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Effects of cigarette smoke on in situ mitochondrial substrate oxidation of slow- and fast-twitch skeletal muscles

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ABSTRACT

Epidemiological and clinical evidence suggests that cigarette smoke exposure alters glucose and fatty acid metabolism, leading to greater susceptibility to metabolic disorders. However, the effects of cigarette smoke exposure on mitochondrial substrate oxidation in the skeletal muscle are still poorly understood. Accordingly, this study aimed to examine the acute effects of cigarette smoke on mitochondrial respiratory capacity, sensitivity, and concurrent utilization of palmitoylcarnitine (PC), a long-chain fatty acid, and pyruvate, a product of glycolysis, in permeabilized gastrocnemius and soleus muscle fibers exposed to an acute (1 h) dose (4 %) of cigarette smoke concentrate. Cigarette smoke decreased both mitochondrial respiratory capacity (CONTROL: 50.4 ± 11.8 $pmol_{O2}/s/mg_{wt}$ and SMOKE: 22.3 ± 4.4 $pmol_{O2}/s/mg_{wt}$, $p < 0.01$) and sensitivity for pyruvate (CONTROL: 0.10 ± 0.04 mM and SMOKE: 0.11 ± 0.04 mM, $p < 0.01$) in the gastrocnemius muscle. In the soleus, only the sensitivity for pyruvate-stimulated mitochondrial respiration trended toward a decrease (CONTROL: 0.11 ± 0.04 mM and SMOKE: 0.23 ± 0.15 mM, $p = 0.08$). In contrast, cigarette smoke did not significantly alter palmitoylcarnitine-stimulated mitochondrial respiration in either muscle. In the control condition, pyruvate-supported respiration was inhibited by the concurrent addition of palmitoylcarnitine in the fast-twitch gastrocnemius muscle (−27.1 ± 19.7 %, $p < 0.05$), but not in the slow-twitch soleus (−9.2 ± 17.0 %). With cigarette smoke, the addition of palmitoylcarnitine augmented the maximal respiration rate stimulated by the concurrent addition of pyruvate in the gastrocnemius (+18.5 ± 39.3 %, $p < 0.05$). However, cigarette smoke still significantly impaired mitochondrial respiratory capacity with combined substrates compared to control ($p < 0.05$). Our findings underscore that cigarette smoke directly impairs mitochondrial respiration of carbohydrate-derived substrates and is a primary mechanism underlying cigarette smoke-induced muscle dysfunction, which leads to a vicious cycle involving excess glucose conversion into fatty acids and lipotoxicity.

1. New & noteworthy

While cigarette smoke exposure is a well-known risk factor for the development of metabolic disorders, it is unclear how mitochondrial substrate metabolism is directly affected by cigarette smoke. Herein, while acute cigarette smoke exposure did not significantly alter fatty acid metabolism, cigarette smoke significantly impaired mitochondrial pyruvate oxidation. Furthermore, acute cigarette smoke exposure augmented mitochondrial respiration in the presence of competing substrates. With this evidence, we demonstrate that pyruvate oxidation is directly impaired by cigarette smoke, thus providing mechanistic insights into the link between cigarette smoking and the development of metabolic diseases.

2. Introduction

Cigarette smoke exposure is accountable for up to 480,000 premature deaths per year in the United States [1], and is considered an independent risk factor for metabolic diseases such as type 2 diabetes [2], metabolic syndrome [3], and non-alcoholic fatty liver disease [4].

Abbreviations: P, Pyruvate; AmA, Antimycin A; Omy, Oligomycin; PC, Palmitoylcarnitine; CSC, Cigarette Smoke Concentrate.

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Phenotypically, tobacco smoke exposure results in higher central adiposity [5,6], ectopic fat accumulation [7,8], and lower muscle mass [8,9]. Despite abundant evidence of its negative impact on health, the mechanisms underlying the metabolic toxicity of cigarette smoke are, however, still poorly understood.

Dyslipidemia is common in cigarette smokers who exhibit higher levels of circulating cholesterol [10], intramuscular saturated or non-saturated triglycerides [8,11], and saturated diacylglycerol [11] compared to non-smokers. In this regard, acute exposure to cigarette smoke has been documented to increase circulating free fatty acids (FFA) [12] and whole-body palmitate rate of appearance and oxidation measured by ^{13}C isotope tracers during fasting conditions in young adults [11]. Similar alterations to lipid metabolism were observed in chronic smokers, which were attenuated upon smoking cessation [13]. Together, these findings suggest that cigarette smoke stimulates adipose tissue lipolysis coupled to incomplete fatty acid oxidation in peripheral tissues, thus resulting in ectopic fat accumulation in the skeletal muscle. However, whether incomplete oxidation of fatty acids is attributable to direct smoke-induced inhibition of mitochondrial capacity to utilize fatty acids is still unknown.

Cigarette smoke is also strongly linked to glucose metabolism abnormalities and the development of skeletal muscle insulin resistance [11,14], which is partly mediated by insulin receptor inhibition on the muscle cell membrane [13]. Interestingly, exposure to cigarette smoke also impairs muscle mitochondrial respiratory capacity using substrates replicating the Krebs cycle (glutamate, malate, and succinate) [15], thus also implicating mitochondrial dysfunction in the pathogenesis of insulin resistance with cigarette smoke. Of note, inhibition of ceramide formation, a fatty acid of the sphingolipid family, by myriocin resulted in higher in situ respiratory capacity in skeletal muscle permeabilized fibers [15], which alluded to a potential role for ceramide accumulation and lipotoxicity in the inhibition of mitochondrial respiration by cigarette smoke. However, mitochondrial fatty acid oxidation was not assessed, and ceramide inhibition improved mitochondrial respiration regardless of smoking status in this study, which suggests that the effects of ceramides were not specific to cigarette smoke exposure. Thus, it is still unclear whether cigarette smoke directly caused ceramide accumulation resulting in mitochondrial dysfunction, or if ceramide accumulation was secondary to a shift in mitochondrial substrate sensitivity and/or impaired respiration with glycolytic products, thus causing glucose conversion into fatty acids and triglycerides.

Considering that the mitochondria are the ultimate site of energy metabolism and substrate oxidation, the investigation into the effects of cigarette smoke on mitochondrial suibstrate oxidation are critical to understanding the mechanisms underlying the cigarette smoke-induced shift in fatty acid and carbohydrate metabolism. However, despite intensive investigations into the effects of cigarette smoke exposure on substrate metabolism at the whole-body and organ levels, no studies to date have investigated the direct effects of cigarette smoke exposure on mitochondrial substrate metabolism. Accordingly, this study aimed to determine cigarette smoke's acute effects on substrate sensitivity, respiratory capacity, and fuel interaction in skeletal muscles with different metabolic profiles. Specifically, we assessed mitochondrial respiration in situ using palmitoylcarnitine (PC), a long-chain fatty acid, and pyruvate, a product of glycolysis, in predominantly fast (white gastrocnemius) and slow-twitch (soleus) skeletal muscle fibers exposed to cigarette smoke concentrate (CSC). We hypothesized that CSC would impair mitochondrial respiration (sensitivity and maximal capacity) supported by pyruvate, whereas fatty acid-linked respiration will remain unaltered. As a result, mitochondrial substrate preference with CSC would shift toward greater lipid oxidation.

3. Methods

3.1. Animals and experimental design

Eleven mature C57BL/6 mice (male/female: 7/4) were used for this study. All animals were maintained on a 12-hour dark/light cycle and fed standard chow *ad libidum*. Protocols were approved by the Institutional Animal Care and Use Committee of UMASS Amherst. Following euthanasia by 5 % isoflurane, the gastrocnemius and soleus were immediately harvested and placed in ice-cold BIOPS preservation solution [16,17]. The gastrocnemius and soleus were specifically chosen to encompass tissues with different metabolic properties [18].

3.2. Preparation of permeabilized muscle fibers

The tissue preparation and respiration measurement techniques were adapted from established methods [16,17] and have been previously described by our group [19]. Briefly, BIOPS-immersed fibers (2.77 mM CaK2EGTA, 7.23 mM K2EGTA, 50 mM K+ MES, 6.56 mM MgCl2, 20 mM Taurine, 5.77 mM ATP, 15 mM PCr, 0.5 mM DTT, 20 mM Imidazole) were carefully separated with fine-tip forceps and subsequently bathed in a BIOPS-based saponin solution (50 μg saponin·mL^{-1} BIOPS) for 30 min at 4 °C. Following saponin treatment, muscle fibers were rinsed twice in ice-cold mitochondrial respiration fluid (MIR05, in mM: 110 Sucrose, 0.5 EGTA, 3 MgCl2, 60 K-lactobionate, 20 taurine, 10KH2PO4, 20 HEPES, BSA 1 g·L^{-1}, pH 7.1) for 10 min each.

Following chemical permeabilization, tissues were incubated for 1 h in a 2 mL solution of MiR05 (control) or MiR05 with 4 % (1600 μg/mL) cigarette smoke concentrate (CSC; Murty Pharmaceuticals, Lexington, KY) at 4 °C. This concentration of cigarette smoke was chosen based on pilot studies indicating that this concentration replicates the mitochondrial perturbations previously documented in mice and humans chronically exposed to cigarette smoke [9,15,20].

After the muscle sample was gently dabbed with a paper towel to remove excess fluid, the wet weight of the sample (1–2 mg) was measured using a standard, calibrated scale. The muscle fibers were then placed in the respiration chamber (Oxygraph O2K, Oroboros Instruments, Innsbruk, Austria) with 2 mL of MIR05 solution warmed to 37 °C. Oxygen was added to the chambers, and oxygen concentration was maintained between 190 and 250 μM. After allowing the permeabilized muscle sample to equilibrate for 5 min, mitochondrial respiratory function was assessed in duplicate. Following the addition of each substrate, the respiration rate was recorded until a steady state of at least 30-s was reached, the average of which was used for data analysis. To assess mitochondrial respiration supported by glycolysis or fatty acids, the rate of O_2 consumption (picomoles per second per milligram of wet weight) was assessed with 2 protocols:

3.3. Protocol 1: pyruvate-stimulated respiration

Saturating concentrations of malate (M; 2 mM) and ADP (D; 5 mM) were added to the chamber followed by a stepwise titration of pyruvate (P) at final concentrations of 0.10, 0.25, 0.50, 1, and 5 mM. Cytochrome c (10 μM) was then added to the chamber to assess mitochondrial membrane integrity [16,17]. Finally, antimycin A (2.5 μM) and oligomycin (5 nM) were added to assess residual, non-mitochondrial oxygen consumption (AmA & Omy).

3.4. Protocol 2: palmitoylcarnitine-stimulated respiration and pyruvate-palmitoylcarnitine respiration interaction

To assess palmitoylcarnitine-stimulated respiration, saturating concentrations of malate (M; 2 mM) and ADP (D; 5 mM) were added to the chamber, followed by a stepwise titration of palmitoylcarnitine (Palm), resulting in final concentrations of 0.0025, 0.005, 0.0125, 0.025, and 0.04 mM. Pyruvate was then titrated in the chambers to assess the

kinetics of pyruvate in the presence of palmitoylcarnitine using Protocol 1. Membrane integrity was tested with cytochrome *c*, followed by the addition of antimycin A and oligomycin to assess residual, non-mitochondrial oxygen consumption.

3.5. Data analysis

Apparent K_m and V_{max} were determined using a 3-parameter model of the Michaelis-Menten equation:

$$V = c + (V_{max} - c)/(1 + (K_m/[S]))$$

where JO_2 is the respiration rate at the concentration of a given substrate [S], and c is JO_2 when [S] = 0.

Samples that demonstrated impaired mitochondrial membrane integrity (more than a 10 % increase in respiration in response to cytochrome *c*) were excluded from the analysis ($n = 3$). All extreme outliers (more extreme than Q1 - 3 * IQR or Q3 + 3 * IQR) were also excluded from the analysis. Normality and homoscedasticity were determined using the Shapiro-Wilk and Levene's tests, respectively. The effects of cigarette smoke and tissue on the K_m and V_{max} for each substrate were determined using a two-way (Smoke x Tissue) Aligned Ranks Transform (ART) ANOVA [21]. If main effects or interaction effects were determined to be statistically significant ($p < 0.05$), post hoc comparisons were performed using Dunn's test with a Holm-Sidak correction. Effect sizes were determined by calculating the partial eta-squared (η^2) and Cohen's *d* (*d*) for both the ART ANOVA and the post hoc comparisons, respectively. For clarity, the results are presented as mean ± SD in text and tables and mean ± SEM in the figures.

4. Results

4.1. Effects of CSC on mitochondrial respiration supported by pyruvate oxidation

Dose-response curve for pyruvate-supported respiration in each tissue is shown in Fig. 1A and B. Smoke and tissue type had significant main (Smoke: $p < 0.001$, partial $\eta^2 = 0.56$; Tissue: $p = 0.008$, partial $\eta^2 = 0.22$) and interaction effects (p = 0.008, partial $\eta^2 = 0.22$) on the apparent sensitivity (K_m) of the mitochondria to pyruvate (Fig. 1C). Post hoc tests indicated that K_m of the control gastrocnemius (0.10 ± 0.04 mM) was significantly lower than the CSC-exposed gastrocnemius (0.45 ± 0.19 mM, adj. $p < 0.001$, $d = 2.51$) and CSC-exposed soleus (0.23 ± 0.15 mM, adj. $p = 0.045$, $d = 1.23$). Likewise, the control soleus (0.11 ± 0.04 mM) had a significantly lower K_m than the CSC-exposed gastrocnemius (adj. $p = 0.001$, $d = 2.42$) while the difference between the control soleus and the CSC-exposed soleus did not reach significance (adj. $p = 0.080$, $d = 1.13$). There was not a significant difference in the sensitivity to pyruvate between the control gastrocnemius and the control soleus (adj. $p = 0.337$, $d = 0.25$), or the CSC-exposed gastrocnemius and the CSC-exposed soleus (adj. $p = 0.106$, $d = 1.24$).

Similar to the K_m, smoke and tissue type had significant main (Smoke: $p < 0.001$, partial $\eta^2 = 0.35$; Tissue: p < 0.001, partial $\eta^2 = 0.52$) and interaction effects ($p = 0.012$, partial $\eta^2 = 0.19$) on the maximal respiration capacity (V_{max}) of the mitochondria to pyruvate (Fig. 1D). Post hoc tests indicated that the V_{max} of the CSC-exposed gastrocnemius (22.3 ± 4.4 $pmol_{O2}/s/mg_{wt}$) was significantly lower than the control gastrocnemius (50.4 ± 11.8 $pmol_{O2}/s/mg_{wt}$; adj. $p = 0.029$, $d = 3.17$), control soleus (66.6 ± 15.2 $pmol_{O2}/s/mg_{wt}$; adj. $p < 0.001$, $d = 3.96$), and CSC-exposed soleus (59.8 ± 15.8 $pmol_{O2}/s/mg_{wt}$;

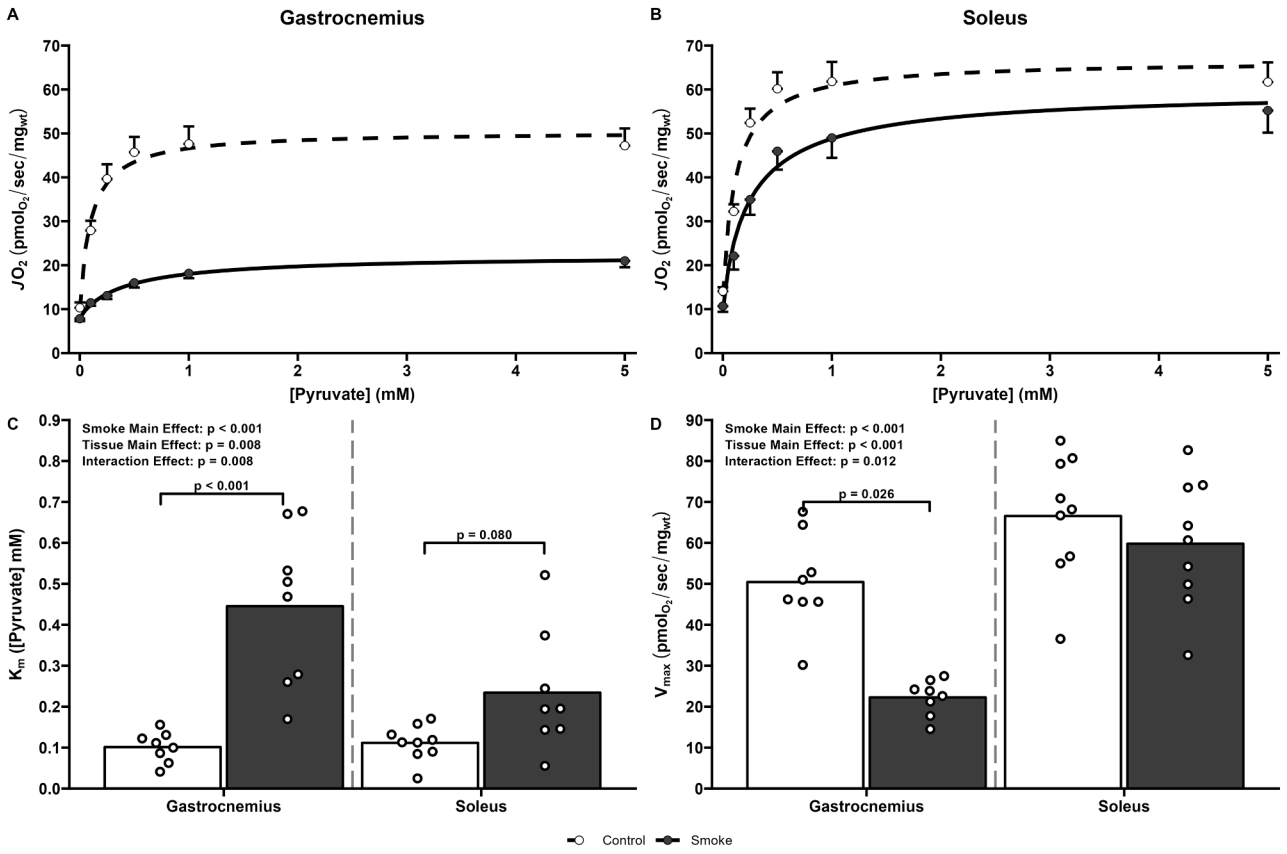

Fig. 1. Dose-response curve for Pyruvate-stimulated mitochondrial respiration in the Gastrocnemius (A; $n = 8$ per group) and Soleus (B; n = 8–9 per group), and the mean estimates for the apparent K_m (C) and V_{max} (D) of mitochondrial respiration to pyruvate for both control (white) and CSC-exposed (grey) fibers. Values are expressed as mean ± SEM, where appropriate.

adj. $p = 0.001$, $d = 3.25$). However, there was not a significant difference between the control gastrocnemius and the control soleus (adj. $p = 0.099$, $d = 1.18$), the control soleus and the smoke-exposed soleus (adj. $p = 0.224$, $d = 0.44$), or the control gastrocnemius and the smoke-exposed soleus (adj. $p = 0.258$, $d = 0.67$).

4.2. Effect of CSC on mitochondrial respiration supported by palmitoylcarnitine oxidation

Dose-response curve for PC supported respiration in each tissue are shown in Fig. 2A and B. While tissue type had a significant main effect on mitochondrial PC sensitivity ($p = 0.002$, partial $\eta^2 = 0.32$), we observed no effect of CSC ($p = 0.220$, partial $\eta^2 = 0.06$) or an interaction effect (p = 0.220, partial $\eta^2 = 0.06$; Fig. 2C). Post hoc analysis of the tissue main effects indicated that the K_m of the gastrocnemius (Control: 0.0030 ± 0.0017; Smoke: 0.0030 ± 0.0027) was significantly lower than the K_m of the soleus (Control: 0.0093 ± 0.0033; Smoke: 0.0155 ± 0.0164) for both the control (adj. $p = 0.014$, $d = 2.38$) and the CSC (adj. $p = 0.009$, $d = 1.07$) conditions.

Furthermore, there was not a main effect of CSC ($p = 0.240$, partial $\eta^2 = 0.05$) or an interaction effect ($p = 0.952$, partial $\eta^2 < 0.01$); however, there was a significant effect of tissue type ($p < 0.001$, partial $\eta^2 = 0.46$) on the maximal rate of PC-stimulated mitochondrial respiration (Fig. 2D). Post hoc analysis indicated that the V_{max} of the control gastrocnemius (14.7 ± 4.7 $pmol_{O2}/s/mg_{wt}$) was significantly lower than the V_{max} of the control soleus (22.8 ± 8.0 $pmol_{O2}/s/mg_{wt}$; adj. $p = 0.010$, $d = 1.23$). Likewise, the CSC-exposed gastrocnemius (11.5 ± 2.6 $pmol_{O2}/s/mg_{wt}$) was significantly lower than the CSC-exposed soleus (21.2 ± 6.2 $pmol_{O2}/s/mg_{wt}$; adj. $p = 0.001$, $d = 2.02$).

4.3. Effect of CSC on mitochondrial respiration with concurrent addition of pyruvate and palmitoylcarnitine

Dose-response curve for pyruvate supported respiration in the presence of 0.04 mM PC in each tissue are shown in Fig. 3A and B. Contrary to pyruvate alone, there was no interaction effect ($p = 0.344$, partial $\eta^2 = 0.03$) on the K_m of pyruvate in 0.04 mM PC, however, there was a significant effect of CSC ($p = 0.038$, partial $\eta^2 = 0.14$; Fig. 3C) and tissue ($p = 0.048$, partial $\eta^2 = 0.13$). Post hoc analysis indicated that there was not a significant difference between the gastrocnemius groups (Gastrocnemius Control: 0.11 ± 0.03, Gastrocnemius Smoke: 0.12 ± 0.03; adj. $p = 0.263$, $d = 0.40$), but there was a trend toward significance between the soleus control fibers (0.12 ± 0.04 mM) and the soleus CSC-exposed fibers (0.20 ± 0.12 mM; adj. $p = 0.066$, $d = 0.85$). No significant post hoc differences were detected between the control fibers ($p = 0.241$, $d = 0.48$) and the CSC-exposed fibers ($p = 0.081$, $d = 0.93$).

Tissue type ($p < 0.001$, partial $\eta^2 = 0.68$) and cigarette smoke ($p = 0.035$, partial $\eta^2 = 0.14$) had significant main effects on the V_{max} of pyruvate in 0.04 mM of PC (Fig. 4D); however, there was not a significant interaction effect ($p = 0.276$, partial $\eta^2 = 0.04$). Post hoc analysis indicated that the control gastrocnemius (35.2 ± 8.1 $pmol_{O2}/s/mg_{wt}$) was significantly different than the control soleus (60.1 ± 17.1 $pmol_{O2}/s/mg_{wt}$; adj. $p = 0.005$, $d = 1.86$). Likewise, the CSC-exposed gastrocnemius (22.6 ± 2.4 $pmol_{O2}/s/mg_{wt}$) was significantly different than the CSC-exposed soleus (55.0 ± 12.3 $pmol_{O2}/s/mg_{wt}$; adj. $p < 0.001$, $d = 3.65$). However, we did not identify any significant post hoc effects between the control gastrocnemius and CSC-exposed gastrocnemius ($p = 0.104$, $d = 2.12$) or between the soleus control and the CSC-exposed soleus ($p = 0.385$, $d = 0.34$).

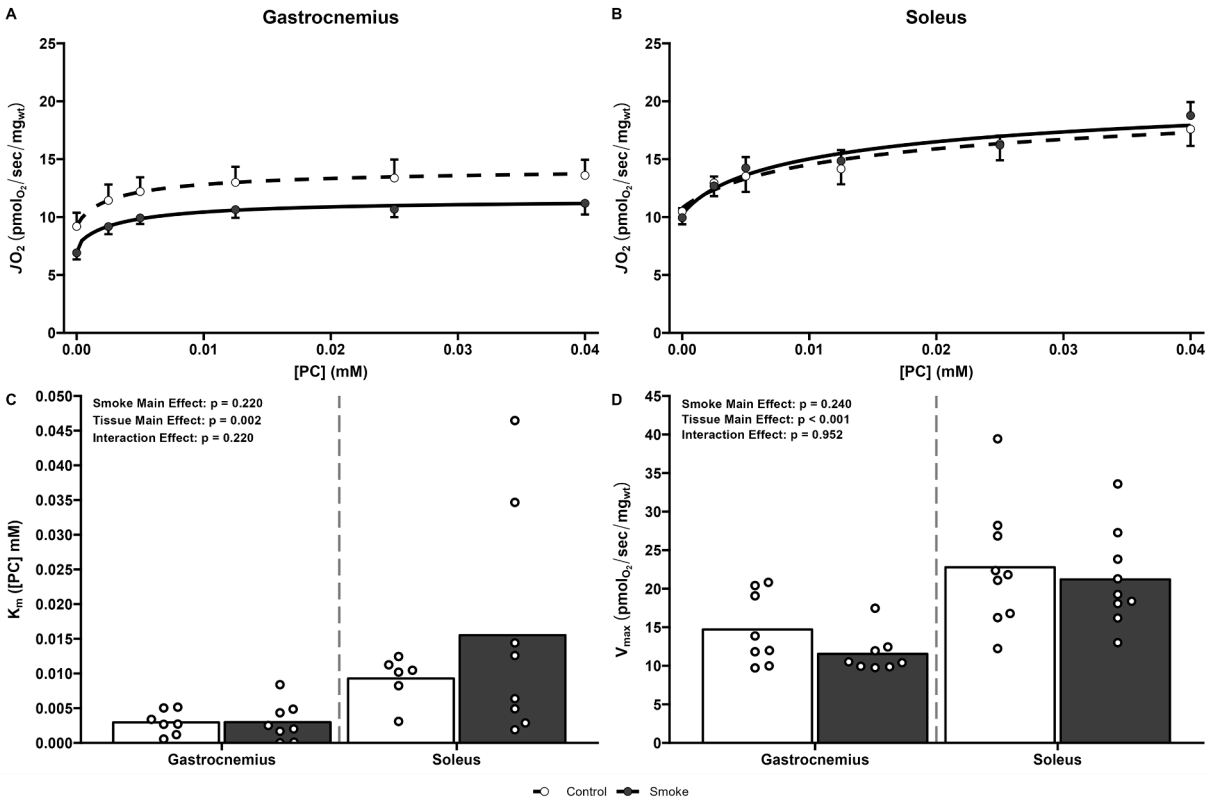

Fig. 2. Dose-response curve for palmitoylcarnitine-stimulated mitochondrial respiration in the Gastrocnemius (A; $n = 7$–8 per group) and Soleus (B; n = 8–9 per group), and the mean estimates for the apparent K_m (C) and V_{max} (D) of mitochondrial respiration to palmitoylcarnitine (PC) for both control (white) and cigarette smoke condensate-exposed (grey) fibers. Values are expressed as mean ± SEM, where appropriate.

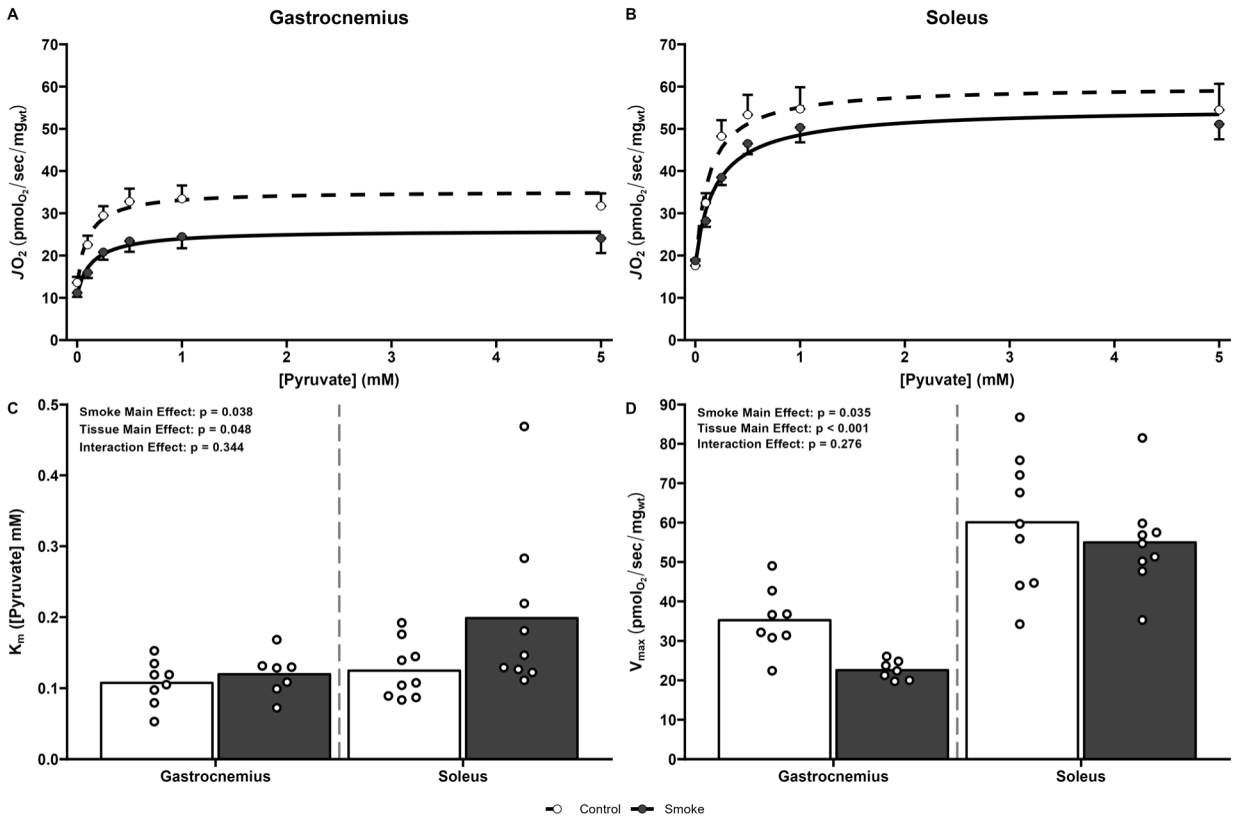

Fig. 3. Dose-response curve for Pyruvate-stimulated mitochondrial respiration in the presence of 0.04 mM PC for the Gastrocnemius (A; n = 7–8 per group) and Soleus (B; n = 7–9 per group), and the mean estimates for the apparent K_m (C) and V_{max} (D) of mitochondrial respiration to pyruvate for both control (white) and cigarette smoke condensate-exposed (grey) fibers. Values are expressed as mean ± SEM, where appropriate.

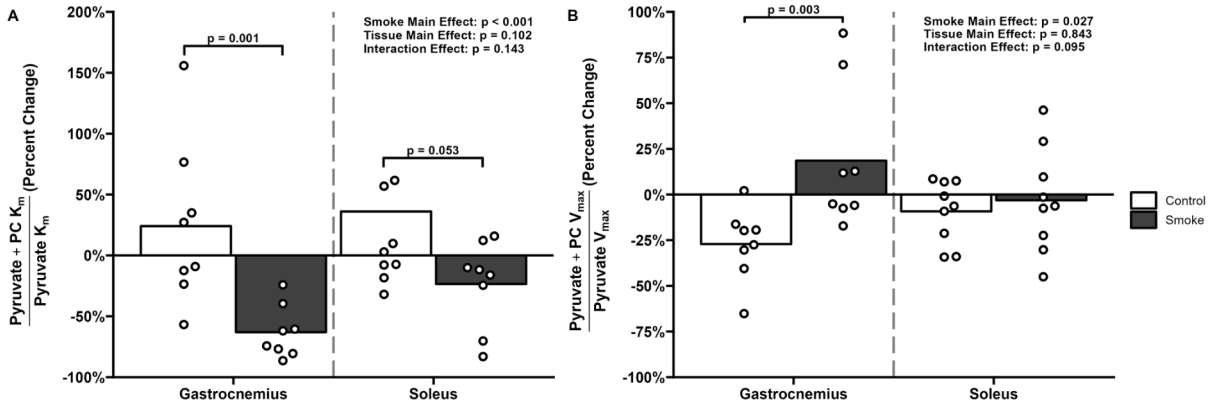

Fig. 4. Change in the K_m (A; n = 8 per group) and V_{max} (B; n = 8–9 per group) of mitochondrial pyruvate oxidation in the presence of saturating (0.04 mM) concentration of palmitoylcarnitine relative to mitochondrial pyruvate oxidation without palmitoylcarnitine in control (white) and cigarette smoke condensate-exposed (grey) white gastrocnemius and soleus fiber bundles.

4.4. Specific effect of CSC on palmitoylcarnitine induced inhibition of pyruvate supported respiration

To determine the effect of smoke on the PC-induced inhibition of pyruvate oxidation, we normalized the K_m and V_{max} of Pyruvate with concurrent addition of PC to the K_m and V_{max} of pyruvate in the absence of PC (Fig. 4). CSC had a significant main effect ($p < 0.001$, partial $\eta^2 = 0.43$) on the change in K_m (Fig. 4A), but there was no main effect of tissue type ($p = 0.250$, partial $\eta^2 = 0.05$) or an interaction effect ($p = 0.156$, partial $\eta^2 = 0.07$). Post hoc analysis indicated that the change in K_m to the CSC-exposed gastrocnemius (−63 ± 22 %) was significantly lower than the control gastrocnemius (24 ± 67 %; $p < 0.001$, $d = 1.75$). However, the change in K_m to the CSC-exposed soleus (−24 ± 36 %) only trended toward being significantly different than the control soleus (8 ± 34 %; $p = 0.053$, $d = 0.35$).

Similarly, CSC had a significant main effect ($p = 0.027$, partial $\eta^2 = 0.15$) on the change in V_{max} (Fig. 4B), but there was no main effect of tissue type ($p = 0.843$, partial $\eta^2 < 0.01$) or an interaction effect ($p = 0.095$, partial $\eta^2 = 0.09$). Post hoc analysis indicated that the differences between the control (−27 ± 20 %) and CSC-exposed gastrocnemius (19 ± 39 %; $p = 0.001$, $d = 1.47$) were significant, but not the differences between the control (−9 ± 17 %) and CSC-exposed soleus (−5 ± 30 %; $p = 0.131$, $d = 0.35$).

5. Discussion

Cigarette smoking is closely linked to the development of metabolic disorders. However, the mechanisms underlying the metabolic toxicity of cigarette smoke remain unclear. Therefore, this study aimed to determine cigarette smoke's acute effects on substrate sensitivity, respiratory capacity, and fuel interaction in skeletal muscles with different metabolic characteristics. Consistent with our hypothesis, cigarette smoke condensate directly impaired mitochondrial respiration supported by pyruvate, a product of glycolysis, in the fast-twitch gastrocnemius and, albeit to a lesser extent, the slow-twitch soleus muscles. Specifically, CSC exposure decreased both muscle respiration sensitivity and maximal capacity linked to pyruvate oxidation. In contrast, the sensitivity and maximal respiration supported by the fatty acid palmitoylcarnitine was unaffected by cigarette smoke in the gastrocnemius or soleus tissues. In the control condition, respiration supported by pyruvate was inhibited by the concurrent addition of palmitoylcarnitine in the fast-twitch gastrocnemius muscle but not in the soleus muscle. With cigarette smoke, the addition of palmitoylcarnitine augmented the maximal respiration rate stimulated by the concurrent addition of pyruvate in the gastrocnemius. However, mitochondrial respiratory capacity with combined substrates remained significantly lower in cigarette smoke than in controls for both muscles, indicating impaired mitochondrial metabolic flexibility.

5.1. CSC impairs skeletal muscle mitochondrial respiration supported by pyruvate

Consistent with our hypothesis, CSC drastically impaired pyruvate-supported skeletal muscle respiration. Specifically, there was more than a 4-fold decrease in the sensitivity (~440 % increase in $K_{m,}$ Fig. 1C), and the maximal rate of mitochondrial respiration linked to pyruvate oxidation was decreased by ~58 % (lower V_{max}, Fig. 1D) in the fast-twitch gastrocnemius muscle. This inhibitory effect of CSC was attenuated in the slow-twitch soleus muscle, for which the sensitivity of mitochondrial respiration to pyruvate was decreased only by 2.5-fold (Fig. 1C), and muscle respiratory capacity was not significantly affected by CSC incubation (Fig. 1D).

The present study's findings align with the results of studies performed by Thatcher et al. [15], which reported a ~60 % decrease in maximal glutamate-and-malate-stimulated respiration in cultured myotubes exposed to smoke-conditioned media for 4 h. This study also reported a ~50 % decrease in maximal ADP-stimulated respiration with complex I substrates, glutamate-and-malate, in the gastrocnemius muscle of mice exposed to mainstream cigarette smoke for 6 weeks. Our results, therefore, confirm and extend these findings to submaximal rates, thus revealing a smoke-induced decrease in sensitivity for pyruvate oxidation in the fast-twitch gastrocnemius muscle, with this effect being attenuated in slow-twitch soleus muscle.

In conjunction with the present results in situ, the findings from Thatcher et al. [15] support the concept of a direct inhibitory effect of cigarette smoke on mitochondrial oxidation of glucose-derived substrates. Further supporting this interpretation, constituents of cigarette smoke, specifically nicotine and o-cresol, have been identified as inhibitors of mitochondrial Complex I, the primary site of NADH oxidation, in isolated mitochondria [22]. As the ultimate energy-producing fate of glucose oxidation is to support the reduction of NAD^+ to NADH to support complex I-driven respiration, the direct inhibition of complex I function by cigarette smoke is likely to be an important mechanism contributing to impaired glucose oxidation, and the greater risk of hyperglycemia and insulin resistance often observed in smokers [2].

5.2. Skeletal muscle mitochondrial respiration supported by fatty acids is unaffected by CSC

Unlike pyruvate oxidation, fatty acid-stimulated mitochondrial respiration was not significantly affected by CSC in either muscle group (Fig. 2A-D). For example, in the gastrocnemius muscle, CSC had almost no (~1 %) effect on the $K_{m,}$ of mitochondrial respiration for fatty acid substrates. Likewise, CSC did not significantly alter the V_{max} in the soleus (−7 %). Overall, these results with different conditions of substrate availability in permeabilized fibers rule out a direct effect of cigarette smoke on the mitochondrial oxidation of fatty acids in the skeletal muscle.

Our findings in an in situ muscle preparation somewhat contrast with whole-body studies in humans, reporting a ~30 % increase in the oxidation of ^{13}C-palmitate in the quadriceps muscles of humans during an active smoking session [11]. Also, Jensen et al. [23] documented a significant correlation ($r = 0.57$) between urine cotinine excretion and fat oxidation measured by indirect calorimetry. A possible explanation for this discrepancy between in situ and in vivo studies might be the calorigenic effects of cigarette smoke and one of its main constituent, nicotine, which can confound the interpretation of the whole-body results. For instance, smoking and nicotine have been demonstrated to increase 24-h [24] and resting energy expenditure [25,26]. A strength of our study design was that our skeletal muscle preparation in situ allowed us to parse out the direct effects of cigarette smoke on mitochondrial respiratory function with fatty acid substrates from the confounding changes in metabolic demand. Therefore, our findings seem to discount a smoke-induced defect in mitochondrial capacity to oxidize lipids as a factor responsible for dyslipidemia and ectopic fat accumulation in chronic smokers, as observed by several epidemiological studies [5–8].

5.3. CSC alters skeletal muscle mitochondrial respiration with competing substrates

A unique feature of the experimental design in the present study was the assessment of mitochondrial respiration rates with competing substrates (fatty acids and pyruvate) using experimental conditions that replicate the transition from fasting to the fed state to examine mitochondrial metabolic flexibility (Fig. 3A-D). In line with work conducted in mitochondria isolated from fast-twitch muscles [27], we observed an inhibitory effect of palmitoylcarnitine on pyruvate-stimulated mitochondrial respiration (increased K_m and lower V_{max}) in both the control gastrocnemius and soleus muscles in situ (Fig. 4). Specifically, the results of these experiments in permeabilized fibers indicated that palmitoylcarnitine decreased the sensitivity of the mitochondria to pyruvate by ~20–40 %, and decreased the maximal respiration capacity (V_{max}) of pyruvate by ~10–25 % in the fast-twitch gastrocnemius and slow-twitch soleus muscles (Fig. 4A-B). Consistent with these results in the skeletal muscle in situ, palmitoylcarnitine inhibited maximal respiration capacity linked to pyruvate oxidation by 34 % in mitochondria isolated from fast-twitch mouse muscles [27].

We then sought to examine whether CSC exposure would alter the inhibition induced by palmitoylcarnitine on pyruvate-stimulated respiration. Interestingly, with CSC, palmitoylcarnitine had an additive effect and improved mitochondrial respiration sensitivity for pyruvate by 20–60 % in the gastrocnemius and soleus muscles. Similarly, the pyruvate-stimulated V_{max} in the gastrocnemius was increased by ~20 % (Fig. 3C-D). However, it is important to note that mitochondrial respiratory capacity with combined substrates remained significantly lower in cigarette smoke than in controls, indicating impaired mitochondrial metabolic flexibility. These findings may reconcile the conflicting results between experiments in humans using whole-body indirect calorimetry and isotope tracers [11,23], which indicated a CSC-induced increase in substrate oxidation, and studies in vitro [15,22], which demonstrated CSC-induced impairment in substrate oxidation by the mitochondria. In light of the present results obtained in permeabilized muscle fibers in situ, it is apparent that the oxidation by the mitochondria of the glycolytic product (e.g., pyruvate) or downstream tricarboxylic acid cycle (TCA) intermediates (e.g., glutamate and malate) is impaired by cigarette smoke exposure. This inhibition was substantial when

depending solely on those substrates (Fig. 1) whereas the effect was attenuated, but still statistically significant, using a combination of glucose- and fatty acid-derived substrates at submaximal amounts (Fig. 3), as is the case in a physiological system. Future studies using simultaneous measurements of the fluxes through the TCA cycle and β-oxidation, combined with direct measurements of the electrochemical potential in conditions of competing substrates, are therefore needed to elucidate the thermodynamic mechanisms underlying these findings.

5.4. Tissue specific effects of cigarette smoke condensate on mitochondrial substrate utilization

The use of permeabilized fibers from predominantly fast (white gastrocnemius) and slow-twitch (soleus) skeletal muscle in the present study provided an opportunity to examine the tissue specific effects of cigarette smoke on respiratory capacity and substrate utilization in muscles with well characterized differences in mitochondrial content and metabolic properties [28]. In the control condition, our results demonstrated greater capacity to oxidize pyruvate or palmitoylcarnitine in the soleus than the gastrocnemius, which was consistent with previous studies [28–30]. Unlike fatty-acid specific respiration, which was unaffected by cigarette smoke exposure, pyruvate-stimulated respiration was modulated both by CSC and the fiber type. Specifically, both the sensitivity and the maximal rate of mitochondrial respiration linked to pyruvate oxidation were attenuated by CSC in the gastrocnemius (Fig. 1C and D). In contrast, only the K_m for pyruvate supported respiration was increased, non-significantly, in the soleus (Fig. 1C). Together, these findings reveal a competitive inhibition of mitochondrial respiration with pyruvate by cigarette smoke condensate in the soleus whereas the response of the gastrocnemius was characteristic of a non-competitive and irreversible inhibition by cigarette smoke. Conceptually, the tissue-specific inhibition of respiration induced by CSC may originate from changes in mitochondrial pyruvate carrier function, pyruvate dehydrogenase activity (PDH), and/or Kreb's cycle enzymes. Interestingly, the compensatory increase in sensitivity and capacity to oxidize pyruvate when combined with palmitoylcarnitnine in the gastrocnemius (Fig. 4A and B) suggest that enzymes of the Kreb's cycle were not affected by CSC as both beta-oxidation and pyruvate-oxidation converge to this energetic pathway. Also, previous studies in patients with Chronic Obstructive Pulmonary Disease, a pathology caused by chronic smoking, documented a higher concentration of pyruvate, but unchanged PDH activity in the vastus lateralis muscle of these patients compared to age-matched controls [31,32] thus suggesting another mechanism to explain the current results. Future studies are therefore warranted to examine the susceptibility of the mitochondrial pyruvate carrier function in response to acute and chronic cigarette smoke exposure both in fast and slow-twitch skeletal muscle.

5.5. Clinical perspectives

Herein, we investigated the impacts of cigarette smoke on substrate oxidation at the mitochondrial level — the final site of substrate oxidation for the generation of ATP. Our study in a skeletal muscle preparation in situ revealed that, while maximal mitochondrial respiration with fatty acid substrates was unaffected by cigarette smoke exposure, mitochondrial respiration supported by carbohydrate-derived substrates, alone or in combination with fatty acids, was significantly impaired in the skeletal muscle. Clinically, these findings of impaired mitochondrial metabolic flexibility could explain both the decline in insulin sensitivity and the increase in triglyceride storage associated with chronic smoking [2,5–8]. Conceptually, the inhibition of pyruvate oxidation in the mitochondria can decrease cellular glucose oxidation. As excess carbohydrates are converted into fatty acids in the liver, those can then be incorporated into triglycerides and cholesterol to be transported in the blood or converted into sphingolipids and/or ceramide [33,34]. These excess lipid species would then be taken up and stored in ectopic fat depots, such as skeletal muscle and liver, with adverse effects (lipotoxicity) on these organs. Therefore, impaired pyruvate oxidation induced by cigarette smoke leads to a vicious cycle whereby the incorporated lipid species further impair mitochondrial respiration and cause further cellular damage, as observed in individuals chronically exposed to cigarette smoke.

5.6. Experimental considerations

One limitation of the present study was that mitochondrial ATP production was not directly assessed. However, methods to simultaneously detect mitochondrial oxygen consumption and ATP production (typically using fluorescent detection of magnesium green) are problematic as these methods are currently not well-validated in permeabilized muscel fibers, nor are they widely used in the literature. Therefore, mitochondrial ATP production would have to be measured from isolated mitochondria of the skeletal muscle. While potentially insightful, a caveat with this approach is that diffusion of cigarette smoke condensate into the mitochondria and the compartmentalization of the energetic pathway would be markedly different between the two preparations, which would complicate the interpretation of such findings.

Numerous previous studies examined the toxicity of cigarette smoke using intermittent respiratory exposure for several days or even months. While this approach has proven useful to improve our understanding of chronic adaptions related to protein and DNA damages induced by cigarette smoke exposure, this methodology did not elucidate the initial insult eliciting those adaptations. Also, these methods of exposure elicit systemic responses (e.g. cytokine released by the lungs and other organs, nicotine-induced hypeactivation of the sympathetic nervous system, …) which may also alter mitochondrial function in the skeletal muscle. Therefore, an advantage of the method used in the present study was to isolate the direct functional effects and the fundamental regulatory changes that occur in response to an acute exposure to cigarette smoke on the skeletal muscle. Without such knowledge our understanding of the chronic adaptions to cigarette smoke exposure would otherwise remain incomplete. Therefore, both chronic respiratory exposure and acute organs/tissues incubation with CSC are complementary and necessary to further our understanding of the toxicity of cigarette smoke.

6. Conclusion

In conclusion, this study revealed that cigarette smoke condensate acutely impaired mitochondrial respiration supported by pyruvate, a product of glycolysis, in the fast-twitch gastrocnemius and, to a lesser extent, the slow-twitch soleus muscle. In contrast, the sensitivity and maximal respiration supported by the fatty acid palmitoylcarnitine were unaffected by cigarette smoke in either the gastrocnemius or soleus tissues. In a condition replicating the transition from fasting to the fed state, respiration supported by pyruvate was inhibited by the concurrent addition of palmitoylcarnitine in the fast-twitch gastrocnemius muscle. Interestingly, palmitoylcarnitine increased pyruvate utilization at submaximal respiration rates in conditions with cigarette smoke in the gastrocnemius. However, this additive effect of fatty acids was insufficient to restore mitochondrial respiration to the level of the control condition, thus still indicating an impaired mitochondrial metabolic flexibility. Our findings underscore that impaired metabolism of carbohydrate-derived substrates are the primary mechanism underlying cigarette smoke-associated muscle mitochondrial dysfunction, which leads to a vicious cycle involving excess glucose conversion into fatty acids and lipotoxicity, further exacerbating skeletal muscle and mitochondrial abnormalities commonly observed in humans chronically exposed to cigarette smoke.

CRediT authorship contribution statement

S.T.D. and G.L. conceived and designed research; S.T.D., A.A.M., S.T.B, J.P.M., and N.A-M. performed experiments; S.T.D., A.A.M, and G.L. analyzed data and interpreted results; S.T.D. drafted manuscript; S.T.D., A.A.M, S.T.B, J.P.M, N.A-M., and G.L. edited and revised manuscript; S.T.D., A.A.M, S.T.B, J.P.M, N.A-M., and G.L. approved final version of manuscript.

Declaration of competing interest

The authors have no conflict of interest to declare.

Data availability

Data are available upon reasonable request by contacting glayec@umass.edu.

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