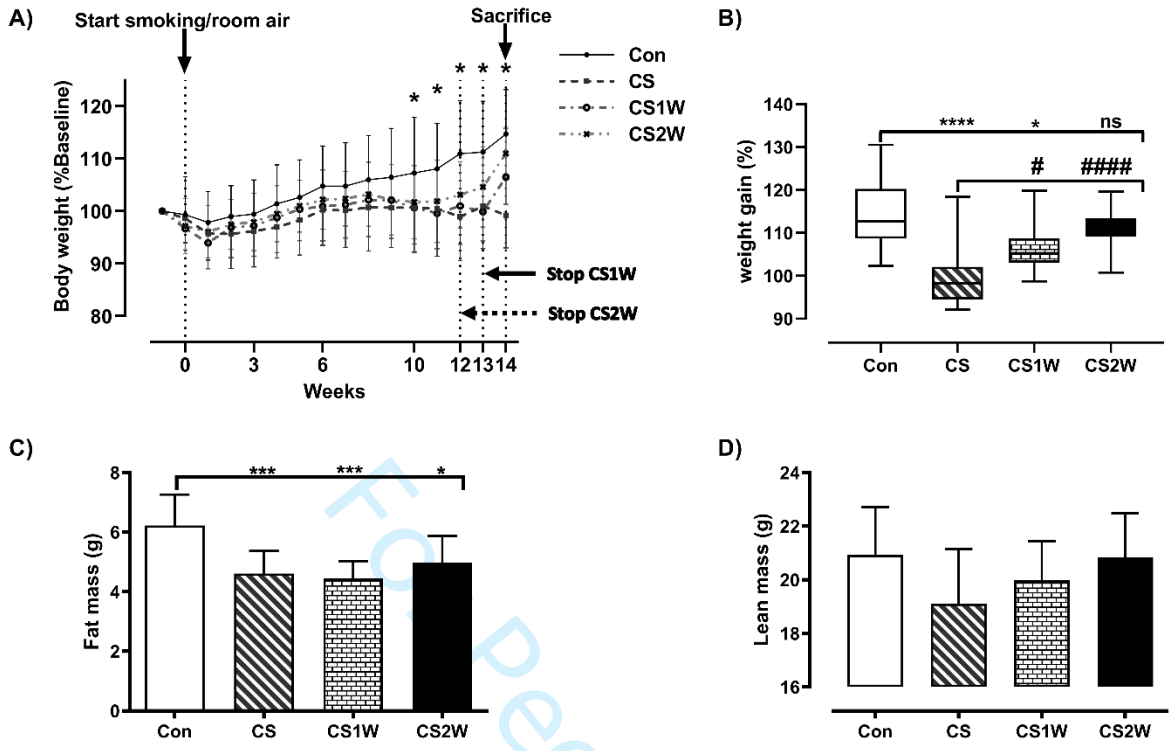

Figure S4. A) Body mass changes (n=19/group) [Full and dashed arrows indicate smoking cessation for CS1W and CS2W respectively. Asterisks indicate statistically significant differences between all CS-exposed animals and the air-exposed group]; B) weight gain (%) from the end of CS or room air exposure in CS1W, CS2W, CS and Con showing clear improvements in the cessation groups (n=19/group); C) fat mass and; D) lean mass changes (n=11/group) showing non-statistically significant improvement after cessation. Con (closed circles and open bars), CS (closed squares and hatched bars), CS1W (open circles and brick bars) and CS2W (crosses and solid black bars). **Con** = air-exposed for 14 weeks; **CS** = CS-exposed for 14 weeks; **CS1W** = CS-exposed for 13 weeks and stop for 1 week; **CS2W** = CS-exposed for 12 weeks and stop for 2 weeks. Values are presented as mean and standard deviation (A, C and D) or median and interquartile range (B). ****p<0.0001, *p<0.05 vs Con; #### p<0.0001, # p<0.05 vs CS, ns: not significant.

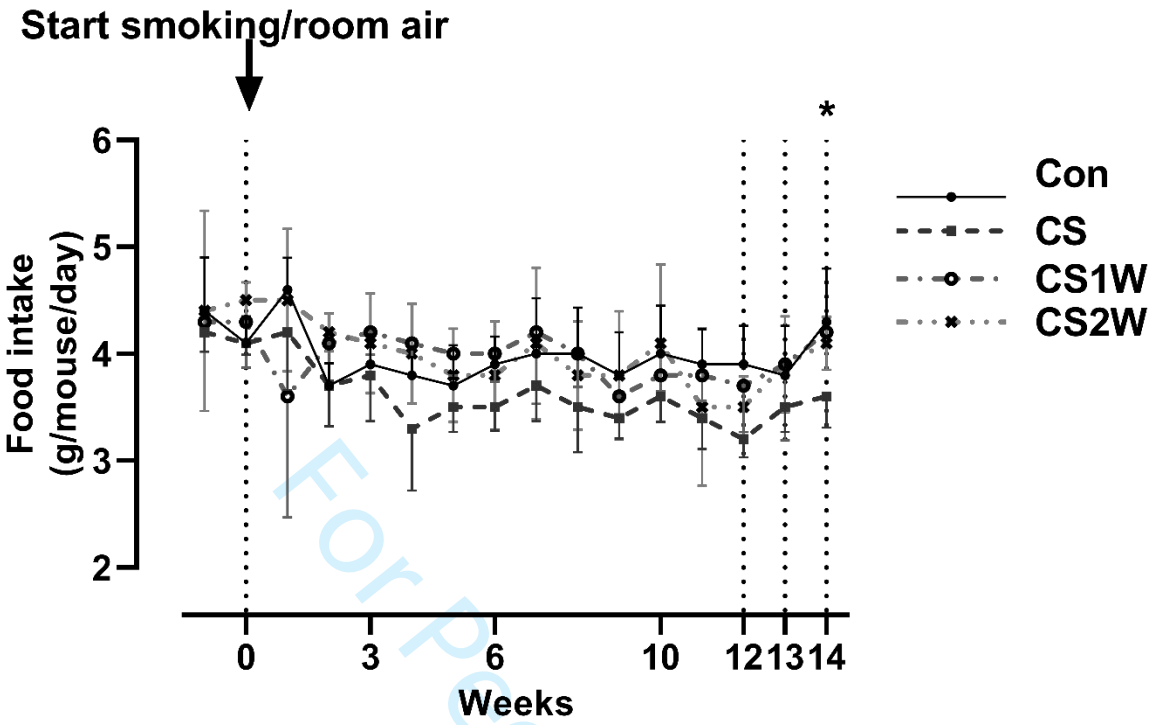

Figure S5. Average weekly food intake during protocol. CS1W and CS2W mice ate significantly more after smoking cessation. Con (closed circles), CS (closed squares), CS1W (open circles) and CS2W (crosses). Data are shown as mean ± SD. **P<0.05 for CS vs others*.

Table S1: Maximal Exercise Capacity, Whole-Body Strength and Spontaneous physical activity

	Con	CS	CS1W	CS2W	ANOVA	Post Hoc
	Maximal exercise capacity (Maximum running speed) [m/min]					
Baseline	19.9 ± 2.6	22.8 ± 2.5	21.8 ± 2.3	21.2 ± 1.8	*p = 0.004*	CS > Con
Terminal	18.8 ± 2.8	20.6 ± 2.2	19.9 ± 1.7	19.3 ± 1.9	*p = 0.28*	ns
p-Value (Bl vs Ter)	*0.18*	*0.02*	*0.05*	*0.01*		
	Whole body strength (Latency-to-fall time [min])					
Baseline	4.4 ± 3.7	5.4 ± 3.6	6.5 ± 5.6	2.8 ± 1.2	*p = 0.30*	ns
Terminal	1.5 ± 1.5	1.9 ± 1.2	1.0 ± 0.3	1.0 ± 0.6	*p = 0.15*	ns
p-Value (Bl vs Ter)	*0.002*	*0.002*	*0.018*	*0.001*		
	Spontaneous physical activity (night activity [m/h])					
Baseline	38.0 ± 10.3	42.2 ± 10.8	40.1 ± 11.0	44.8 ± 10.0	*p = 0.50*	ns

Start smoking or air	16.9 ± 4.8	18.2 ± 3.9	18.0 ± 3.9	21.8 ± 3.8	*p = 0.09*	ns
p-Value (Bl vs start)	*0.18*	0.06	*0.006*	*0.07*		
11 - 12 weeks	14.9 ± 6.9	14.4 ± 3.3	17.6 ± 6.0	14.7 ± 5.1	*p = 0.78*	ns
p-Value (Bl vs 11-12)	*0.009*	*0.049*	*0.003*	*0.03*		
Week 13	11.8 ± 4.3	11.6 ± 4.4	12.7 ± 6.0	14.0 ± 4.2	*p = 0.74*	ns
p-Value (Bl vs Wk 13)	*0.0004*	*0.01*	*0.0009*	*0.03*		
Week 14 (Terminal)	14.7 ± 5.6	12.6 ± 3.0	16.9 ± 4.3	16.5 ± 4.9	*p = 0.22*	ns
p-Value (Bl vs Wk 14)	*0.04*	*0.047*	*0.0009*	*0.02*		

Con = air-exposed for 14 weeks; CS = CS-exposed for 14 weeks; CS1W = CS-exposed for 13 weeks and stop for 1 week; CS2W = CS-exposed for 12 weeks and stop for 2 weeks (n = 13/group). Values expressed as mean ± SD. Maximal exercise capacity at baseline (BL) and at sacrifice (Ter)[n = 13 mice/ group]. Whole body strength at sacrifice compared to baseline (n = 13 mice/ group). Spontaneous physical activity expressed as distance in meters covered per hour obtained from night activity at baseline, start of CS or room air exposure, at 11-12 weeks of exposure and at week 13 and 14 of the study representing 1 or 2 weeks smoking cessation (n = 9 mice/ group). There was no effect of CS-exposure or cessation on functional physical activity measurements. Baseline is the same for all these parameters and refers to measurements done before the mice were exposed to cigarette smoke or air

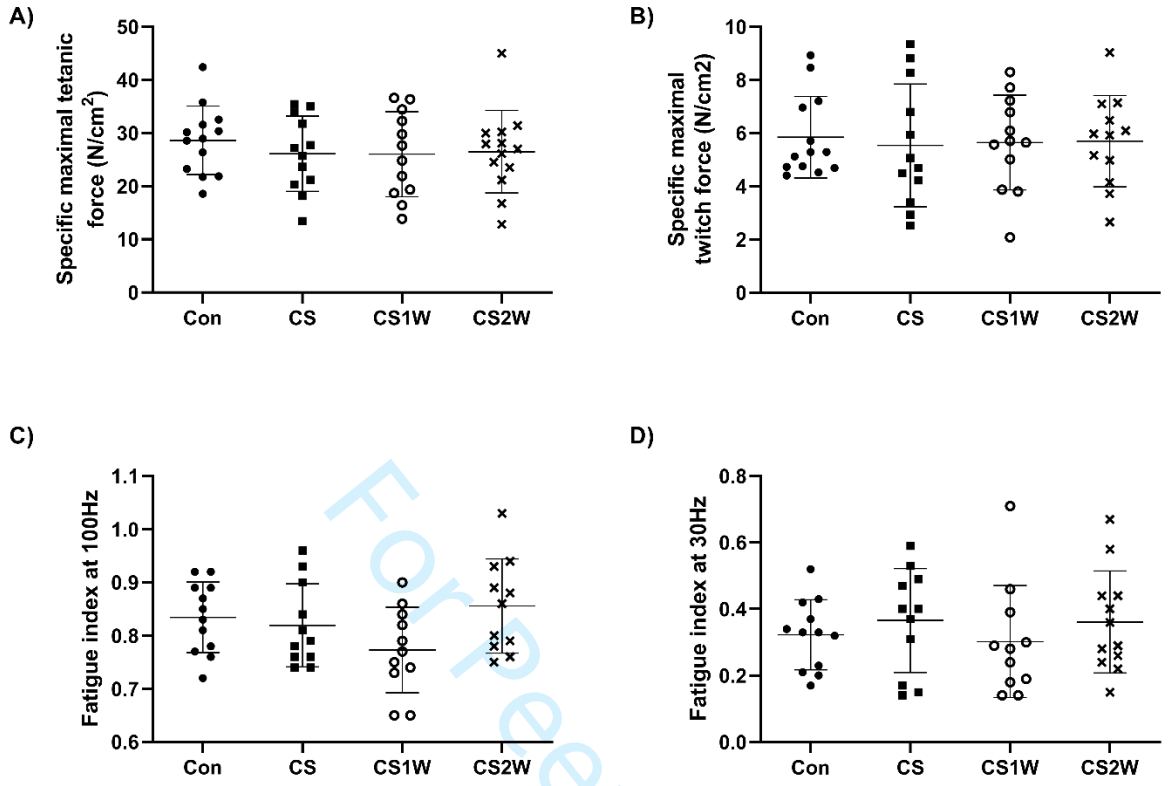

Figure S6. *In situ* contractile properties of the plantaris muscle. Specific tetanic (A) and twitch (B) force. Fatigue index at high (FI100) [C] and low (FI30) [D] stimulation frequency. Con (*closed circles*), CS (*closed squares*), CS1W (*open circles*) and CS2W (*crosses*). Values are presented as mean and SD.

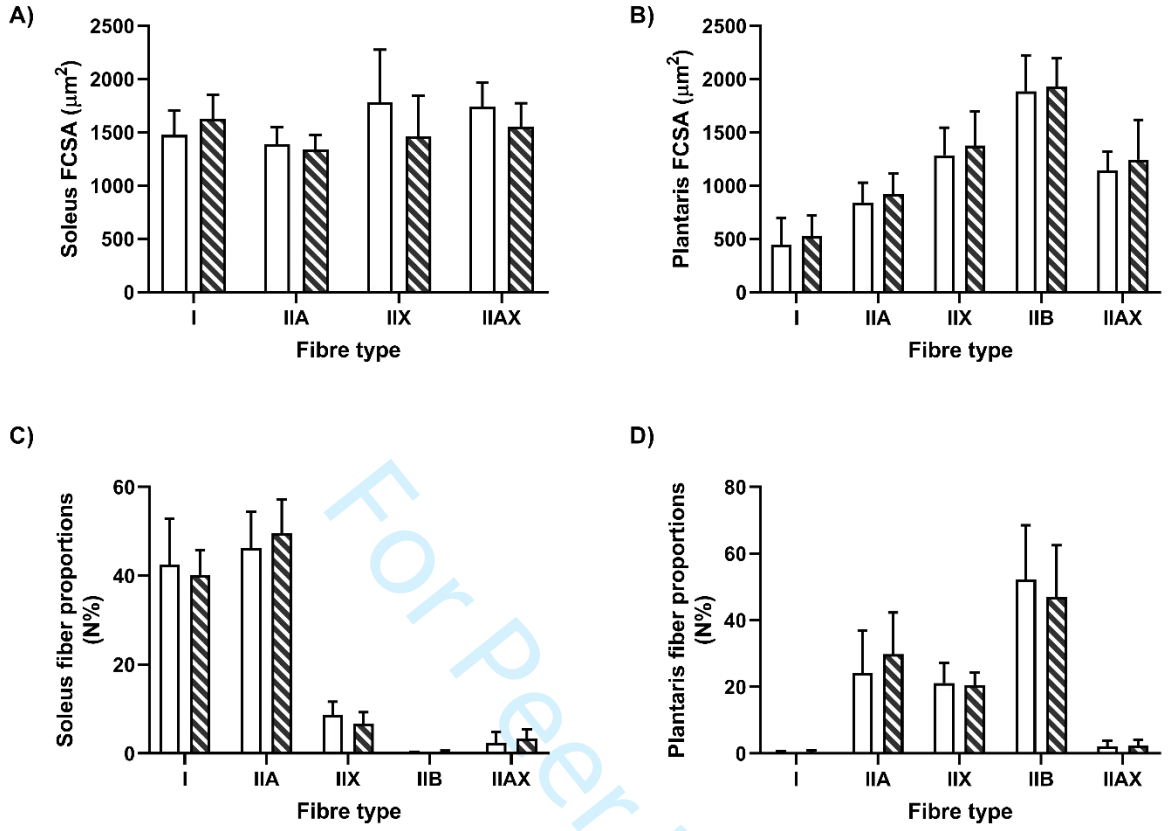

Figure S7. Fiber dimensions (FCSA, [A – B]) and proportions (C – D) in the soleus and plantaris muscles in Con (open bars) and CS (hatched bars). Values are presented as mean and SD.

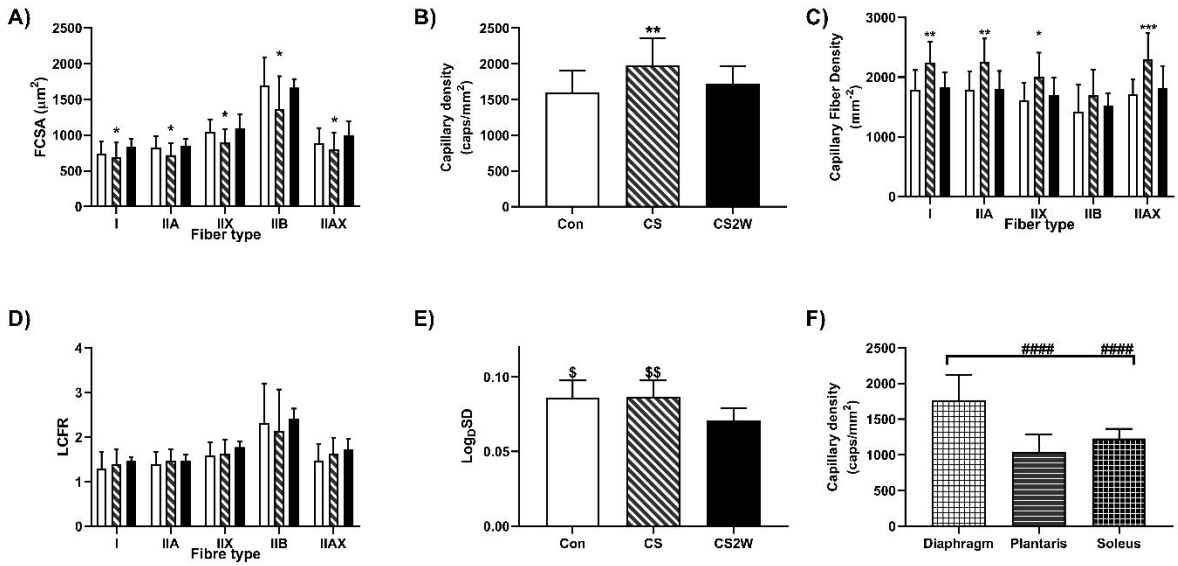

Figure S8. Diaphragm fiber cross-sectional area (FCSA) (A), capillary density (B) and capillary fiber density in the different fiber types (C) are altered by CS-exposure and similar to control with cessation, though LCFR remained unchanged in CS and CS2W (D), indicative of no angiogenesis. Improved diaphragm capillary distribution in CS2W (E). Higher capillary density of the diaphragm compared to the plantaris and soleus muscles (F).Con (open bars), CS (hatched bars) and CS2W (solid black bars) (n = 13 mice/group). ****P<0.001; **P<0.01; *P<0.05 vs control and smoking, ####P<0.0001 vs diaphragm, $$P=0.008; $P<0.05 vs CS2W)*. $Log_D SD$ = logarithmic standard deviation of the domain areas Values presented as mean and SD.

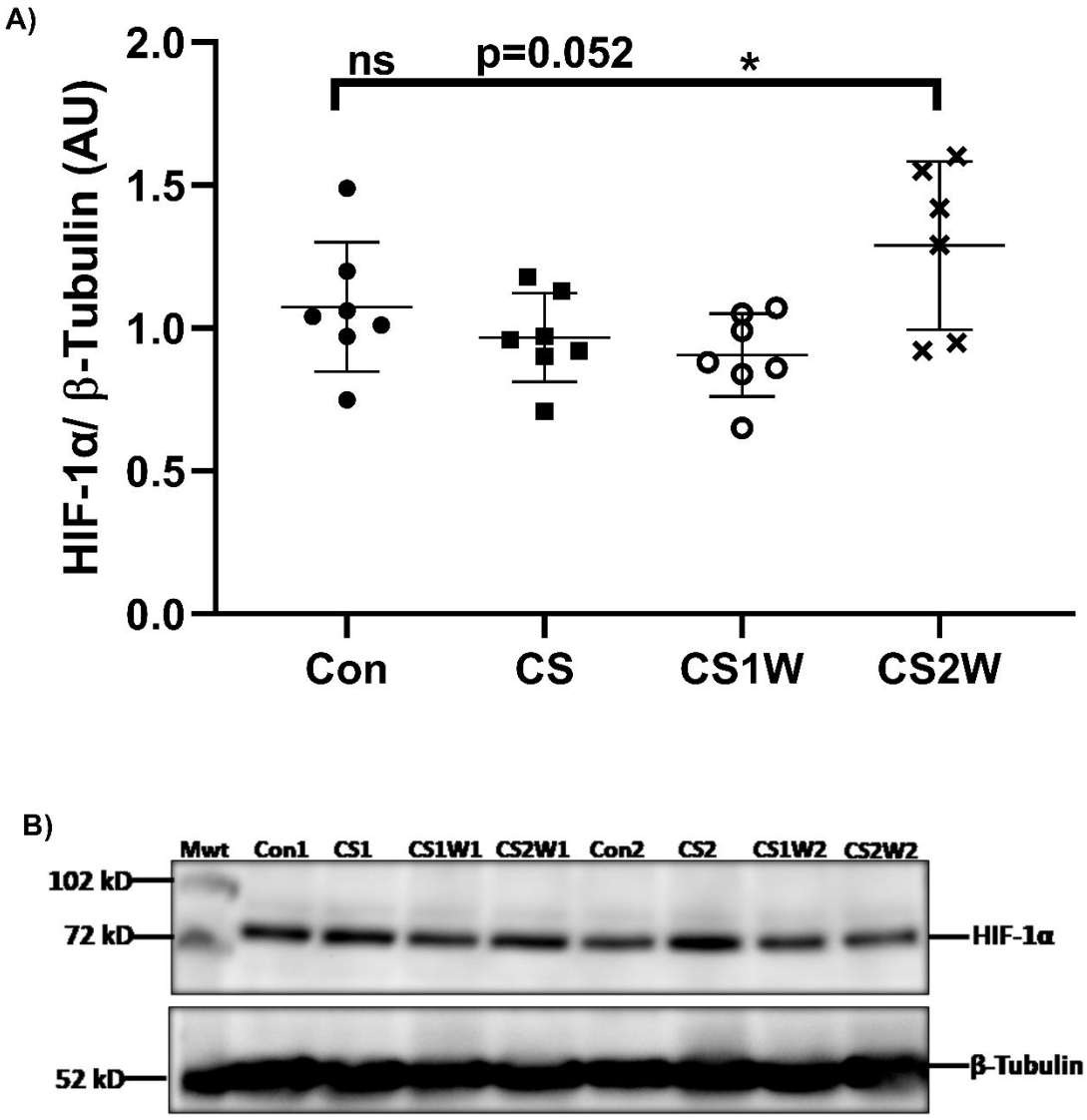

Figure S9. HIF-1α western blots. Cytoplasmic protein levels of HIF-1α normalized by β-tubulin in arbitrary units (AU) increased significantly in CS2W (n = 7 mice/group) [A]. Typical western blot with bands corresponding to HIF-1α and β-tubulin cytoplasmic protein levels (B). Each point represents an individual value, horizontal bars represent mean values while vertical lines represent standard deviation. **P<0.05 vs CS2W, ns: not significant*.