Ceramides mediate cigarette smoke-induced metabolic disruption in mice

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Thatcher MO, Tippets TS, Nelson MB, Swensen AC, Winden DR, Hansen ME, Anderson MC, Johnson IE, Porter JP, Reynolds PR, Bikman BT. Ceramides mediate cigarette smoke-induced metabolic disruption in mice. Am J Physiol Endocrinol Metab 307:E919–E927, 2014. First published October 2, 2014; doi:10.1152/ajpendo.00258.2014.—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 comorbidities. Although cigarette smoke contains chemicals that reportedly increase energy expenditure and promote weight loss (29), smoke exposure is associated with increased visceral adipose accumulation (46, 48) that increases with greater smoking frequency (46).

Two lines of evidence may support the theory that cigarette smoke causes metabolic disruption leading to fat gain. First, cigarette smoke exposure is known to disturb mitochondrial function and biogenesis (3, 37), which may compromise the cell’s ability to use fat for fuel. Second, insulin resistance and hyperinsulinemia are common features attending regular smoking (4, 15, 20, 43, 44), and insulin is a potent inducer of adipocyte expansion and fat gain (30, 36, 50). How smoke exposure causes both of these deleterious metabolic consequences is unclear. Of the myriad mediators that may disrupt healthy metabolic function in response to cigarette smoke exposure, the particular effects of the sphingolipid ceramide on 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. C2C12 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 C2C12 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 underwent intraperitoneal glucose (G7021; Sigma-Aldrich, St. Louis, MO) and insulin (Actrapid; Novo Nordisk, Plainsboro, NJ) tolerance tests. For both tests, 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.

![Fig. 1. Cigarette smoke extract (CSE) increases ceramide biosynthesis.](image)

Fig. 1. Cigarette smoke extract (CSE) increases ceramide biosynthesis. Alveolar type 2 cells (A549) were treated with PBS or CSE without (CSE) or with myriocin (CSE + Myr; 10 µM) suspended in growth medium. Following treatment period, ceramides (A; n = 6) and serine palmitoyltransferase 2 (Sptlc2) gene (B; n = 6), and protein (C; n = 3) levels were measured. *P < 0.05 for CSE vs. other treatments.

![Fig. 2. A549 cells secrete ceramide.](image)

Fig. 2. A549 cells secrete ceramide. A549 cells were treated with PBS or CSE without (CSE) or with myriocin (CSE + Myr; 10 µM) suspended in growth medium. Following 8 h, treatment medium was replaced with fresh growth medium for another 16 h, after which lipids were isolated from the medium and analyzed for ceramides. *P < 0.05 for CSE vs. other treatments; n = 5.
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. 4. Inhibition of ceramide biosynthesis prevents lung cell conditioned medium-induced loss of muscle cell insulin signaling. Skeletal muscle cells (C2C12) were treated with conditioned medium from PBS- and CSE-treated A549 cells. C2C12 myotubes were treated with serum-free conditioned medium from CSE-treated A549 cells + myriocin (10 μM) to block ceramide biosynthesis. At the end of the treatment period, cells were treated with insulin (100 nM, 10 min) before being lysed for immunoblotting (A; n = 4) and glycogen analysis (B; n = 6). *P < 0.05 for treatment vs. control.
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expression were 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 SC+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 GMS2, as well as FCCP addition to measure uncoupled respiration (GMSE). 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-

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, SC+Myr, respectively). Following treatment, muscle cell mitochondrial O2 consumption was determined according to the protocol outlined in MATERIALS AND METHODS (n = 6). GMc2, glutamate (10 mM) + malate (2 mM); GMp, +ADP (2.5 mM); GMS2, +cytochrome c (10 μM); GMS2, +succinate (10 mM); 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.
completely 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; smoke-exposed mice had significantly elevated 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 determine mitochondrial respiration (Fig. 6C). We found that ADP-supported complex I-mediated respiration (GM$_P$) was significantly blunted in smoke animals. This difference was further exaggerated with succinate addition (GMS$_P$) to introduce complex II but was absent with uncoupling by the addition of FCCP (GMS$_E$), 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$_P$). Myriocin injections failed
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).

![Graph](http://ajpendo.physiology.org/)

**Fig. 7.** Effects of myriocin on metabolic parameters with smoking and diet. Twelve-week-old mice were placed on 1 of 4 treatments [SD, standard diet; SD+CS, + daily CS exposure; high-fat, high-sugar (HFHS) diet; and HFHS+CS] while receiving vehicle or myriocin (0.3 mg/kg ip) injections every other day. Body weight (A), soleus ceramides (B), and fasting blood glucose and insulin (C) were measured at the conclusion of the study period. *P < 0.05 for treatment vs. SD within each group; #P < 0.05 for comparable treatment between myriocin vs. vehicle; n = 6–12.

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The protocol outlined in the Methods section (A; myriocin (0.3 mg/kg) I.P. injections every other day. Following treatment, muscle cell mitochondrial oxygen consumption was determined according to the protocol outlined in the Methods section (A; n = 6). GMc, glutamate (10 mM) + malate (2 mM); GMc, + ADP (2.5 mM); GMc, + cytochrome c (10 μM); GMS, + succinate (10 mM); GMS, + FCCP (0.05 μM + ); S(Rot), + rotenone (0.5 μM); and + antimycin A (2.5 μM) as baseline. Western blot was performed on sample lysates to determine levels of mitochondrial complexes (B; n = 4). *P < 0.05 for treatment vs. SD within each group; #P < 0.05 for comparable treatment between myriocin vs. vehicle.

**DISCLOSURES**

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


REFERENCES