Ceramides mediate cigarette smoke-induced metabolic disruption in mice

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Cigarette smoke exposure increases lung ceramide biosynthesis and alters metabolic function. We hypothesized that ceramides are released from the lung during cigarette smoke exposure and result in elevated skeletal muscle ceramide levels, resulting in insulin resistance and altered mitochondrial respiration. Employing cell and animal models, we explored the effect of cigarette smoke on muscle cell insulin signaling and mitochondrial respiration. Muscle cells were treated with conditioned medium from cigarette smoke extract (CSE)-exposed lung cells, followed by analysis of ceramides and assessment of insulin signaling and mitochondrial function. Mice were exposed to daily cigarette smoke and a high-fat, high-sugar (HFHS) diet with myriocin injections to inhibit ceramide synthesis. Comparisons were conducted between these mice and control animals on standard diets in the absence of smoke exposure and myriocin injections. Muscle cells treated with CSE-exposed conditioned medium were completely unresponsive to insulin stimulation, and mitochondrial respiration was severely blunted. These effects were mitigated when lung cells were treated with the ceramide inhibitor myriocin prior to and during CSE exposure. In mice, daily cigarette smoke exposure and HFHS diet resulted in insulin resistance, which correlated with elevated ceramides. Although myriocin injection was protective against insulin resistance with either smoke or HFHS, it was insufficient to prevent insulin resistance with combined CS and HFHS. However, myriocin injection restored muscle mitochondrial respiration in all treatments. Ceramide inhibition prevents metabolic disruption in muscle cells with smoke exposure and may explain whole body insulin resistance and mitochondrial dysfunction in vivo.

Cigarette smoke; ceramide; mitochondria; metabolic disruption; insulin resistance

Obesity and cigarette smoking are two of the largest causes of preventable deaths worldwide, increasing the risk of multiple illnesses such as heart disease, stroke, airway infections, and diabetes (31, 52). The incidences of smoking and obesity mirror each other in nations across the globe; where one risk factor is increasing, the other follows (31, 52). This correlation has elicited speculation of a causative association between toxin exposure, such as cigarette smoke, and metabolic disruption (6, 11, 42), which adds another layer of complexity to the already convoluted search for a treatment for obesity and its consequences. Of the myriad mediators that may disrupt cellular insulin sensitivity and mitochondrial function elucidate a novel mechanism and potential therapeutic target (9).

The lung produces ceramides in response to cigarette smoke exposure (24, 35), which may explain the altered metabolic function evident with smoking (7, 18). Ceramide is a well-established mediator of both insulin resistance (12, 49) and compromised mitochondrial function (25, 47). Based on these observations, the purpose of our study was to test the hypothesis that cigarette smoke exposure leads to ceramide accumulation in systemic tissues, particularly skeletal muscle, and mediates insulin resistance and altered mitochondrial function. Additionally, by using a rodent smoke exposure model, these studies provide evidence that long-term cigarette smoke exacerbates weight gain.

MATERIALS AND METHODS

Cell culture. Cigarette smoke extract (CSE) was generated as previously described, with slight modifications (45). Briefly, one 2RF4 research cigarette (University of Kentucky, Lexington, KY) was continuously smoked by connecting the filtered end of the cigarette to a vacuum pump, pulling the particles into 5 ml of DMEM-F12, and the resulting medium was defined as 100% CSE and diluted with culture medium to 10%. The total particulate matter content of 2RF4 cigarettes is 11.7 mg/cigarette, tar is 9.7 mg/cigarette, and nicotine is 0.85 mg/cigarette. Human type II-like pulmonary adenocarcinoma cells (A-549) were maintained in DMEM-F12 supplemented with 10% FBS (Invitrogen, Grand Island, NY) and antibiotics. Cells were split into six-well dishes and grown to 80% confluence. C5C12 muscle cells were maintained in DMEM + 10% FBS. For differentiation into myotubes, myoblasts were grown to confluence, and the medium was replaced with DMEM + 10% horse serum (Invitrogen). Myotubes were used for experiments on day 4 of differentiation. A-549 cultures were exposed to serum-free medium supplemented with 10% CSE or medium alone for 4 h, after which the medium was transferred to differentiated C5C12 myotubes (termed “conditioned medium”) for 12 h. Where indicated, cells were treated with myriocin (10 μM; Sigma, M1177). Muscle cells were harvested for RNA, protein, and lipid isolation following treatments.
Animals. Male C57Bl/6 mice were housed in a conventional animal house and maintained on a 12:12-h light-dark cycle. Two animal studies were conducted. In the first study, animals received standard diet chow (Harlan-Teklad 8604) and water ad libitum. At 12–14 wk of age, animals were randomly divided into room air- and cigarette smoke (CS)-exposed groups. Mice were placed in soft restraints and connected to the exposure tower of a nose-only exposure system (InExpose System; Scireq, Canada). Animals were nasally exposed to mainstream CS generated by research cigarettes where a computer-controlled puff was generated every minute, leading to 10 s of CS exposure followed by 50 s of fresh air. The CS-exposed group inhaled CS from two consecutive cigarettes per day for 3 wk, at which point the dosage was increased to two cigarettes twice daily. Control animals were similarly handled and restrained in fresh air for the same duration. After the 6-wk course, mice were fasted for 6 h and received an injection of either glucose (1 g/kg body wt) or insulin (0.75 U/kg body wt). Blood glucose was determined at the times indicated in the figures, using the Bayer Contour glucose meter. For the second study, 12-wk-old mice were separated into one of several treatment groups for 8 wk. Each of the following groups was duplicated to have one group receive vehicle or myriocin injections (0.3 mg/kg) every other day for a total of eight groups: 1) control: standard diet chow, no smoke; 2) cigarette smoke (CS) exposure (two cigarettes, twice daily) with standard diet chow; 3) high-fat, high-sugar (HFHS) diet (Harlan-Teklad 45F30S); 4) HFHS diet with CS exposure. Tissues were harvested at the conclusion of the study period and following a 6-h fast. Insulin resistance was assessed using fasting blood glucose and insulin (MP Biomedicals; Insulin RIA kit, 07260102) to compute a homeostatic model assessment (HOMA-IR) score, as previously described (8). Studies were conducted in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee (IACUC) at Brigham Young University.
RESULTS

A549 cells produce and secrete ceramide in response to CS extract. Compared to PBS-treated cells, ceramide was significantly elevated in alveolar type 2 A549 cells following treat-

Fig. 3. Smoke-treated lung cell conditioned medium increases muscle cell ceramide biosynthesis. Skeletal muscle cells (C2C12) were treated with conditioned medium from A549 cells. A: schema indicating culture medium handling. B: muscle cells treated with conditioned medium from A549 cells following incubation with control (CCM), cigarette smoke extract medium (SCM), and SCM with myriocin (SCM + Myr; 10 μM). *P < 0.05 for CSE vs. other treatments; n = 5.

Lipid analysis. Lipids were extracted and quantified as described previously (47). Briefly, lipids were isolated with chloroform-methanol (1:2), and after addition of water, the organic phase was collected and dried. After resuspension, lipids were quantified using a shotgun lipidomics technique on a Thermo Scientific LTQ Orbitrap XL mass spectrometer.

Protein quantification and quantitative real-time PCR. Protein and message were quantified as described previously (21, 47). Primers were used as listed previously (21). β-Actin reactions were performed side by side with every sample analyzed. The following antibodies were used: β-actin (Cell Signaling, no. 4967), Akt (no. 9272), p-Akt-Ser473 (no. 9271), GSK3β (no. 9315), p-GSK3β-Ser21 (no. 9323), GSK3β/Akt ratio (relative to control).

Changes in the mRNA level of each gene following treatments were normalized to the β-actin control mRNA according to Pfaffl (39).

Cell and muscle fiber bundle permeabilization. Cells and tissue were prepared for the mitochondrial respiration assay as described previously (47). Following the respiration protocol (outlined below), samples were removed from the chambers and used for further analysis, including protein quantification.

Mitochondrial respiration protocol. High-resolution O2 consumption was determined at 37°C in permeabilized cells and fiber bundles, using the Oroboros O2K Oxygraph (Innsbruck, Austria) with MiRO5 respiration buffer as described previously (38, 47). Respiration was determined by all or parts of the following substrate-uncoupler-inhibitor-titration (SUIT) protocol (32): electron flow through complex I was supported by glutamate + malate (10 and 2 mM, respectively) to determine O2 consumption from proton leak (GMp). Where indicated, outer mitochondrial membrane integrity was tested by adding cytochrome c (10 μM, GMCp). Succinate was added (GMS, p) for complex I-+II electron flow into the Q-junction. To determine full electron transport system (ETS) capacity over oxidative phosphorylation in cells, the chemical uncoupler carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) was added (0.05 μM followed by 0.025 μM steps until maximal O2 flux was reached). Complex II-supported ETS was then measured by inhibiting complex I with rotenone (Rot; 0.5 μM). Last, residual O2 consumption was measured by adding antimycin A (2.5 μM) to block complex III action, effectively stopping any electron flow. This value provides a rate of respiration that is used as a baseline.

Glycogen assay. Glycogen was measured from cells where indicated, according to the manufacturer’s instructions (BioVision, Milpitas, CA).

Statistics. Data are presented as mean ± SE. Data were compared by ANOVA with Tukey’s post hoc analysis (Graphpad Prism, La Jolla, CA). Significance was set at P < 0.05.
ment with cigarette smoke extract (CSE) for 4 h (Fig. 1A). However, this effect was prevented when CSE treatment was supplemented with myriocin (CSE + Myr; Fig. 1A). Myriocin inhibits the initial and rate-limiting step in de novo ceramide biosynthesis, serine palmitoyltransferase (SPT) by preventing condensation of palmitoyl-CoA and serine. The relevance of SPT was further determined by measuring the gene expression of SPT2. CSE treatment elicited an over twofold increase in SPT2 gene expression and protein (Fig. 1, B and C), providing evidence of the role of the de novo pathway in CSE-induced ceramide biosynthesis. In addition to ceramide production, we sought to determine the ability of A549 cells to secrete ceramide. Accordingly, lipids were isolated and ceramides measured following 8-h treatment with PBS and CSE medium (± myriocin; Fig. 2). Medium from CSE-treated cells contained ~60% more ceramide than PBS-treated cells.

Muscle cells treated with lung cell conditioned medium have higher ceramide levels. To determine the effect of lung cell-secreted ceramides on muscle cell ceramide metabolism, conditioned medium from PBS- and CSE-treated cells (± myriocin) was harvested from A549 cells and placed on C2C12 myotubes for 12 h, as outlined in Fig. 3A. Myotubes treated with conditioned medium from CSE-treated cells (SCM) had significantly higher levels of ceramide than both myotubes treated with PBS-conditioned medium (CCM) and addition of myriocin to CSE treatment (SCM + Myr; Fig. 3B).

Muscle cells experience metabolic disruption following SCM treatment. Two important aspects of metabolic disruption that may mediate obesity-related morbidities and/or exacerbate weight gain are the reduction of muscle insulin sensitivity and altered mitochondrial respiration. Similarly to the above, C2C12 myotubes were treated with conditioned medium from A549 cells previously treated with normal medium, CSE, or CSE + Myr. Following 12-h serum-free conditioned medium treatment, myotubes were stimulated with insulin for 10 min. As an indicator of insulin signaling, Akt and GSK3β phosphorylation was determined. Whereas CCM myotubes experienced a marked increase in both Akt and GSK3β phosphorylation in response to insulin, myotubes treated with SCM failed to respond to insulin (Fig. 4A). However, when myotubes were treated with SCM + Myr, insulin responsiveness was restored (Fig. 4A). These results were strengthened by determining glycogen accrual with similar treatments (Fig. 4B).

To determine mitochondrial respiration, following similar treatments indicated above, myotubes were permeabilized to undergo a substrate-uncoupler-inhibitor titration protocol (protocol details provided in MATERIALS AND METHODS). No difference in rates of mitochondrial O2 was observed between treatment conditions with addition of glutamate and malate (Leak state; Fig. 5A). However, addition of ADP to measure respiration with complex I-mediated oxidative phosphorylation (GMp) revealed a marked contrast, where O2 consumption failed to increase in myotubes treated with SCM. Similar results were found with addition of succinate to introduce complex II-mediated respiration (GMSp), as well as FCCP addition to measure uncoupled respiration (GMS_E). Addition of rotenone (complex I inhibitor) resulted in a general reduction and equalization of respiration rates regardless of treatment [S(Rot)E]. Addition of myriocin to CSE treatment com-

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**Fig. 5.** Inhibition of ceramide biosynthesis protects muscle cell mitochondrial respiration. Skeletal muscle cells (C2C12) were treated with conditioned medium from A549 cells. A: myotubes were treated PBS- and CSE-A549-conditioned medium ± myriocin (CCM, SCM, SCM + Myr, respectively). Following treatment, muscle cell mitochondrial O2 consumption was determined according to the protocol outlined in MATERIALS AND METHODS (n = 6). GML, glutamate (10 mM) + malate (2 mM); GMp, +ADP (2.5 mM); GMCP, +cytochrome c (10 μM); GMSp, + succinate (10 mM); GMS_E, +FCCP (0.05 μM); S(Rot)E, + rotenone (0.5 μM); and +antimycin A (2.5 μM) as baseline. B: Western blot and quantification were performed on sample lysates to determine levels of mitochondrial complexes (n = 3). *P < 0.05 for SCM vs. other treatments.
pletely protected mitochondrial respiration (SCM+Myr), and rates were comparable to control (CCM) throughout the assay. All together, these findings suggest a severely compromised ability of muscle cell mitochondria to respire in the midst of the high-ceramide state caused by CS. The smoke-induced decrease in mitochondrial respiration may at least partly result from the slight yet significant ceramide-dependent reduction in complex III and IV protein evident in SCM-treated cells (Fig. 5B).

CS exposure causes metabolic disruption in mice. In an effort to determine whether CS causes insulin resistance, adult male mice were exposed to CS daily for 6 wk. Treatment period was determined by the length of time necessary to observe differences in glucose tolerance and insulin sensitivity. We found that smoke-exposed mice became less glucose tolerant (Fig. 6A) and less insulin sensitive (Fig. 6B) than control mice. Furthermore, the insulin resistance was attended by altered ceramides in multiple tissues, including lung (Fig. 6D), blood (Fig. 6E), and skeletal muscle (Fig. 6G), but not in liver (Fig. 6D). A portion of the gastrocnemius was permeabilized to assess mitochondrial respiration (Fig. 6C). We found that ADP-supported complex I-mediated respiration (GM<sub>p</sub>) was significantly blunted in smoked animals. This difference was further exaggerated with succinate addition (GMS<sub>p</sub>) to introduce complex II but was absent with uncoupling by the addition of FCCP (GMS<sub>E</sub>), suggesting that maximal, but not submaximal, mitochondrial respiration rate in the muscle is unaffected by CS exposure.

Ceramide inhibition prevents smoke-induced metabolic disruption, but not with combined CS and HFHS diet. The previous animal experiment provided proof of concept that CS exposure causes insulin resistance. To elucidate the role of ceramides in smoke-induced metabolic disruption, mice received vehicle or myriocin injections every other day to inhibit ceramide biosynthesis. Additionally, mice received a longer smoking regimen with the addition of a dietary intervention. Namely, animals received standard diet (SD) chow or the HFHS diet. The rationale for the dietary intervention was to more closely mimic current real world conditions, considering that smokers tend to engage in less healthy eating habits than nonsmokers (28). Interestingly, CS exposure (SD+CS) alone increased body weight (Fig. 7A), despite no apparent difference in eating habits (Sup. Fig. 1). The HFHS diet also resulted in significantly higher body weight compared with animals fed SD, although the HFHS+CS combination resulted in the highest weight gain among all groups (Fig. 7A). However, myriocin injection prevented CS-induced weight gain and mitigated HFHS-induced weight gain but was insufficient to prevent weight gain with combined HFHS+CS (Fig. 7A). Ceramides increased in soleus with all interventions (Fig. 7B). Myriocin injection prevented significant ceramide gains with all treatments. At the conclusion of the study, fasting blood glucose and insulin levels were used to determine HOMA-IR score following the 8-wk treatment period (Fig. 7C). HOMA-IR scores suggested pronounced insulin resistance with smoke (SD+CS) and dietary intervention (HFHS, HFHS+CS), but this was significantly mitigated with myriocin treatment. Last, red gastrocnemius fibers were separated and permeabilized to determine mitochondrial respiration (Fig. 8). With vehicle injections, CS exposure, regardless of diet, blunted mitochondrial respiration throughout the protocol, although inhibition with HFHS diet alone became apparent only after addition of succinate (GMS<sub>p</sub>). Myriocin injections failed

![Fig. 6](http://ajpendo.physiology.org/Downloadedfromhttp://ajpendo.physiology.org/) Cigarette smoke (CS) increases tissue ceramides and causes insulin resistance and reduced mitochondrial respiration. Adult male C57Bl/6 mice were exposed to restraint (Control) or sidestream CS (Smoke) daily for 6 wk. Glucose (A; 1 g/kg body wt ip) and insulin (B; 0.75 U/kg body wt ip) tolerance tests were performed and mitochondrial respiration analyzed in red gastrocnemius according to the protocol outlined in MATERIALS AND METHODS (C). Ceramides were determined in lung (D; P < 0.05, n = 4), blood (E; P < 0.05, n = 4), liver (F; P = 0.07, n = 4), and soleus (P < 0.05, n = 8) and gastrocnemius (P < 0.05, n = 7) (G). *P < 0.05 for smoke vs. control.
to prevent reduced respiration with the various treatments, but it offered some protection, considering that respiration was generally higher with myriocin than the comparable vehicle-treated conditions.

DISCUSSION

Dr. Gerald Reaven was the first to identify a relationship between smoking and insulin resistance over 20 years ago (22), and his initial findings have been corroborated by multiple studies since then (4, 10, 19). Although previous findings may reveal a mechanism (i.e., insulin resistance) whereby smoking results in systemic diseases (17, 27), the ensuing decades have failed to implicate a mediating molecular process linking smoking and insulin resistance. In addition to insulin resistance, smoking compromises mitochondrial function (5, 34, 37). The purpose of this report was to determine the role of the sphingolipid ceramide as a mediator of CS-induced metabolic disruption, i.e., insulin resistance and reduced mitochondrial function.

Our first step was to determine whether lung cells, specifically alveolar type 2 cells or bronchial epithelial cells (data not shown), produce and secrete ceramide in response to CSE. We observed that both cells secrete ceramides with smoke exposure, but the response is greater in alveolar type 2 cells. Previous studies exploring the source of blood ceramides have revealed a prominent role for the liver in producing and packaging ceramides into lipoproteins for transport from the liver throughout the body (16). However, ceramides are able to cross the cell membrane without lipoproteins (16), which is our predicted mechanism for ceramides exiting lung cells. However, once in the blood, ceramides are likely transported via albumin or, more likely, lipoproteins (51). Upon confirming ceramide secretion from lung cells, we utilized a cell culture system of treating myotubes with conditioned medium from alveolar type 2 cells that were treated with CSE with or without myriocin to inhibit ceramide synthesis. Our findings of a protective effect on insulin signaling and mitochondrial respiration with myriocin-treated lung cells suggest that ceramides are necessary for smoke-induced metabolic disruption in vitro. Results from our animal studies are noteworthy, considering that chronic smoking caused insulin resistance. Importantly, the dose and method of cigarette smoke exposure we used has been previously shown to yield similar levels of blood carboxyhemoglobin seen in human smokers (53). To determine the role of ceramides in insulin signaling and mitochondrial function in vivo, we performed a second and similar smoking regimen that prolonged the smoke exposure (from 6 to 8 wk), and included a HFHS diet with regular myriocin injections. The rationale for the HFHS diet was to more closely mimic real world conditions, considering smokers tend to engage in less healthy eating habits than nonsmokers (28). In general, the combination of CS and HFHS diet tended to exacerbate the effects of smoke alone, including body weight, ceramides, and HOMA-IR. Ceramide inhibition with myriocin treatment tended to mitigate these negative effects. Together, our findings build upon the initial observations of smoking and disturbed metabolic function (13, 22, 44) and allow, for the first time, a definitive implication of ceramides as a mediator of CS-induced metabolic disruption.

Similarly to insulin resistance, CS elicits a host of negative effects on mitochondria, including inhibition of respiration (34, 37) and increased ROS generation (5). It is tempting to speculate these effects may be a result of altered mitochondrial dynamics, which is affected by smoke exposure (23). We have
recently shown that mitochondrial fission is necessary for ceramide-induced changes in mitochondrial function, including varied respiration and ROS production (47).

CS is a common environmental toxin, with half of the US population being regularly exposed (40, 41) and ~20% of young children living with someone who smokes in the home (2). Everyday, almost 4,000 young adults smoke their first cigarette and 1,000 become habitual smokers (1). We intentionally utilized sidestream (i.e., second-hand) smoke in this study, given that it affects both smokers and bystanders. Our findings, in conjunction with numerous observational studies (4, 15, 20, 43, 44) linking smoking with insulin resistance are relevant to current metabolic syndrome and obesity concerns, given that insulin resistance is the crux of the pathologies that constitute the metabolic syndrome (26). Previous animal studies have indicated weight loss in smoke-exposed mice (14), although the treatment length was considerably shorter than that used in this study (4 days vs. 8 wk). However, whether smoking exerts an anti- or pro-obesogenic effect in humans is unclear. Smoking is traditionally considered to prevent fat gain, but these observations are exclusively cross-sectional (15). In contrast, prospective studies following large cohorts reveal that initiation of smoking promotes greater fat gain compared with nonsmokers (48) and heavy smoking increases the fat gain (33). Given insulin’s potent actions in increasing fat mass (30, 36, 50), the hyperinsulinemia that accompanies insulin resistance may be an important regulator of smoke-induced fat gain. Importantly, while cross-sectional studies suggest that smokers may indeed weigh less (e.g., lower BMI), they also tend to have a higher waist-to-hip ratio (15), suggesting increased visceral fat, which is an additional feature of the metabolic syndrome. Our data suggest that ceramide is an important mediator of the metabolic disruption and weight gain that accompanies chronic smoke exposure.

This study presents the perspective that smoking disrupts healthy metabolic function and, via ceramide accrual, increases risk of weight gain, especially when smoking is combined with a typical Western diet. Future efforts are currently focused on exploring the role of ceramide in other smoke-related pathologies, including the considerable smoke-induced cardiovascular burden. These findings reveal a new metabolic concern for smokers who struggle to quit, but we consider the concern even greater for those exposed to cigarette smoke as bystanders, particularly at early ages. In the end, the results suggest a potential use for anti-ceramide therapies in preventing at least some of the metabolic consequences of smoke exposure.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).
AUTHOR CONTRIBUTIONS

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