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Cardiomyocyte mitochondrial respiration is reduced by receptor for advanced glycation end-product signaling in a ceramide-dependent manner

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Nelson MB, Swensen AC, Winden DR, Bodine JS, Bikman BT, Reynolds PR. Cardiomyocyte mitochondrial respiration is reduced by receptor for advanced glycation end-product signaling in a ceramide-dependent manner. Am J Physiol Heart Circ Physiol 309: H63–H69, 2015. First published May 8, 2015; doi:10.1152/ajpheart.00043.2015.—Cigarette smoke exposure is associated with an increased risk of cardiovascular complications. The role of advanced glycation end products (AGEs) is already well established in numerous comorbidities, including cardiomyopathy. Given the role of AGEs and their receptor, RAGE, in activating inflammatory pathways, we sought to determine whether ceramides could be a mediator of RAGE-induced altered heart mitochondrial function. Using an in vitro model, we treated H9C2 cardiomyocytes with the AGE carboxy-methyllysine before mitochondrial respiration assessment. We discovered that mitochondrial dysfunction was significantly impaired in AGE-treated cells, but not when cotreated with myriocin, an inhibitor of de novo ceramide biosynthesis. Moreover, we exposed wild-type and RAGE knockout mice to secondhand cigarette smoke and found reduced mitochondrial respiration in the left ventricular myocardium from wild-type mice, but RAGE knockout mice were protected from this effect. Finally, conditional overexpression of RAGE in the lungs of transgenic mice elicited a robust increase in left ventricular ceramides in the absence of smoke exposure. Taken together, these findings suggest a RAGE-ceramide axis as an important contributor to AGE-mediated disrupted cardiomyocyte mitochondrial function.

receptor for advanced glycation end products; ceramide; cigarette smoke; mitochondria; cardiomyopathy

RECEPTORS FOR ADVANCED GLYCATION END-PRODUCTS (RAGES) are important mediators of numerous chronic complications. Many of these conditions, including peripheral neuropathy (2), retinopathy (1, 8), nephropathy (48), and cardiomyopathy (6, 15), reduce quality of life (32) and often involve macrovascular and microvascular complications (7). RAGE was originally characterized for its ability to bind advanced glycation end products (AGEs) and for its role as a prominent feedforward receptor involved in inflammation (25). AGEs are derived via the nonenzymatic combination of amino groups and reducing sugars (19), and hyperglycemia-mediated induction of protein glycosylation has been implicated in the production of systemic AGEs (13, 46). However, independent of blood glucose, cigarette smoke exposure increases AGE formation through Malliard chemical reactions involving smoke components and plasma proteins (20). Whether through chronic hyperglycemia or secondhand cigarette smoke exposure, cellular responses provide abundant AGEs for RAGE signaling that causes enhanced expression of proinflammatory cytokines and deleterious inflammatory responses (17). Two AGEs often found in increased abundance with diabetics and smokers are Nε-carboxy-methyllysine (CML) (34) and Nε-carboxy-ethyllysine (45).

In addition to RAGE, sphingolipids are another factor increasingly recognized to play a role in the progression of inflammatory disorders (3, 16). Ceramides, in particular, are a subset of sphingolipid that have been linked to cardiometabolic disruption (42), altered mitochondrial dynamics and function (4, 36, 39), increasing atheroma development (5), and mediating insulin resistance (41). We have previously shown that ceramides are actively synthesized in the lung with cigarette smoke exposure (42).

Since activation of inflammatory signaling pathways has been shown to upregulate ceramide biosynthesis (3), it is plausible that RAGE-mediated inflammation can stimulate the synthesis and elaboration of ceramides. RAGE functions as other pattern recognition receptors, including Toll-like receptor (TLR)4, which increases ceramide biosynthesis upon activation (14). This paradigm involving a connection between RAGE and ceramide, however, has yet to be fully investigated. Based on this intersection, we hypothesized that RAGE-AGE signaling would increase cardiomyocyte ceramide accrual, thereby increasing ceramide-mediated cardiomyocyte mitochondrial dysfunction.

MATERIALS AND METHODS

Cell culture. Immortalized rat H9C2 cardiac myocytes were obtained from the America Type Cell Culture (Manassas, VA) and used at passages 9–12. CML-BSA was purchased from MBL (Woburn, MA), and myriocin was obtained from Sigma-Aldrich (St. Louis, MO). Cells were split into six-well plates and grown to confluency. Myriocin-treated cells were pretreated for 1 h with 1 µM of 10 mM myriocin. Cells were subsequently cotreated with either 30 µM CML-BSA/mL growth media or normal growth media for 24 h. All subsequent analysis took place after the exposure period.

Mice. Wild-type (WT) C57BL/6 mice are in house and were obtained from Jackson Laboratories (Bar Harbor, ME). RAGE knockout (KO) mice lacking membrane and soluble RAGE were generated on a C57BL/6 background. Conditional RAGE transgenic mice were also generated, which overexpress RAGE in the alveolar epithelium when fed doxycycline (28, 37). Doxycycline incorporation into mu-
rime diets caused the upregulation of RAGE in the lungs of transgenic mice from postnatal day 20 until the date of death at postnatal day 110 (38). Mice were kept on normal light-dark cycles and had free access to food and water. Housing and treatment of all mice were in accord with approved Institutional Animal Care and Use Committee protocols at Brigham Young University.

Smoke exposure. For one study, WT and RAGE KO mice were randomly assigned to control and smoke-exposed groups and acutely treated using an in-house nose-only smoke exposure system (InExpose System, Scireq). Over the course of 1 wk, mice were restrained daily and connected to an exposure tower for 10 min, where they were nasally exposed to secondhand cigarette smoke from two cigarettes. Computer-generated puffs resulted in 10 s of secondhand exposure followed by 50 s of fresh air. After exposure, mice were then allowed to rest for 10 min before repeating the process two additional times until they had been exposed to a total of 6 cigarettes/day. The smoke challenge chosen in the present study was associated with a good tolerance of mice to the smoke sessions and an acceptable level of particulate density concentration according to literature (29, 44). Control animals were similarly handled and restrained but kept under a smoke-free environment. In a second chronic exposure study, WT mice were similarly assigned to control and smoke-exposed groups and exposed to secondhand smoke. The procedure, however, included exposure to 4 cigarettes/day for 8 wk. Studies were performed in accordance with principles outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Immunohistochemistry. Heart tissue from control and cigarette smoke-exposed mice was fixed in 4% paraformaldehyde, processed, embedded in paraffin wax, sectioned, and stained according to standard immunohistochemical procedures (27, 30). The ceramide primary antibody used for immunohistochemical detection was obtained from Sigma-Aldrich (C8104-50TST, 1:500). Development in 3,3'-diaminobenzidine revealed enhanced brown chromogen in tissues positive for ceramide expression.

Mass spectrometry. In isolating lipids, pellets were suspended in ice-cold chloroform-methanol (1:2), incubated for 15 min on ice, and then briefly vortexed. Aqueous and organic phases were separated by the addition of ice-cold water and chloroform. The organic phase was collected in a fresh vial and dried via vacuum centrifugation (Eppendorf Concentrator Plus). Lipids were then characterized and quantified using shotgun lipidomics on a Thermo Scientific LTQ Orbitrap XL mass spectrometer, as previously described (12).

Mitochondrial respiration. O2 consumption from H9C2 cardiomyocytes and cardiac muscle obtained from mice was determined using an O2K oxigraph (Oroboros Instruments). Cells [digoxin (1 mg/ml)] and tissue [saponin (50 µg/ml)] were then permeabilized. After permeabilization, samples were transferred to respiration chambers. Respiration was determined using a substrate-uncoupler-inhibitor titration protocol. Electron flow through complex 1 was assessed by supporting the system with glutamate (G; 10 mM) and malate (M; 2 mM). After stabilization, ADP was added to determine oxidative phosphorylation capacity (2.5 mM). Succinate (S; 10 mM) was then added to assess complex 2 electron flow. To determine full electron transport system capacity, the chemical uncoupler FCCP (E; 0.05 µM) was added. To assess complex 2 electron flow, complex 1 was then inhibited by including rotenone (0.5 µM).

Real-time PCR. RNA from cells was isolated using TRIzol (Invitrogen, Grand Island, NY) and quantified via optical density measurement. Reverse transcription of RNA was performed using a Superscript III First-Strand Synthesis System to obtain cDNA for PCR. Primers for serine palmitoyltransferase (SPT)1, SPT2, and ceramide synthase (CerS)2 were obtained and diluted according to the manufacturer’s protocol. Bio-Rad iQ SYBR Green Supermix was used to perform real-time PCR along with previous CDNA, primers, and water. Values were assessed using the ΔΔCt method (where Ct is threshold cycle), and comparisons were made with amplified actin. Control wells lacking template or reverse transcriptase were included to identify primer-dimer products and to exclude possible contaminants.

Immunoblot analysis. Total protein from H9C2 cardiac myocytes and heart tissue was obtained after homogenization with RIPA buffer supplemented with protease inhibitors (Fisher Scientific, Waltham, MA). Protein was then quantified using a BCA Protein Assay Kit (Fisher Scientific), and 20 µg of the resulting lysate were blotted using a mouse polyclonal antibody (AF1179, R&D Systems, Pittsburg, PA) against RAGE (1:1,000), as previously described (45). Western blots were visualized and quantified using a LI-COR C-DiGit Blot Scanner (LI-Cor Biosciences). Quantification of RAGE was performed by densitometry, and normalization with actin provided comparisons between samples.

Statistics. In vitro data presented are representative of experiments performed in triplicate and animal experiments involved at least 4 samples/group. Data are presented as means ± SE. Data were compared by ANOVA with Tukey’s post hoc analysis (Graphpad Prism, La Jolla, CA). Significance was set at P < 0.05.

RESULTS

AGES reduced cardiomyocyte mitochondrial function in a ceramide-dependent manner. CML is one of the most physiologically relevant AGEs in a diabetic context. To assess the effects of AGES on cardiomyocytes, we treated H9C2s with growth media containing CML-BSA with and without myriocin, an inhibitor of SPT, the rate-limiting enzyme in de novo ceramide biosynthesis. Compared with control cells, gene expression levels of SPT1, SPT2, and CerS2 were significantly elevated in CML-treated cells (Fig. 1A). Similarly, compared with control conditions, cells exposed to CML-BSA showed a robust increase in ceramide production by mass spectrometry but not when pretreated with myriocin (Fig. 1B, CML + myriocin). The increase in SPT and ceramides plausibly implicate RAGE as a factor contributing to de novo ceramide biosynthesis.
To determine the effect of RAGE-mediated inflammation on heart cell function, we similarly exposed H9C2 cardiomyocytes to the same conditions described above and assessed mitochondrial respiration using a substrate-uncoupler-inhibitor titration protocol (see MATERIALS AND METHODS). A significant reduction in respiration was elicited in cells treated with CML-BSA upon the addition of glutamate and malate [leak state (GM_L); Fig. 2A]. Pretreatment with myriocin, however, abolished the effects of CML-BSA and restored respiration to control levels. Similar results were also found upon stimulation of complex I-mediated oxidative phosphorylation with ADP addition (GM_P). The addition of succinate to introduce complex II-mediated respiration (GMS_P) revealed a slight departure from the trend; whereas CML-treated cells experienced a comparable increase in respiration as control cells, CML treatment appeared to blunt the normal response to succinate addition. We further observed reduced complex II function when analyzing the complex II control factor (GMSP; Fig. 2B). Similar to succinate addition, the addition of FCCP (respiration uncoupler; GMS_E) failed to increase respiration with CML treatment to a level seen with control or myriocin treatment. Taken together, these findings suggest that respiration in cardiomyocytes is impaired by RAGE signaling and is mediated through increased ceramide accrual.

RAGE signaling contributed to cigarette smoke-induced ceramide accrual in cardiomyocytes. Because AGEs and smoke impact ceramide levels in vitro, we next assessed whether similar effects occurred in vivo. Specifically, as RAGE expression is known to be elevated with inflammation and exposure to cigarette smoke (24, 44), we sought to ascertain the expression profile of RAGE in cardiac tissue under both control conditions and after smoke exposure. To establish its expression in the heart, WT mice were restrained and exposed to either room air or secondhand cigarette smoke for 1 or 8 wk. After the treatment, we assessed RAGE protein expression in the left ventricular myocardium. Compared with control animals, RAGE expression was significantly elevated in animals after 1 wk (Fig. 3A) or 8 wk of secondhand cigarette smoke exposure (Fig. 3B). We next analyzed ceramide levels by mass spectrometry to assess whether ceramides are also elevated in cardiomyocytes after secondhand smoke exposure. WT mice exposed to acute cigarette smoke showed a significant increase in ceramides (Fig. 4A); however, this effect was diminished in RAGE KO mice after 1 wk of exposure. To qualitatively visualize these differences, we also performed immunohistochemistry on heart samples for ceramides. WT mice exposed to secondhand cigarette smoke expressed pronounced 3,3-diaminobenzidone-mediated staining for ceramides in heart tissue (Fig. 4B, arrow), but ceramides were qualitatively diminished in exposed RAGE KO mice (Fig. 4B).

RAGE signaling was necessary for cigarette smoke-mediated reductions in myocardial mitochondrial respiration. After exposure to secondhand cigarette smoke, the left ventricular myocardium from WT and RAGE KO mice was permeabilized and assessed for mitochondrial respiration. WT mice showed altered mitochondrial respiration in response to acute cigarette smoke exposure (Fig. 5A). However, RAGE deletion was protective against secondhand smoke-induced respiration de-

![Image](attachment://fig2.png)

**Fig. 2.** RAGE signaling reduces cardiomyocyte mitochondrial respiration through ceramide accrual. Cardiac myocytes (H9C2, n = 3) were treated for 24 h with either normal growth media or growth media containing CML-BSA with and without Myr. After treatment, heart cell mitochondrial O2 consumption was assessed using a substrate-uncoupler-inhibitor titration protocol (A). Complex (C)II control factor was used to determine the specific effect on succinate addition on respiration (B). A description of the protocol is shown in C. ETS, electron transport chain. *P < 0.05 for CML vs. other treatments.
fects (Fig. 5A). As with cells, we observed reduced complex II function when analyzing the complex II control factor (GMS, less GMP; Fig. 5B).

Conditional pulmonary upregulation of RAGE increased biosynthesis of cardiomyocyte ceramides. The experiments shown in Figs. 3–5 considered the biology of RAGE and ceramide in cardiomyocytes after controlled pulmonary exposure to secondhand smoke. To further assess the role of RAGE in promoting cardiac accumulation of ceramide, experiments were designed in the absence of smoke that compared control mice and conditional transgenic mice that geneti-
cally overexpress RAGE in the peripheral lung. Upregulation of RAGE in these mouse models has been validated by both immunoblot analysis and quantitative PCR (27, 29). Lipids were purified from heart tissue obtained from RAGE transgenic mice after 90 days of RAGE upregulation and age-matched nontransgenic control mice before subjection to mass spectrometry analysis. Compared with control mice, hearts from transgenic mice showed a significant increase in ceramides (Fig. 6), further supporting the notion that RAGE is a sufficient mediator of de novo ceramide biosynthesis in cardiac tissue.

Fig. 3. Cigarette smoke increases RAGE expression in the heart with secondhand cigarette smoke exposure. Wild-type (WT) mice were restrained and exposed to either room air (n = 3) or secondhand cigarette smoke (n = 3, see MATERIALS AND METHODS) for 1 (A) or 8 (B) wk. After the exposure period, Western blot analysis and quantification after normalization with actin were performed on heart tissue lysates to determine relative expression levels of RAGE. *P < 0.05 for smoke vs. room air.

Fig. 4. RAGE knockout (KO) prevents heart ceramide accrual with cigarette smoke. WT and RAGE KO mice were restrained and exposed to either room air or acute secondhand cigarette smoke for 1 wk. After the exposure period, lipids were isolated from heart tissue and analyzed for ceramides (A). Immunohistochemical staining for ceramides was also performed to qualitatively determine any differences in heart tissue between WT and RAGE KO mice under the different exposure conditions (B). Ceramides were significantly increased in WT mice exposed to tobacco smoke (arrows) compared with exposed RAGE KO mice. Scale bars = 200 nm. *P < 0.05 for smoke vs. room air within each group.
This study assessed the effects of RAGE stimulation on cardiomyocyte ceramide accrual and mitochondrial function. Because AGEs are established factors in the progression of cardiovascular complications (15, 21), our goal was to assess whether RAGE signaling leads to mitochondrial disruption in heart tissue and whether ceramide is an obligate mediator of these effects. Our results demonstrated that cardiomyocytes respond to AGE treatment with a robust increase in ceramide accrual. This increase may be due, at least in part, to increased expression of the initial and rate-limiting enzymes of ceramide biosynthesis (i.e., SPT) as well as CerS2. To our knowledge, our results are the first to establish a RAGE-ceramide axis within heart cells and tissue.

Under normal physiological circumstances, RAGE serves a protective role by generating a nonspecific inflammatory response to a host of heterogeneous compounds, but this process can become pathological with sustained activation and chronic inflammation (18). Although the scope of our study was limited to the effects of RAGE and ceramides on cardiomyocyte mitochondrial function, both have been implicated in a host of diabetic and smoke-induced cardiovascular complications. However, AGEs inhaled by smokers may be chemically and functionally different compared with de novo AGE synthesis in uncontrolled diabetics. Ma et al. (15) revealed that AGE-RAGE ablation prevents diabetes-induced altered cardiovascular function, including cardiomyopathy. Moreover, Park et al. (23) found that ceramide inhibition through SPT ablation is protective against dilated diabetes-induced cardiomyopathy. Thus, in light of our results of increased ceramides with RAGE activation in heart tissue, future work will seek to determine whether ceramides are necessary for altered heart function orchestrated by RAGE signaling. Importantly, mitochondrial dysfunction may play a critical role in the pathogenesis of cardiomyopathy (11). This is relevant given our previous findings that ceramides directly disrupt mitochondrial physiology, reducing respiration and increasing oxidative stress (42), through a mitochondrial fission-dependent process (36). We have previously found that ceramide accrual exhibited a widespread inhibition of mitochondrial respiration, but the effect appeared more selective to inhibiting complex II-mediated respiration. We note similar findings in this report: CML-treated cells did not experience the succinate-induced respiration increase evidenced in both control and myriocin-treated cells. Our demonstration that AGES induce ceramide-dependent impairment in mitochondrial O2 flux may explain, at least in part, why diabetic hearts are characterized by contractile impairment from anomalous energy metabolism.

Our rationale for targeting RAGE as an activator of ceramide accrual in the heart stems from two observations: 1) as noted earlier, RAGE mediates cardiovascular complications
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(15); and 2) RAGE shares common signaling intermediates in pathways known to activate ceramide biosynthesis, namely, TLR4. We reported in 2011 that TLR4 is required for lipiddendotoxin-induced ceramide biosynthesis (14). Interestingly, not only do TLR4 and RAGE share common downstream signaling (e.g., IL-1 receptor-associated kinase (33), but also common ligand activators (e.g., high-mobility group protein B1) (10, 35). Our use of cigarette smoke exposure as an intervention to stimulate RAGE expression is based on our earlier reports of smoke exposure eliciting a robust increase in lung RAGE expression (26, 31), which is a necessary event in transducing inflammation. However, the increase in heart RAGE expression is novel and conveys the possible importance of RAGE modulation in heart complications. To our knowledge, while Denis et al. (9) were the first to find a ceramide-RAGE connection when they noted a roughly two-fold increase in ceramides in cultured bovine pericytes with AGE exposure, our data may be the first to reveal this phenomenon in whole tissue. Moreover, our evidence of RAGE-mediated increased ceramides in animals exposed to cigarette smoke and in the lungs of RAGE transgenic mice speaks to the influence of lung RAGE signaling on heart ceramide accrual. Despite the clear implication of RAGE in ceramide accrual, important questions remain, such as to what extent RAGE signaling intermediates participate in ceramide biosynthesis and mitochondrial function and how pulmonary RAGE up-regulation impacts ceramide accrual in heart tissue.

Given the considerable cardiovascular burden inherent to cigarette smoke exposure (40, 43), our results provide a possible mechanism whereby smoke exposure leads to cardiovascular complications. Ceramide has been shown to mediate cardiomyopathy (22) and atherosclerosis (5) and inhibit nitric oxide-induced vasodilation (47). Thus, our results may have broad implications for cardiovascular therapies.

In conclusion, our results demonstrate that RAGE signaling reduces heart mitochondrial respiration in a ceramide-dependent manner. Considering the dependence of the myocardium on normal mitochondrial function, these results provide evidence for the utility of anticeramide therapies in the treatment or prevention of multiple cardiovascular complications.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


REFERENCES


