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Bowen, T, Aakerøy, L, Eisenkolb, S et al. (10 more authors) (2017) Exercise Training Reverses Extrapulmonary Impairments in Smoke-exposed Mice. Medicine & Science in Sports & Exercise, 49 (5). pp. 879-887. ISSN 0195-9131

https://doi.org/10.1249/MSS.0000000000001195

Exercise training reverses extrapulmonary impairments in smoke-exposed mice

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Running title: Smoke-induced extrapulmonary impairments

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Abstract

Purpose: Cigarette smoking is the main risk factor for chronic obstructive pulmonary disease and emphysema. However, evidence on the extrapulmonary effects of smoke exposure that precede lung impairments remains unclear at present, as are data on non-pharmacological treatments such as exercise training.

Methods: Three groups of mice including control (n=10), smoking (n=10), and smoking with 6 weeks of high-intensity interval treadmill running (n=11) were exposed to 20 weeks of fresh air or whole-body cigarette smoke. Exercise capacity (peak oxygen uptake) and lung destruction (histology) were subsequently measured, while the heart, peripheral endothelium (aorta), and respiratory (diaphragm) and limb (extensor digitorum longus and soleus) skeletal muscles were assessed for *in vivo* and *in vitro* function, *in situ* mitochondrial respiration, and molecular alterations.

Results: Smoking reduced body weight by 26% ($P<0.05$) without overt airway destruction ($P>0.05$). Smoking impaired exercise capacity by 15% while inducing right ventricular dysfunction by ~20%, endothelial dysfunction by ~20%, and diaphragm muscle weakness by ~15% (all $P<0.05$), but these were either attenuated or reversed by exercise training ($P<0.05$). Compared to controls, smoking mice had normal limb muscle and mitochondrial function (cardiac and skeletal muscle fibers), however diaphragm measures of oxidative stress and protein degradation were increased by 111% and 65% ($P<0.05$), but these were attenuated by exercise training ($P<0.05$).

Conclusions: Prolonged cigarette smoking reduced exercise capacity concomitant with functional impairments to the heart, peripheral endothelium, and respiratory muscle that preceded the development of overt emphysema. However, high-intensity exercise training was able to reverse these smoke-induced extrapulmonary impairments.

Key words: Diaphragm; Skeletal muscle; Endothelium; High-intensity interval training; Mitochondrial function; COPD

Introduction

Cigarette smoking is the main risk factor for chronic obstructive pulmonary disease (COPD). This is characterized not only by airway destruction and airflow limitation but also extrapulmonary impairments to the heart (12, 25), peripheral vasculature (6, 27), and skeletal muscles (2, 3, 18, 24), which collectively exacerbate the key symptom of exercise intolerance. Interestingly, initial evidence suggests that smokers at risk of COPD may also suffer extrapulmonary impairments similar to those demonstrated in smokers with CODP (11, 31). However, data detailing the extrapulmonary effects of prolonged smoke exposure in the period preceding COPD remain unclear at present, as are data on potential favorable therapeutic interventions. For example, it still remains unknown whether smoke-induced extrapulmonary impairments in the heart, endothelium, and respiratory and limb skeletal muscle occur in parallel as a direct consequence to cigarette smoke (i.e., primary effect) or as an indirect consequence following the subsequent development of emphysema (i.e., secondary effect) (2, 3, 7, 13). In addition, data on whether prolonged smoke exposure induces skeletal muscle dysfunction in the lower limbs remains controversial, with some (24, 28) but not all (7, 34) studies reporting impairments. Further, whether smoking directly induces respiratory (i.e., diaphragm) fiber contractile dysfunction remains unknown, despite the diaphragm playing a direct role in the pathogenesis of dyspnea in smokers (11) and also not being affected by disuse (as is common for the lower limb muscles (7)). As such, more evidence is needed to clarify the role of cigarette smoke on respiratory and limb skeletal muscle contractile function.

Interestingly, physical inactivity has recently been demonstrated to exacerbate the extrapulmonary impairments associated with prolonged cigarette smoking (7), while exercise training has been shown to reverse smoke-induced cardiac dysfunction in mice (15). Yet whether exercise training can also reverse numerous smoke-induced systemic impairments related not only to cardiac

function but also to the peripheral endothelium as well as respiratory and locomotor skeletal muscles remains unknown. In this study, therefore, we exposed mice to cigarette smoke followed by the intervention of high-intensity exercise training and used an integrative approach by combining measures of the lung and exercise capacity in parallel with multiple-extrapulmonary organs. We hypothesized that cigarette exposure would impair cardiac, endothelial, respiratory and limb muscle function prior to the development of overt emphysema, and that exercise training would be able to attenuate such impairments.

Materials and Methods

Animals and smoke exposure

Ten week old A/JOlaHsd female mice (Harlan Laboratories, UK) were used in this study, as approved by the Norwegian Animal Research Authority and in accordance with ACSM's animal care standards. This study lasted a total of 26 weeks: 0-20 weeks mice were exposed to either fresh-air or cigarette smoke; 20-26 weeks mice performed exercise training or remained sedentary; week 26 mice were sacrificed. Briefly, mice were randomly separated into three groups to receive either: (1) fresh air (control mice; n=10); (2) cigarette smoke (smoking mice; n=10); and (3) cigarette smoke and high-intensity interval exercise training (smoking+HIIT mice; n=11). The exposure period consisted of 6 hours per day for 5 days a week and this was achieved by placing mice in a chamber connected to a smoking device (TE-10, Teague Enterprises, California, US), which provided fresh air or cigarette smoke (Research Cigarettes 3R4F, University of Kentucky, Lexington, US). The total particulate matter (TPM) was 100 mg/m^3 for the first 2 weeks and 200 mg/m^3 for the remaining exposure time (a level of 250 TPM has been suggested to be equivalent to humans smoking around 15 packs per day (21)), which was monitored weekly and measured with gravimetric method as previously described (20). The exposure period was terminated after 20 weeks and the exercise training intervention was then performed.

Exercise training and exercise capacity

High-intensity interval training (HIIT) was performed on a treadmill (25° incline) 5 times per week over 6 weeks. Each session consisted of 10 intervals (4 min at ~90 % peak work rate) separated by 2 min recovery (~60% peak work rate). Exercise capacity was assessed in all mice by measuring peak oxygen uptake ($\dot{V}O_{2peak}$) in ml/min via a closed metabolic chamber, as previously described (17).

In vivo cardiac function and hemodynamics

Echocardiography was performed as previously described (15). Briefly, in the week before sacrifice mice were anesthetized with 2 % isoflurane adjusted to maintain similar heart- and respiration rates between animals. The echocardiography system with a 24 MHz probe was used to determine both right and left ventricular function (Vevo 2100 Visual Sonics, Toronto, Canada). Right ventricular systolic function was assessed by measuring the tricuspid annular plane systolic excursion (TAPSE), which was defined as the differences in systolic and diastolic displacement of the right ventricular base, as obtained from the apical four chamber view in B-mode. This included the M-mode cursor being oriented to the junction of the tricuspid valve plane in the right ventricular free wall and through the apex. Right ventricular systolic velocity (RVS') was obtained from the apical four chamber view using tissue Doppler from the lateral wall close to the tricuspid inflow. Pulmonary acceleration time (PAP), defined as the onset of pulmonary flow to the peak flow, was measured using pulsed-wave Doppler in the short-axis view at the level of the aortic valve. In addition left ventricular function was assessed using B-mode images in the parasternal short axis view to calculate left ventricular ejection fraction (LVEF), while left ventricular fractional shortening (LVFS) was calculated in M-mode. *In vivo* hemodynamics of the right ventricular systolic pressure was also determined as previously described (33). Briefly, immediately prior to sacrifice the mice were anesthetized with 2 % isoflurane under spontaneous breathing and the external jugular vein was dissected free and a 1.2 F pressure catheter inserted to determine right ventricular systolic pressure (Scisense pressure catheter FTH-1211B-0018, Scisense Inc, Canada). Data were acquired with Advantage PV System ADV500 (Transonic Inc, Canada). Body temperature was measured with a rectal probe, which was kept constant at 37.5°C during the experiment. The animal was observed until stable readings before recording. The animals were

killed immediately following the recordings by removal of the heart. Data were analyzed with LabChart version 7 (ADInstruments, UK). Right ventricular systolic pressure was used an index of pulmonary hypertension, which is usually developed during emphysema.

Lung emphysema

The right lung was inflated by instilling a 4 % formaldehyde phosphate buffered solution via the trachea at a pressure of 25 cm H_2O. It was maintained to fixate like this for a minimum of 15 min before ligating the right main bronchus and putting the whole specimen in formaldehyde solution. Later, it was paraffin-embedded, sectioned, and stained with hematoxylin, eosin and saffron. Light microscopy was then used to identify suitable areas free from artefacts and 4-8 images per animal were selected on a random basis. A reference grid was placed on images in order to count the points of intercepts between the grid lines and alveolar walls, followed by dividing this number by the total length of all grid lines, in order to determine the mean linear intercept length (Lm), as previously detailed (10).

In vitro skeletal muscle function

A diaphragm strip, the soleus, and the extensor digitorum longus (EDL) were isolated and stimulated across a range of frequencies for measurement of *in vitro* isometric contractile function, as previously described (5). Muscle force (N) was normalized to muscle cross-sectional area (cm^2) by dividing muscle mass (g) by the product of L_o (cm) and estimated muscle density (1.06) (8), which allowed specific force in N/cm^2 to be calculated.

In vitro peripheral vascular endothelial function

To provide an index of vascular function, aortic rings (n=4) were dissected from mice and mounted in a buffer-filled (in mmol/L: 118 NaCl, 25 $NaHCO_3$, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 2.5 $CaCl_2$, 5.5 glucose) organ bath between a hook and a force transducer, as previously described (1). After 30 min of equilibrium, the maximal constriction was achieved by adding KCl (final concentration 100 mmol/L) to the buffer. After several rinses, aortic rings were pre-constricted by adding increasing concentrations of phenylephrine (Sigma, Taufkirchen Germany; 10^{-9} to 10^{-3} mol/L) to around 70 % of maximal KCl constriction. Relaxation to increasing concentrations of acetylcholine (Sigma, Taufkirchen Germany; 10^{-9} to 10^{-2} mol/L) and sodium nitroprusside (Sigma; 10^{-9} to 10^{-2} mol/L) were recorded until no further changes were observed in order to determine endothelial-dependent and endothelial-independent vasodilation, respectively.

In situ mitochondrial respiration

Mitochondrial respiration was measured *in situ* from permeabilized cardiac and diaphragm fibers using high resolution respirometry (Oxygraph-2k: Oroboros Instruments, Innsbruck, Austria), as previously described (5). Dissected fibers were immediately placed in ice-cold BIOPS (2.77 mM CaK_2EGTA buffer, 7.23 mM K_2EGTA buffer, 20 mM imidazole, 20 mM taurine, 50 mM 2-(N-morpholino)ethanesulfonic acid hydrate, 0.5 mM dithiothreitol, 6.56 mM $MgCl2 \cdot 6H_2O$, 5.77 mM Na_2ATP and 15 mM Na_2PCr (pH 7.1)) and under a microscope carefully dissected in 2 ml BIOPS at 4°C and permeabilized in saponin (50 µg/ml) for 30 min. Fiber bundles were then transferred to 2 ml ice cold mitochondrial respiration medium (MiR05; 0.5 mM EGTA, 3mM MgCl2·6H2O, 60mM Lactobionic acid, 20mM taurine, 10mM KH_2PO4, 20mM HEPES, 110mM D-Sucrose, 1 g/L BSA essentially fatty acid free, pH 7.1) and gently agitated for 10 minutes at 4°C before placed in a 2 mL oxygraph chamber incubated in MIR05 at 37°C oxygenated at ~300-400 µM (Oxygraph-2k, Oroboros, Innsbruck, Austria). All experiments were performed in duplicates. A protocol

evaluating both leak (*L*) and oxidative phosphorylation (*P*) respiration states in complex I (C_I) and complex I+II (C_{I+II}) was assessed by addition of malate (2mM) and glutamate (10mM), followed by saturating ADP (5mM), and then succinate (10mM), respectively. All measurements were normalized to muscle wet weight and are presented as pmol $O_2 \cdot sec^{-1} \cdot mg^{-1}$. In addition, the respiratory control ratio (RCR) was calculated as the ratio of maximal C_I (*P* C_I) to leak state (*L* C_I) respiration, and also a substrate control ratio (SRC) was calculated as *P* C_I to *P* C_{I+II}.

Tissue analyses

Fiber typing and cross sectional-area analyses were performed on diaphragm sections, as previously described (5). Protein expression of MuRF1, MAFbx, NADPH oxidase (subunit $gp91^{phox}$), myosin heavy chain, and eNOS (phosphorylated and acetylated) was assessed by western blot, while enzyme activity of NADPH oxidase, catalase, and superoxide dismutase was quantified in a photometric manner, as previously described (5). All data are presented as fold change relative to sham.

Statistical analyses

Data are presented as mean±SEM. Between-group differences in terms of physical characteristics, cardiac function, $\dot{V}O_{2peak}$, mitochondrial function, protein expression, and enzyme activity were assessed by one-way ANOVA (Bonferroni post hoc) or non-parametric Kruskal-Wallis (Dunn's post hoc). Endothelial function (group X concentration) as well as force-frequency (group X frequency) and fatigue protocols (group X time) from each skeletal muscle were assessed by two-way repeated measures ANOVA with Bonferroni post hoc analysis. Statistical significance was accepted as $P<0.05$.

Results

Smoke exposure in mice reduced body weight by 26 % ($P<0.05$; Fig. 1A) and impaired exercise capacity by 15 % ($P<0.05$; Fig. 1B), however the latter was normalized following exercise training ($P>0.05$; Fig. 1B). In addition, in line with our hypothesis cigarette smoke exposure did not cause the development of overt emphysema, as demonstrated by no discernable alveolar destruction (i.e., mean linear intercept remained unchanged; $P>0.05$; Fig. 1C) and by the absence of pulmonary hypertension (i.e., right-ventricular systolic pressures were unchanged; $P>0.05$; Fig. 1D). Similarly, no differences were found between groups in terms of tibia length or skeletal muscle wet weights ($P>0.05$).

Smoke-induced extrapulmonary impairments

Heart: Heart weight remained unaltered following cigarette smoking ($P>0.05$). In addition, while echocardiography revealed that left ventricular function was not impaired by smoking ($P>0.05$; Table 1), right ventricular systolic function was reduced compared to controls by ~20 % as assessed by TAPSE ($P<0.05$; Table 1). To determine whether mitochondrial dysfunction may underlie this impairment, we further assessed mitochondrial respiration in fibers from the right and left ventricle, but found no differences ($P>0.05$; Fig. 2A-B).

Peripheral endothelium: The effect of smoking on peripheral endothelial function was assessed from isolated aortic rings for both endothelial-dependent and -independent vasodilation (i.e., relaxation). Stimulation of aortic rings with increasing concentrations of acetylcholine revealed that smoking was associated with impaired endothelial-dependent vasodilation ($P<0.05$; Fig. 3A), with smoke-exposed mice achieving only 70±7 % of their maximal dilation compared to control mice values of 91±3 % ($P<0.01$). In contrast, smoke exposure did not impair endothelium-independent vasodilation ($P>0.05$; Fig. 3B), with maximal vasodilation values of 100% achieved

in both groups (P>0.05). To determine potential mechanisms underlying these functional impairments, we further assessed protein expression of phosphorylated eNOS and acetylated eNOS in the aorta but found no differences between control and smoke-exposed mice (P>0.05).

Respiratory and limb skeletal muscles: Compared to controls, diaphragm fiber bundles from smoking mice were weaker by an average of 15±2 % (range: 11 - 19%; P<0.05; Fig. 4A), which occurred at the lower and more clinically-relevant frequencies of 1, 15, 30, and 50 Hz. However, no differences were discerned during the fatigue protocol between groups. In contrast to the diaphragm, smoking had no adverse effect on limb muscle contractile function for either the glycolytic EDL muscle (Fig. 4B) or oxidative soleus muscle (Fig. 4C), or on relative force during the fatigue protocol (P>0.05; data not shown). As these data suggested the diaphragm was more susceptible to cigarette smoke-induced impairments, we focused thereafter on this specific skeletal muscle. Histological analyses of the diaphragm between control and smoke-exposed mice revealed no structural changes in terms of fiber cross-sectional area (fast and slow twitch: 1077±102 vs. 1222±82 μm^2 and 895±123 vs. 877±76 μm^2, respectively; P>0.05) or fiber proportions (fast and slow twitch: 92±1 vs. 92±1 % and 8±1 vs. 8±1 %, respectively; P>0.05). In contrast, while the protein expression of NADPH oxidase, a marker of oxidative stress, was unaltered between groups (P>0.05; 5A), the more functional measure of enzyme activity was increased by 111 % (P<0.05; Fig. 5B). Similarly, the protein expression of MAFbx (a key marker of protein degradation) was also increased by 65 % in smokers compared to controls (P<0.05; 5C). In contrast, however, the protein expression of MuRF1 (another marker of protein degradation) was not different between groups (P>0.05; 5D), as was the case for the contractile protein myosin heavy chain (1.00±0.17 vs. 0.90±0.15; P>0.05). We further assessed whether mitochondrial dysfunction could be contributing to the diaphragm muscle weakness following smoke exposure, however *in situ* mitochondrial respiration of permeabilized fibers was not different between groups (P>0.05; Fig. 2C).

Effects of high-intensity exercise training in smoke-exposed mice

Six weeks of exercise training normalized the smoke-induced reduction in exercise capacity (Fig. 1B), while also improving left ventricular systolic function (i.e., ejection fraction and fractional shortening) when compared to sedentary smoke-exposed mice ($P<0.05$; Table 1). In addition, exercise essentially reversed the smoke-induced impairment of TAPSE, suggesting right ventricular function was normalized ($P<0.05$; Table 1), while also reversing peripheral endothelial dysfunction such that maximal endothelium-dependent dilation was normalized to control values ($P>0.05$; Fig. 3A). In line with this, we found the protein expression of phosphorylated eNOS to be increased in the aorta of smoke-trained mice compared to both control and smoke-exposed sedentary mice ($P<0.05$). Importantly, smoke-induced diaphragm contractile dysfunction was also reversed by exercise training to similar values of that observed in control mice ($P>0.05$; Fig. 4A), which was also the case for both the enzyme activity of NADPH oxidase ($P>0.05$; Fig. 5B) and the protein expression of MAFbx ($P>0.05$; Fig. 5C). Nevertheless, no differences were subsequently found between control, smoking, and smoke-trained mice in terms of the anti-oxidative enzyme activity of superoxide dismutase (1.00±0.09 vs. 1.22±0.22 vs. 1.30±0.23, respectively; $P>0.05$) or catalase (1.00±0.13 vs. 1.01±0.13 vs. 0.83±0.12, respectively; $P>0.05$). Furthermore, exercise training had no influence on the expression of other proteins (e.g., myosin heavy chain, MuRF1, NADPH oxidase), on fiber properties (i.e. cross-sectional area and type), or on mitochondrial function (Fig. 2C; all $P>0.05$).

Discussion

The main findings of this study include: 1) cigarette smoking induced functional impairments to the heart, peripheral endothelium, and respiratory muscle, which occurred in the absence of overt emphysema and these likely converged to reduce exercise capacity; and 2) high-intensity exercise training reversed these smoke-induced extrapulmonary impairments in line with normalizing exercise capacity. Collectively, therefore, our findings may have important clinical implications as they suggest that cigarette smokers in the absence of emphysema likely develop simultaneous extrapulmonary impairments to the heart, peripheral endothelium, and diaphragm that exacerbate exercise intolerance (11, 31), but such maladaptations could be reversed by the non-pharmacological intervention of exercise training.

Smoke exposure and skeletal muscle function

Skeletal muscle dysfunction is a key component of COPD (19) and is associated with numerous adaptations (e.g., fiber atrophy, mitochondrial dysfunction, and increased ROS) (2, 3, 18, 24), yet numerous studies have yielded contradictory results with some (24, 28), but not all (7, 34), showing contractile impediments in the lower limbs. In these previous studies, however, overt emphysema had either developed (7, 24) or was not reported (18, 28), leaving it difficult to discriminate whether the cigarette smoke or emphysema was the causal factor underlying skeletal muscle dysfunction. Our study indicates that cigarette exposure in the absence of emphysema does not induce limb muscle contractile dysfunction, as supported by findings in both highly oxidative (i.e. soleus) and glycolytic (i.e., EDL) muscles. In addition, muscle disuse may have also confounded previous studies where limb muscle dysfunction was reported, as recent data demonstrated physical inactivity combined with smoke-induced emphysema significantly exacerbates limb muscle impairments (7). In the current experiments, therefore, we also assessed the main muscle of

respiration, the diaphragm, which is not only constantly active (i.e., thus attenuating any potential confounding factors associated with disuse) but also is thought to play a direct role in the pathogenesis of dyspnea (a key symptom in smokers even without overt COPD (11)). In the present study, we provide novel evidence that prolonged smoking induces diaphragm contractile dysfunction that is independent of lung structural damage, with fiber bundles ~15-20 % weaker compared to control mice. These data suggest, therefore, a greater sensitivity of respiratory rather than locomotor striated muscle to cigarette smoke-induced dysfunction. Furthermore, this loss of function was not due to muscle atrophy as force was normalized to muscle density, which suggests fiber weakness induced by smoking was the result of intracellular impairments (i.e., contractile dysfunction).

While diaphragm muscle weakness in emphysema has previously been observed in diaphragm fibers from humans (23) and animals (32), we are the first to provide direct evidence that cigarette smoke exposure can lead to diaphragm fiber weakness in the period preceding lung structural damage. The mechanisms underlying the smoke-induced diaphragm muscle weakness may be related to an increase in oxidative stress and protein degradation, as NADPH oxidase activity (but not protein content) and MAFbx protein expression was increased in the diaphragm of smoking mice, but these were attenuated in the smoke-exercise trained mice in parallel with normalized contractile function. These findings compliment previous studies, which also reported increased levels of oxidative stress and oxidative protein modifications in the diaphragm following prolonged cigarette smoking in mice (2, 3). However, contrary to data from the soleus muscle (29), mitochondrial dysfunction was not present in diaphragm fibers following prolonged smoke exposure, which suggests a low oxidative phosphorylation capacity was unlikely mediating the smoke-induced respiratory muscle weakness. Taken together, therefore, our data support a role for

318 cigarette smoking as a causal mechanism in diaphragm muscle weakness, potentially mediated by
319 elevated oxidative stress levels and activation of proteolytic pathways, which likely predisposes
320 many smokers to respiratory dysfunction in the period preceding emphysema (11).
321
322 *Smoke exposure and cardiovascular function*
323 Following prolonged smoke exposure cardiac dysfunction is known to develop, with right
324 ventricular function particularly compromised (25), while peripheral vascular endothelial
325 dysfunction is also manifest (27). Our study confirmed that right ventricular function is reduced
326 and peripheral endothelial function is impaired following cigarette exposure, which occurred in the
327 absence of overt emphysema. In addition, we also confirmed previous findings that right
328 ventricular dysfunction can be reversed by exercise training (15). In order to better understand the
329 mechanisms of these smoke-induced cardiac impairments, we assessed mitochondrial function in
330 cardiac fibers. However, and in contrast to a previous study reporting impaired respiration in fibers
331 from the left ventricle (30), we found no differences between groups in either the left or right
332 ventricle, which suggests mitochondrial dysfunction is unlikely a key mechanism of smoke-
333 induced right ventricular dysfunction, at least in relation to the present animal model. In terms of
334 the peripheral endothelium, we provide novel data that high-intensity exercise training can reverse
335 smoke-induced peripheral vascular impairments, as endothelium-dependent vasodilation in aortic
336 rings was normalized to control values following exercise training. We also found a significant
337 increase in the protein expression of phosphorylated eNOS in smoke-trained mice, which may help
338 explain how exercise mediated its benefits, as smoking is well known to reduce NO bioavailability
339 and eNOS activity (4). However, why endothelial dysfunction was only observed at the higher
340 rather than the lower acetylcholine concentrations in smoke-exposed mice is unclear, but it may be
341 related to differences in membrane receptor sensitivity or density. Nevertheless, that data collected

342 from both animals (27) and humans (16, 26) show a similar trend after smoke exposure supports
343 the physiological relevance of the present findings.
344
345 If we take an integrative approach, it could be speculated that diaphragm muscle weakness induced
346 by smoke-exposure is mediated, in part, by upstream impairments related to cardiac and peripheral
347 endothelial dysfunction that limit blood flow to induce local hypoxia, with the latter suggested as
348 a key mechanism underpinning skeletal muscle dysfunction in COPD (19). Hypoxia has been
349 shown in diaphragm fiber bundles to potentiate ROS production which, in turn, directly impair
350 subsequent force generation (22). As such, our overall findings of impaired central and peripheral
351 vascular function coupled with diaphragm muscle weakness in concert with elevated markers of
352 ROS suggest hypoxia could be a putative mechanism for the smoke-induced respiratory muscle
353 weakness.
354
355 *Animal model of cigarette smoke exposure*
356 The uncertainty related to the role of cigarette smoke exposure on extrapulmonary impairments
357 had previously been clouded by the fact that emphysema was usually developed secondary to
358 cigarette exposure (2, 3, 7, 13) or that lung indices of emphysema were not measured (18, 28). In
359 the current study we selected a period of 20 weeks for smoke exposure so animals would not
360 develop overt emphysema, which is generally documented after 24 weeks in mice (9, 13).
361 Furthermore, the A/J mouse strain we studied is moderately susceptible to lung destruction
362 following smoke exposure (21), but not as sensitive (e.g., C57BL/6J) or resistant as other strains
363 (e.g., NZWLac/J) (14, 35). Collectively, therefore, the present data support our objective and
364 reinforce the clinical translation of our data, as prolonged smoke exposure resulted in no signs of
365 emphysema, while numerous multi-organ impairments converged concomitant with an impaired

366 exercise capacity of ~20%, with the latter value similar to that reported in patients at high risk of
367 developing emphysema (11). Nevertheless, we acknowledge the possibility our mouse model may
368 have still induced lung injury and airflow limitation despite the absence of emphysema
369 histologically. As such, the addition of lung functional measurements including measuring airway
370 resistance would have provided further clarity on this issue. Furthermore, as the present study
371 assessed female mice only, it also remains unclear whether the observed multi-organ impairments
372 would have been reduced or even exacerbated in males exposed to the same amount of cigarette
373 smoke.
374
375 *Conclusion*
376 High-intensity exercise training reversed cigarette smoke-induced extrapulmonary impairments to
377 the heart, the peripheral vascular endothelium, and the diaphragm as well normalizing exercise
378 capacity, which occurred before the onset of overt emphysema. These findings suggest, therefore,
379 that cigarette smokers in the absence of emphysema likely develop extrapulmonary impairments
380 that exacerbate exercise intolerance, but exercise training can reverse these detrimental
381 consequences.

Acknowledgments

None

Conflict of interest

The authors have no conflicts of interest to disclose in relation to professional relationships with companies or manufacturers who will benefit from the results of the present study. The authors state that the results of the present study do not constitute endorsement by ACSM. The authors declare that the results of the study are presented clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation

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Figure Legends

Fig. 1 Body weight (A), exercise capacity (B), mean linear intercept (Lm; C) and right ventricular systolic pressure (RVSP; D) of control, smoking, and smoking mice that performed high-intensity interval training (HIIT). That lung destruction (C) or pulmonary hypertension (D) did not differ between groups supports the contention that overt emphysema was not developed in smoke-exposed mice. *P<0.05 vs. Control.

Fig. 2 *In situ* mitochondrial respiration states and the respective control ratios in permeabilized fibers of the left ventricle (A), right ventricle (B), and diaphragm (C), as assessed from control, smoking, and smoking mice that performed high-intensity interval training (HIIT). Respiration states were assessed, in turn, including: C_I leak, complex I leak respiration following addition of malate and glutamate; C_I, complex I phosphorylated state following addition of ADP; C_{II}, complex II phosphorylated state following addition of succinate. No significant differences were found between groups for all measures.

Fig. 3 Peripheral *in vitro* endothelial-dependent (A) and -independent (B) function in response to increasing acetylcholine and sodium nitroprusside (SNP) concentrations, as assessed in isolated aortic rings from control, smoking, and smoking mice that performed high-intensity interval training (HIIT). *P<0.05 vs. Control and Smoking+HIIT; $^{\#}$P<0.05 vs. Smoking+HIIT

Fig. 4 *In vitro* respiratory and lower limb skeletal muscle contractile function assessed across a range of stimulation frequencies in the diaphragm (A), EDL (B), and soleus (C) for control, smoking, and smoking mice that performed high-intensity interval training (HIIT). *P<0.05 vs. Smoking.

Fig. 5 Protein expression (A) and enzyme activity (B) of NADPH oxidase as well as protein expression of MAFbx (C) and MuRF1 (D) in the diaphragm from control, smoking, and smoking mice that performed high-intensity interval training (HIIT). Representative blots for each protein are also provided in the upper panel, which were normalized to the loading control GAPDH for control (c), smoking (s), and smoking mice that performed HIIT (H). *P<0.05 vs. Control and Smoking+HIIT; $^{\#}$P<0.05 vs. Control.

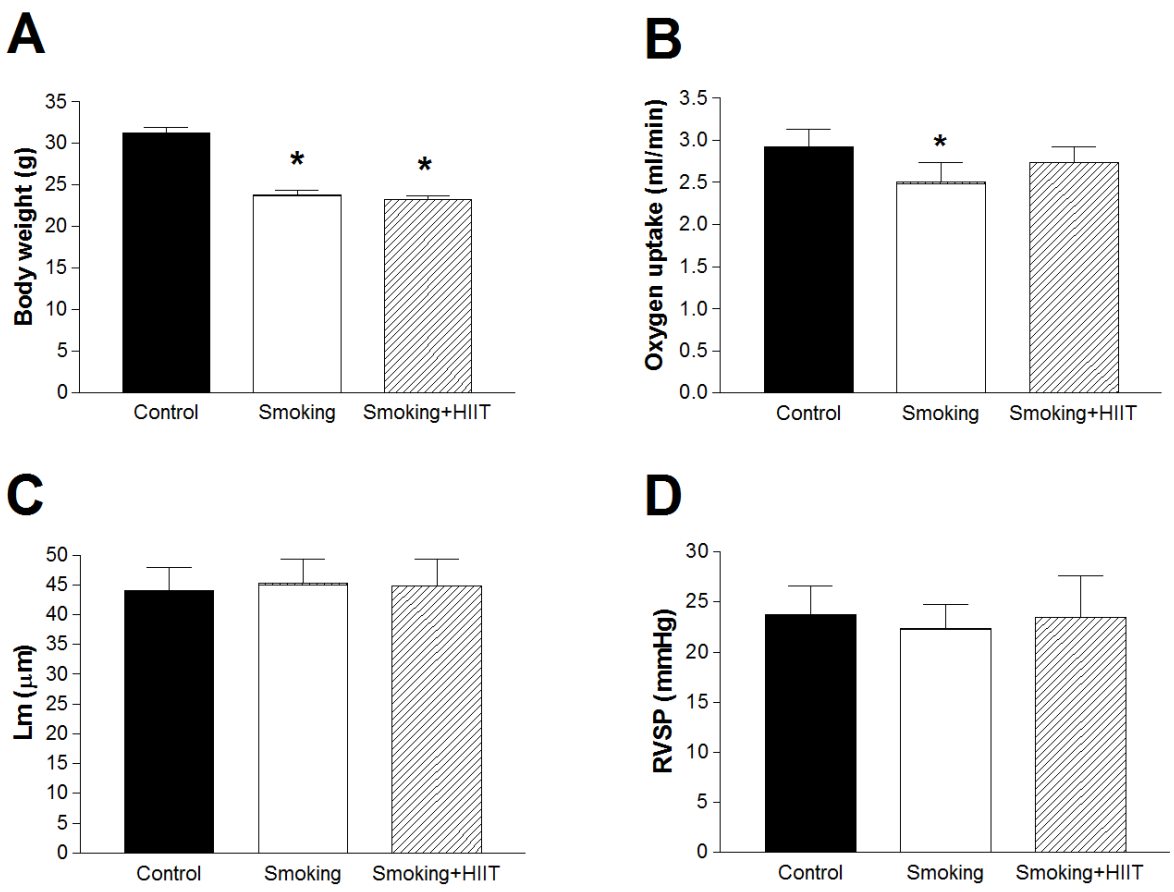

A
Body weight (g)
35
30
25
20
15
10
5
0
*
*
Control
Smoking
Smoking+HIIT
B
Oxygen uptake (ml/min)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
Control
Smoking
Smoking+HIIT
C
Lm (μm)
50
45
40
35
30
25
20
15
10
5
0
Control
Smoking
Smoking+HIIT
D
RVSP (mmHg)
30
25
20
15
10
5
0
Control
Smoking
Smoking+HIIT

Fig. 1.

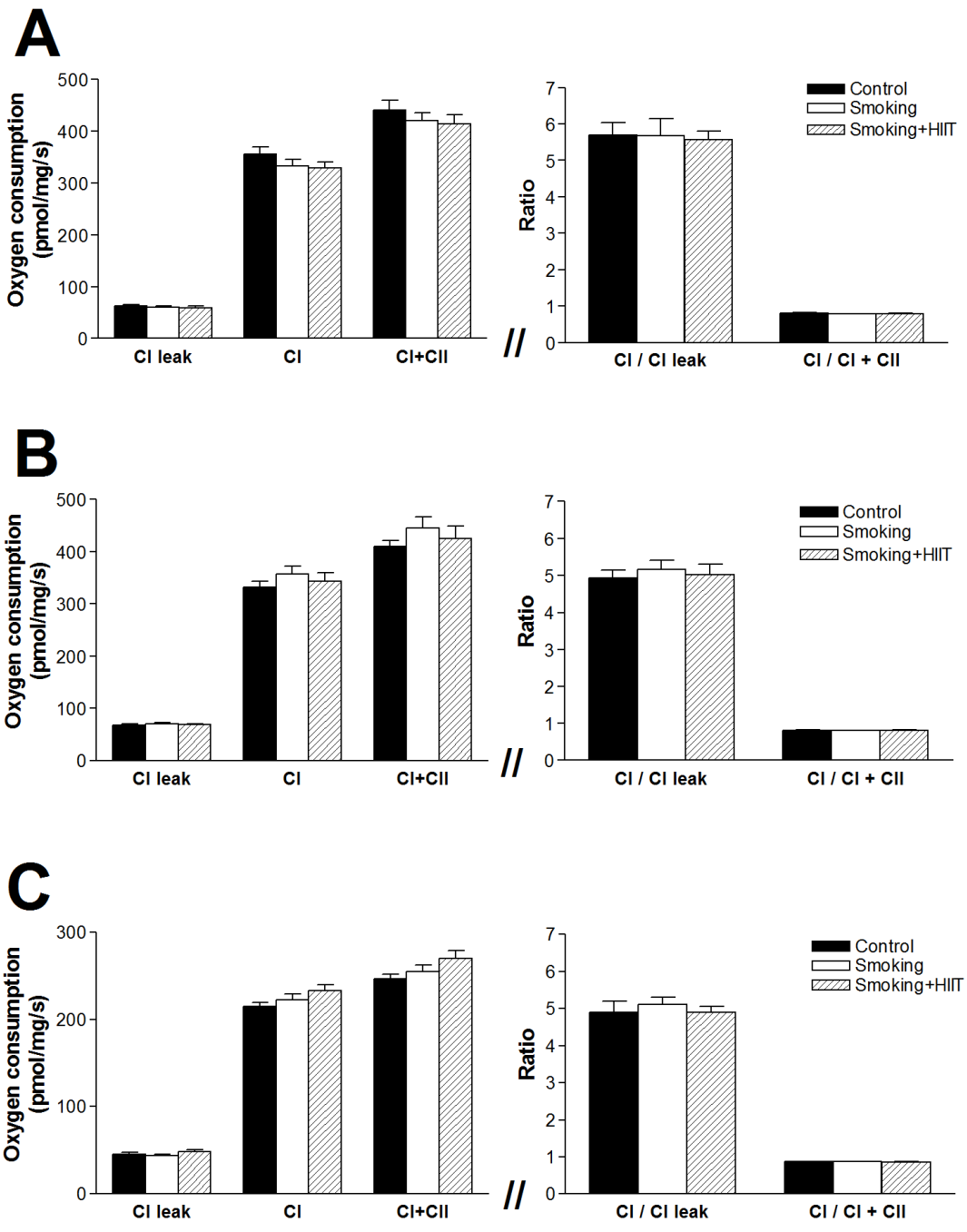
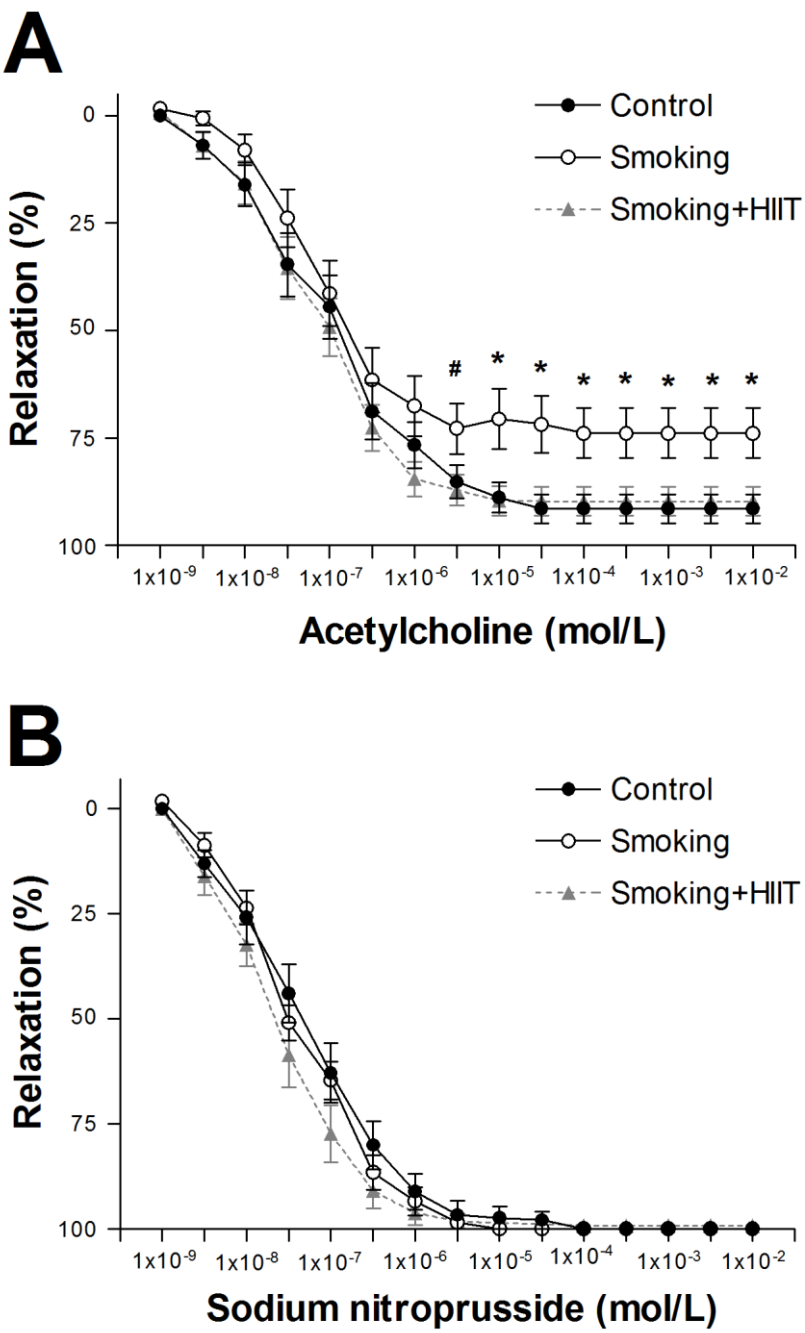

A
Oxygen consumption (pmol/mg/s)
500
400
300
200
100
0
CI leak
CI
CI+CII
//
Ratio
7
6
5
4
3
2
1
0
CI / CI leak
CI / CI + CII
Control
Smoking
Smoking+HIIT
B
Oxygen consumption (pmol/mg/s)
500
400
300
200
100
0
CI leak
CI
CI+CII
//
Ratio
7
6
5
4
3
2
1
0
CI / CI leak
CI / CI + CII
Control
Smoking
Smoking+HIIT
C
Oxygen consumption (pmol/mg/s)
300
200
100
0
CI leak
CI
CI+CII
//
Ratio
7
6
5
4
3
2
1
0
CI / CI leak
CI / CI + CII
Control
Smoking
Smoking+HIIT

Fig. 2



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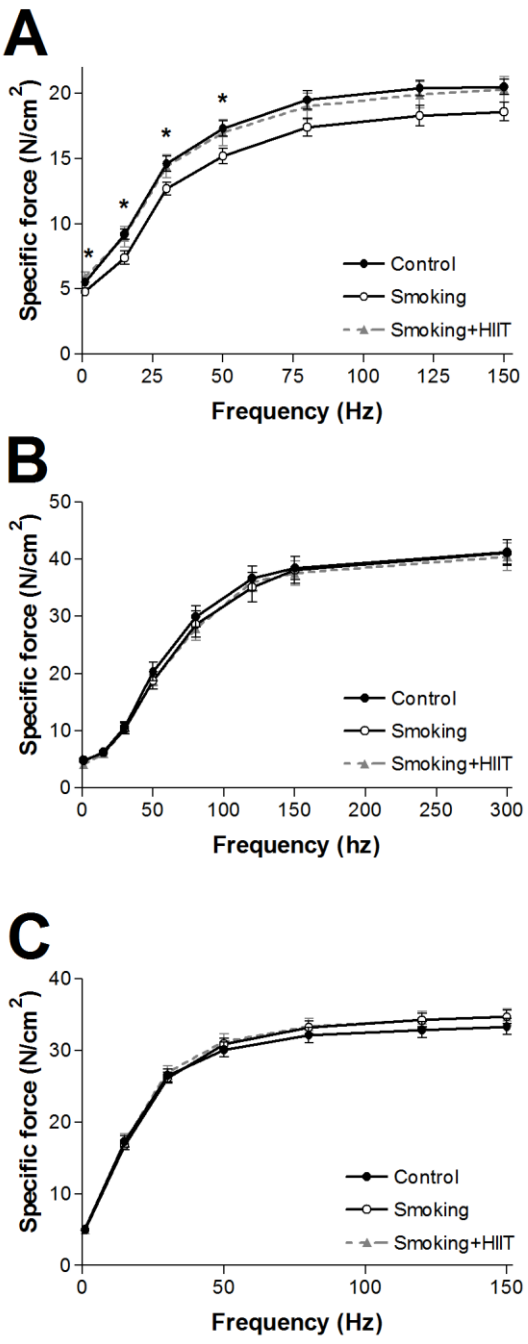
536 Fig. 3

537


A
Specific force (N/cm 2)
Frequency (Hz)
Control
Smoking
Smoking+HIIT
B
Specific force (N/cm 2)
Frequency (hz)
Control
Smoking
Smoking+HIIT
C
Specific force (N/cm 2)
Frequency (Hz)
Control
Smoking
Smoking+HIIT

538

539 Fig. 4

Fig. 4

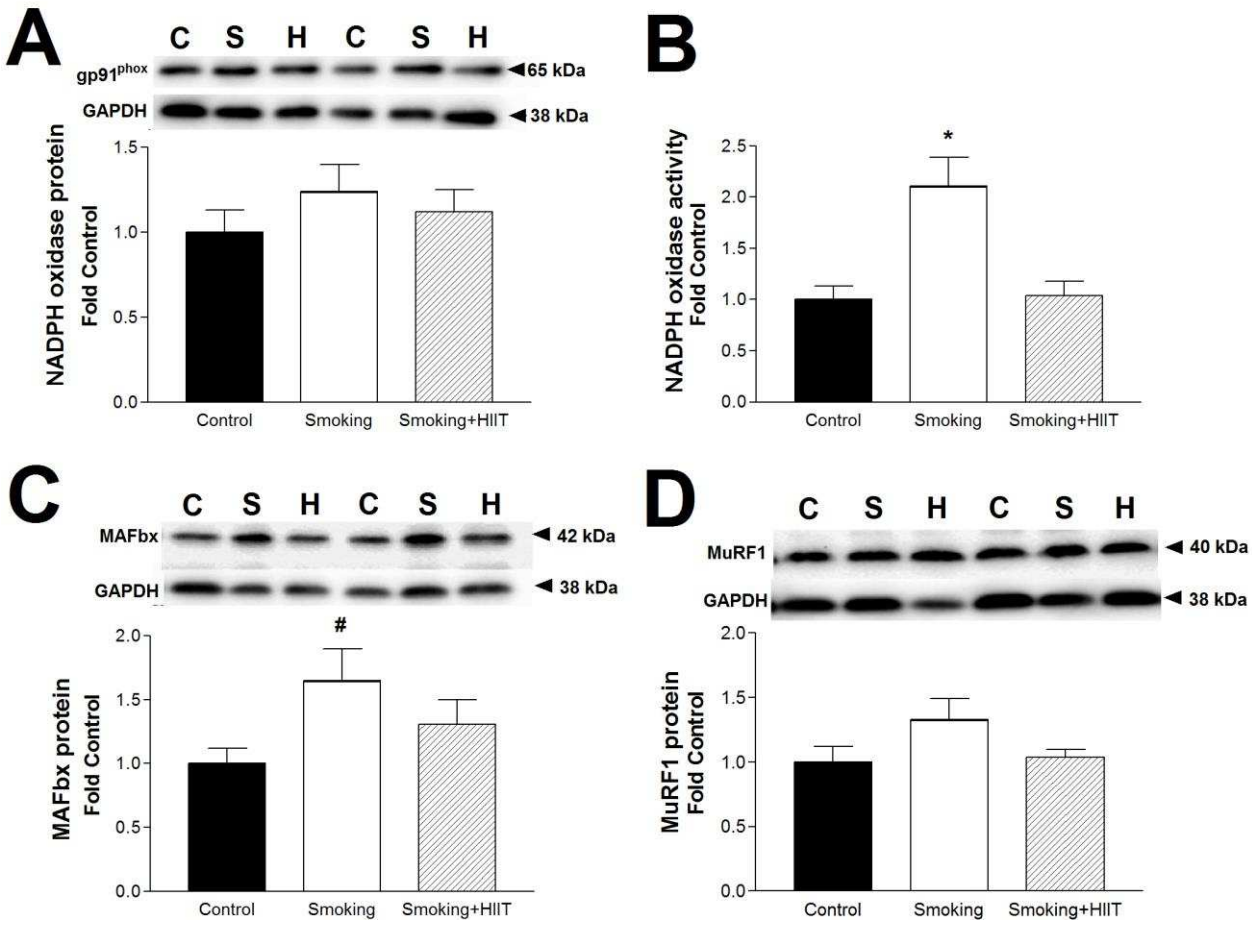

A
C S H C S H
gp91phox
65 kDa
GAPDH
38 kDa
NADPH oxidase protein
Fold Control
1.5
1.0
0.5
0.0
Control
Smoking
Smoking+HIIT
B
NADPH oxidase activity
Fold Control
2.5
2.0
1.5
1.0
0.5
0.0
*
Control
Smoking
Smoking+HIIT
C
C S H C S H
MAFbx
42 kDa
GAPDH
38 kDa
MAFbx protein
Fold Control
2.0
1.5
1.0
0.5
0.0
#
Control
Smoking
Smoking+HIIT
D
C S H C S H
MuRF1
40 kDa
GAPDH
38 kDa
MuRF1 protein
Fold Control
2.0
1.5
1.0
0.5
0.0
Control
Smoking
Smoking+HIIT

Fig. 5.