2015-03-01

The Role of Receptors for Advanced Glycation End-Products (RAGE) and Ceramide in Cardiovascular Disease

Michael Bruce Nelson
Brigham Young University - Provo

The Role of Receptors for Advanced Glycation End-Products (RAGE)

and Ceramide in Cardiovascular Disease

Michael Bruce Nelson

A thesis submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of

Master of Science

Paul R. Reynolds, Chair
Benjamin T. Bikman
Steven M. Johnson

Department of Physiology and Developmental Biology

Brigham Young University

March 2015

ABSTRACT

The Role of Receptors for Advanced Glycation End-Products (RAGE)
and Ceramide in Cardiovascular Disease

Michael Bruce Nelson
Department of Physiology and Developmental Biology, BYU
Master of Science

Type 2 diabetes and cigarette smoke exposure are 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 carboxy-methyl lysine-BSA, followed by mitochondrial respiration assessment. We found that mitochondrial respiration was significantly impaired in AGE-treated cells, but not when co-treated with myriocin, an inhibitor of *de novo* ceramide biosynthesis. Moreover, we exposed WT and RAGE KO mice to side-stream cigarette smoke and found reduced mitochondrial respiration in the left ventricle myocardium from WT mice, but the RAGE KO mice were protected from this effect. Finally, conditional over-expression of RAGE in the lungs of mice also elicited a robust increase in left ventricular ceramides. Altogether, these findings suggest a RAGE-ceramide axis as an important contributor to cardiomyopathy.

Keywords: RAGE, ceramide, cigarette smoke, diabetes, mitochondria, cardiomyopathy

ACKNOWLEDGMENTS

I would like to convey my most sincere thanks to:

- My advisor, Dr. Paul Reynolds. During my undergraduate and graduate experience, his guidance and expertise have motivated me to strive for excellence in all aspects of my life. He is a wonderful example of devotion, charity, and lifelong learning. I could not have asked for a better mentor, and I am grateful for the unique opportunities that he provided by allowing me to work in his lab.

- Dr. Benjamin Bikman, for his friendship and continual support. This work would not have been possible without his knowledge, patience, and collaborative efforts with the Reynolds lab. His expertise on physiology and dedication to healthy lifestyle habits have inspired me to live better. I am also thankful for the support of his graduate and undergraduate students.

- Dr. Steven Johnson, for his continued support and insight.

- The Department of Physiology and Developmental Biology at Brigham Young University, for providing a learning-conducive environment and exceptional facilities to conduct research.

- Graduate and undergraduate students of the Reynolds lab, for their assistance, support, and friendship through the years.

- My family, and especially my parents, for their continual love, support, and dedication. I treasure my memories of being with them, and am grateful for their example of selflessness, charity, and devotion.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1: Introduction

According to the Centers for Disease Control and Prevention, cardiovascular disease is currently the number-one leading cause of death in the United States (CDC; 2010). In 2010, just under 600,000 reported deaths were attributed to heart disease, with an additional 216,000 stemming from chronic lower respiratory disease and diabetic complications (CDC, 2010). Although there are genetic aspects to cardiovascular disease, it's well-established that many of the risk factors stem from lifestyle components including diet and body mass index (Jones, 2010). Chief among those risk factors are whether or not the individual is diabetic (Lorber, 2014) and whether or not they smoke on a regular basis (Messner & Bernhard, 2014). Indeed, many of the additional 216,000 deaths may have carried cardiovascular comorbidities.

These risk factors play a role in the lives of many Americans. They often reduce the quality of life, increase the burden of medical costs, and significantly shorten life-span (Sicras-Mainar, Navarro-Artieda, & Ibanez-Nolla, 2013). Current estimates suggest that over 26 million Americans are diabetic as defined by their HbA1C level, and its predicted that approximately half of Americans will be diagnosed by the year 2020 (Hawks, 2013). Although the prevalence of cigarette smoke usage has declined over the last 50 years, one recent report also suggested that approximately one-fifth of working adults still smoke (Burns, 2014). Although diet, exercise, and healthy lifestyles are undoubtedly the best preventative measures, a greater understanding of the mechanistic pathology could provide novel methods to ameliorate and treat cardiovascular complications as individuals work to treat the underlying causes of their conditions. Where we currently stand, two players that merit further investigation for the role they play in cardiovascular disease are the receptor of advanced glycation end-products (RAGE) and the sphingolipid ceramide.

Receptor of Advanced Glycation End Products (RAGE)

RAGE is a pattern-recognition receptor of the immunoglobulin family (Robinson, Johnson, Bennion, & Reynolds, 2012). Under normal physiological circumstances, membrane-bound RAGE (mRAGE) serves a protective role by generating non-specific inflammatory responses to a host of heterogeneous compounds, but can introduce complications with sustained activation and chronic inflammation (Tobon-Velasco, Cuevas, & Torres-Ramos, 2014). It contains a variable extracellular V-region-like immunoglobulin domain, two constant C-region-like domains, a single-pass transmembrane domain, and a cytosolic domain that is responsible for mediating signal transduction (Robinson, Stogsdill, Lewis, Wood, & Reynolds, 2012). Other forms of RAGE, however, including soluble RAGE (sRAGE) and endogenous secretory RAGE (esRAGE), are also found in the body, but are incapable of generating inflammatory responses (Huang, Que, & Shen, 2014).

The receptor is highly expressed in the lung alveoli (Reynolds et al., 2008), and much of our lab's research on RAGE has focused on its role in the context of lung exposure to cigarette smoke, and to a lesser degree, environmentally available diesel particulate matter (Reynolds, Wasley, & Allison, 2011). Because of its expression profile, it is thought to play a key role during embryonic lung development and to contribute significantly to the progression of COPD (Stogsdill et al., 2013; Winden et al., 2013). Numerous studies, however, have also affirmed that RAGE is expressed in peripheral tissues including vasculature and cardiac tissue (Yu et al., 2013). In many of these other tissues, RAGE may be involved in promoting macrovascular and microvascular complications (Chawla et al., 2014) and contribute to the onset of cardiomyopathy (Bodiga, Eda, & Bodiga, 2014). Indeed, the contribution of RAGE to cardiovascular disease has been well-documented (Yan, Ramasamy, & Schmidt, 2009).

RAGE binds to a host of ligands including S100/calgranulin polypeptides, HMGB1, and advanced glycation end products (AGEs) derived through the non-enzymatic combination of amino groups and reducing sugars (Ibrahim, Armour, Phipps, & Sukkar, 2013; Xue et al., 2014). Ligands of RAGE, however, are also found abundantly in the environment. Dietary AGEs are present in the foods we ingest, and they can be readily absorbed from the gut (Poulsen et al., 2013). They also form a major constituent of cigarette smoke, and are generated through Malliard chemical reactions involving smoke components and plasma proteins (Vlassara & Palace, 2002). Indeed, previous studies have revealed that serum levels of AGEs are higher in smokers compared to non-smokers (Cerami et al., 1997). Finally, AGEs can also be produced endogenously within our bodies. Chronic hyperglycemia can generate oxidative stress and activate several mechanisms leading to increased production of advanced glycation end-products from methylglyoxal derivatives (Gaens, Stehouwer, & Schalkwijk, 2013). Chief among those AGEs are proteins with N(ε)-carboxy-methyl-lysine (CML) and N(ε)-carboxy-ethyl-lysine adducts (Schmitt, Linder, Standker, Hammes, & Preissner, 2008; Xue et al., 2011). As these AGEs accumulate, they can interact with RAGE in vasculature, lung, and heart where they activate signaling cascades that increase expression of pro-inflammatory cytokines that mediate inflammatory responses (Brett et al., 1993). Of note, interaction between CML and RAGE is thought to promote signaling through NF-kB to increase expression of cytokines including IL-1β, IL-6, and TNFα (Tobon-Velasco et al., 2014). These cytokines may, in turn, regulate endogenous production of sphingolipids.

Ceramide

For years, the role of sphingolipids was unknown. In fact, the very word "sphingolipid" is rooted in reference to the mythological sphinx of Greek tradition (Merrill et al., 1997).

Nowadays, however, sphingolipids are known to be a necessary component of cell membranes (Ohta et al., 2009) and to modulate numerous processes including inflammation, apoptosis and autophagy (Schilling et al., 2013). As such, they play a crucial role in heart health and the development of cardiovascular disease (de Faria Poloni, Chapola, Feltes, & Bonatto, 2014). Ceramides fall into this family of lipid, and they can be generated through one of several pathways (Kogot-Levin & Saada, 2014). One such pathway is the *de novo* pathway in which palmitoyl-CoA is converted to ceramide through several enzymes including serine palmitoyltransferase, 3-ketosphingosine reductase, ceramide synthase and ceramide desaturase (Brice & Cowart, 2011). Another method, known as the recycling pathway, involves the cleavage of sphingomyelin by acid- and neutral-sphingomyelinase enzymes to generate ceramide (Claus, Dorer, Bunck, & Deigner, 2009). Many of these enzymes involved in ceramide production can be targeted pharmacologically; however, SPT2 is often the main target and widely recognized as the rate-limiting enzyme of ceramide biosynthesis (Batheja, Uhlinger, Carton, Ho, & D'Andrea, 2003). Despite the important role ceramide plays, it's increasingly believed that excessive accumulation of this particular sphingolipid in heart and blood vessels may be an important factor in the etiology of various disease states.

Hyperlipidemia and the accumulation of lipotoxic metabolites in heart may increase the risk of cardiovascular disease (Kang, Kim, Lee, & Park, 2013). Ceramides have been shown to interfere with signaling pathways involved with various metabolic and physiological processes. For example, it has been shown to induce insulin resistance in skeletal muscle by interfering with insulin signaling pathways (Russo, Ross, & Cowart, 2013), promote hypertension by blocking phosphorylation of eNOS in vascular tissue (Zhang et al., 2012), and even exacerbate emphysema-like symptoms in lung (Petrache et al., 2005). Other studies have asserted that

ceramide can induce cardiomyocyte death by altering mitochondrial metabolism (Parra et al., 2013). One plausible explanation for the latter observation is that ceramide has been shown to stimulate mitochondrial fission (Smith et al., 2013). This paradigm of ceramide-mediated mitochondrial fission in heart, however, has yet to be fully investigated. Because activation of inflammatory signaling pathways has been shown to upregulate ceramide biosynthesis (Bikman, 2012), it's possible that inflammation mediated by RAGE may play a role in the *de novo* biosynthesis of ceramide by lung and heart.

Summary of Research

Previous research conducted in our lab has reinforced the notion of RAGE and ceramide as key players in cardiovascular disease. Our lab has previously demonstrated that A549 cells synthesize and actively secrete ceramides in response to treatment with growth media infused with cigarette smoke extract (CSE) (Thatcher et al., 2014). Following this observation, we hypothesized that, in living organisms, the lungs can actively secrete ceramides into the blood in response to cigarette smoke. Following secretion, these sphingolipids can then travel systemically and accumulate in peripheral tissues where they disturb regular metabolic processes. Specifically, we have demonstrated that cigarette smoke exposure increases ceramide accumulation in both skeletal muscle and heart (Thatcher et al., 2014; Tippetts et al., 2014). Furthermore, we've shown that ceramides stimulate mitochondrial fission and reduce mitochondrial respiration by upregulating dynamin related protein 1 (Smith et al., 2013). Despite these observations, however, we have yet to offer a mechanism accounting for the increase in ceramides.

Interestingly, RAGE shares some similarities with another receptor that is known to increase ceramide biosynthesis. Several studies have shown that the interaction of both palmitic

acid and LPS with Toll-like Receptor 4 (TLR4) in cellular macrophages results in increased production of ceramide (Schilling et al., 2013). RAGE and TLR4 both share similar ligands and activate similar signaling pathways (Ullah et al., 2014; Veloso et al., 2011); therefore, RAGE may exacerbate TLR4-mediated effects by inciting parallel signaling pathways in both cardiac and lung tissue. Both RAGE and TLR4 signal through the canonical NF-kB pathway to increase expression of inflammatory cytokines (Ibrahim et al., 2013; Reynolds, Kasteler, Schmitt, & Hoidal, 2011). These inflammatory cytokines, in turn, may act to activate or increase expression of enzymes involved in either the *de novo* pathway or recycling pathway of ceramide biosynthesis (Medler et al., 2008). Since RAGE is expressed in heart and lung, there is a high likelihood that activation of RAGE by advanced glycation end-products promotes cardiomyopathy by enhancing cardiac ceramide accumulation and reducing the metabolic efficacy of cardiomyocytes.

To our knowledge, the study outlined in the following chapter is the first to test the possibility of a RAGE-ceramide axis in both cardiac and lung tissue. This research helps to identify mechanisms of increased cardiac ceramide accumulation and to what extent ceramide mediates the effects of RAGE in diabetic and smoke-induced cardiovascular disease.

References

Batheja, A. D., Uhlinger, D. J., Carton, J. M., Ho, G., & D'Andrea, M. R. (2003). Characterization of serine palmitoyltransferase in normal human tissues. *J Histochem Cytochem, 51*(5), 687-696.

Bikman, B. T. (2012). A role for sphingolipids in the pathophysiology of obesity-induced inflammation. *Cell Mol Life Sci, 69*(13), 2135-2146. doi: 10.1007/s00018-012-0917-5

Bodiga, V. L., Eda, S. R., & Bodiga, S. (2014). Advanced glycation end products: role in pathology of diabetic cardiomyopathy. *Heart Fail Rev, 19*(1), 49-63. doi: 10.1007/s10741-013-9374-y

Brett, J., Schmidt, A. M., Yan, S. D., Zou, Y. S., Weidman, E., Pinsky, D., . . . et al. (1993). Survey of the distribution of a newly characterized receptor for advanced glycation end products in tissues. *Am J Pathol, 143*(6), 1699-1712.

Brice, S. E., & Cowart, L. A. (2011). Sphingolipid metabolism and analysis in metabolic disease. *Adv Exp Med Biol, 721*, 1-17. doi: 10.1007/978-1-4614-0650-1_1

Burns, D. (2014). How far we have come in the last 50 years in smoking attitudes and actions. *Ann Am Thorac Soc, 11*(2), 224-226. doi: 10.1513/AnnalsATS.201308-258PS

Centers for Disease Control and Prevention. (2010). Deaths and Mortality. Retrieved from http://www.cdc.gov/nchs/fastats/deaths.htm.

Cerami, C., Founds, H., Nicholl, I., Mitsuhashi, T., Giordano, D., Vanpatten, S., . . . Cerami, A. (1997). Tobacco smoke is a source of toxic reactive glycation products. *Proc Natl Acad Sci U S A, 94*(25), 13915-13920.

Chawla, D., Bansal, S., Banerjee, B. D., Madhu, S. V., Kalra, O. P., & Tripathi, A. K. (2014). Role of advanced glycation end product (AGE)-induced receptor (RAGE) expression in diabetic vascular complications. *Microvasc Res, 95*, 1-6. doi: 10.1016/j.mvr.2014.06.010

Claus, R. A., Dorer, M. J., Bunck, A. C., & Deigner, H. P. (2009). Inhibition of sphingomyelin hydrolysis: targeting the lipid mediator ceramide as a key regulator of cellular fate. *Curr Med Chem, 16*(16), 1978-2000.

de Faria Poloni, J., Chapola, H., Feltes, B. C., & Bonatto, D. (2014). The importance of sphingolipids and reactive oxygen species in cardiovascular development. *Biol Cell, 106*(6), 167-181. doi: 10.1111/boc.201400008

Gaens, K. H., Stehouwer, C. D., & Schalkwijk, C. G. (2013). Advanced glycation endproducts and its receptor for advanced glycation endproducts in obesity. *Curr Opin Lipidol, 24*(1), 4-11. doi: 10.1097/MOL.0b013e32835aea13

Hawks, J. H. (2013). Obesity and type 2 diabetes: epidemics that now require a population health approach. *Urol Nurs, 33*(5), 216-217.

Huang, M., Que, Y., & Shen, X. (2014). Correlation of the plasma levels of soluble RAGE and endogenous secretory RAGE with oxidative stress in pre-diabetic patients. *J Diabetes Complications*. doi: 10.1016/j.jdiacomp.2014.12.007

Ibrahim, Z. A., Armour, C. L., Phipps, S., & Sukkar, M. B. (2013). RAGE and TLRs: relatives, friends or neighbours? *Mol Immunol, 56*(4), 739-744. doi: 10.1016/j.molimm.2013.07.008

Jones, P. H. (2010). Management of obesity in the prevention of cardiovascular disease. *Methodist Debakey Cardiovasc J, 6*(4), 33-36.

Kang, S. C., Kim, B. R., Lee, S. Y., & Park, T. S. (2013). Sphingolipid metabolism and obesity-induced inflammation. *Front Endocrinol (Lausanne), 4*, 67. doi: 10.3389/fendo.2013.00067

Kogot-Levin, A., & Saada, A. (2014). Ceramide and the mitochondrial respiratory chain. *Biochimie, 100*, 88-94. doi: 10.1016/j.biochi.2013.07.027

Lorber, D. (2014). Importance of cardiovascular disease risk management in patients with type 2 diabetes mellitus. *Diabetes Metab Syndr Obes, 7*, 169-183. doi: 10.2147/dmso.s61438

Medler, T. R., Petrusca, D. N., Lee, P. J., Hubbard, W. C., Berdyshev, E. V., Skirball, J., . . . Petrache, I. (2008). Apoptotic sphingolipid signaling by ceramides in lung endothelial cells. *Am J Respir Cell Mol Biol, 38*(6), 639-646. doi: 10.1165/rcmb.2007-0274OC

Merrill, A. H., Jr., Schmelz, E. M., Dillehay, D. L., Spiegel, S., Shayman, J. A., Schroeder, J. J., . . . Wang, E. (1997). Sphingolipids--the enigmatic lipid class: biochemistry, physiology, and pathophysiology. *Toxicol Appl Pharmacol, 142*(1), 208-225. doi: 10.1006/taap.1996.8029

Messner, B., & Bernhard, D. (2014). Smoking and cardiovascular disease: mechanisms of endothelial dysfunction and early atherogenesis. *Arterioscler Thromb Vasc Biol, 34*(3), 509-515. doi: 10.1161/atvbaha.113.300156

Ohta, E., Ohira, T., Matsue, K., Ikeda, Y., Fujii, K., Ohwaki, K., . . . Sasaki, M. (2009). Analysis of development of lesions in mice with serine palmitoyltransferase (SPT) deficiency - Sptlc2 conditional knockout mice. *Exp Anim, 58*(5), 515-524.

Parra, V., Moraga, F., Kuzmicic, J., Lopez-Crisosto, C., Troncoso, R., Torrealba, N., . . . Lavandero, S. (2013). Calcium and mitochondrial metabolism in ceramide-induced cardiomyocyte death. *Biochim Biophys Acta, 1832*(8), 1334-1344. doi: 10.1016/j.bbadis.2013.04.009

Petrache, I., Natarajan, V., Zhen, L., Medler, T. R., Richter, A. T., Cho, C., . . . Tuder, R. M. (2005). Ceramide upregulation causes pulmonary cell apoptosis and emphysema-like disease in mice. *Nat Med, 11*(5), 491-498. doi: 10.1038/nm1238

Poulsen, M. W., Hedegaard, R. V., Andersen, J. M., de Courten, B., Bugel, S., Nielsen, J., . . . Dragsted, L. O. (2013). Advanced glycation endproducts in food and their effects on health. *Food Chem Toxicol, 60*, 10-37. doi: 10.1016/j.fct.2013.06.052

Reynolds, P. R., Kasteler, S. D., Cosio, M. G., Sturrock, A., Huecksteadt, T., & Hoidal, J. R. (2008). RAGE: developmental expression and positive feedback regulation by Egr-1 during cigarette smoke exposure in pulmonary epithelial cells. *Am J Physiol Lung Cell Mol Physiol, 294*(6), L1094-1101. doi: 10.1152/ajplung.00318.2007

Reynolds, P. R., Kasteler, S. D., Schmitt, R. E., & Hoidal, J. R. (2011). Receptor for advanced glycation end-products signals through Ras during tobacco smoke-induced pulmonary inflammation. *Am J Respir Cell Mol Biol, 45*(2), 411-418. doi: 10.1165/rcmb.2010-0231OC

Reynolds, P. R., Wasley, K. M., & Allison, C. H. (2011). Diesel particulate matter induces receptor for advanced glycation end-products (RAGE) expression in pulmonary epithelial cells, and RAGE signaling influences NF-kappaB-mediated inflammation. *Environ Health Perspect, 119*(3), 332-336. doi: 10.1289/ehp.1002520

Robinson, A. B., Johnson, K. D., Bennion, B. G., & Reynolds, P. R. (2012). RAGE signaling by alveolar macrophages influences tobacco smoke-induced inflammation. *Am J Physiol Lung Cell Mol Physiol, 302*(11), L1192-1199. doi: 10.1152/ajplung.00099.2012

Robinson, A. B., Stogsdill, J. A., Lewis, J. B., Wood, T. T., & Reynolds, P. R. (2012). RAGE and tobacco smoke: insights into modeling chronic obstructive pulmonary disease. *Front Physiol, 3*, 301. doi: 10.3389/fphys.2012.00301

Russo, S. B., Ross, J. S., & Cowart, L. A. (2013). Sphingolipids in obesity, type 2 diabetes, and metabolic disease. *Handb Exp Pharmacol*(216), 373-401. doi: 10.1007/978-3-7091-1511-4_19

Schilling, J. D., Machkovech, H. M., He, L., Sidhu, R., Fujiwara, H., Weber, K., . . . Schaffer, J. E. (2013). Palmitate and lipopolysaccharide trigger synergistic ceramide production in primary macrophages. *J Biol Chem, 288*(5), 2923-2932. doi: 10.1074/jbc.M112.419978

Schmitt, S., Linder, M., Standker, L., Hammes, H. P., & Preissner, K. T. (2008). Identification of CML-modified proteins in hemofiltrate of diabetic patients by proteome analysis. *Exp Clin Endocrinol Diabetes, 116*(1), 26-34. doi: 10.1055/s-2007-985370

Sicras-Mainar, A., Navarro-Artieda, R., & Ibanez-Nolla, J. (2013). Clinical and economic characteristics associated with type 2 diabetes. *Rev Clin Esp*. doi: 10.1016/j.rce.2013.11.002

Smith, M. E., Tippetts, T. S., Brassfield, E. S., Tucker, B. J., Ockey, A., Swensen, A. C., . . . Bikman, B. T. (2013). Mitochondrial fission mediates ceramide-induced metabolic disruption in skeletal muscle. *Biochem J*. doi: 10.1042/bj20130807

Stogsdill, M. P., Stogsdill, J. A., Bodine, B. G., Fredrickson, A. C., Sefcik, T. L., Wood, T. T., . . . Reynolds, P. R. (2013). Conditional overexpression of receptors for advanced glycation end-products in the adult murine lung causes airspace enlargement and induces inflammation. *Am J Respir Cell Mol Biol, 49*(1), 128-134. doi: 10.1165/rcmb.2013-0013OC

Thatcher, M. O., Tippetts, T. S., Nelson, M. B., Swensen, A. C., Winden, D. R., Hansen, M. E., . . . Bikman, B. T. (2014). Ceramides mediate cigarette smoke-induced metabolic disruption in mice. *Am J Physiol Endocrinol Metab*, ajpendo.00258.02014. doi: 10.1152/ajpendo.00258.2014

Tippetts, T. S., Winden, D. R., Swensen, A. C., Nelson, M. B., Thatcher, M. O., Saito, R. R., . . . Bikman, B. T. (2014). Cigarette smoke increases cardiomyocyte ceramide accumulation and inhibits mitochondrial respiration. *BMC Cardiovasc Disord, 14*, 165. doi: 10.1186/1471-2261-14-165

Tobon-Velasco, J. C., Cuevas, E., & Torres-Ramos, M. A. (2014). Receptor for AGEs (RAGE) as Mediator of NF-kB Pathway Activation in Neuroinflammation and Oxidative Stress. *CNS Neurol Disord Drug Targets, 13*(9), 1615-1626.

Ullah, M. A., Loh, Z., Gan, W. J., Zhang, V., Yang, H., Li, J. H., . . . Sukkar, M. B. (2014). Receptor for advanced glycation end products and its ligand high-mobility group box-1 mediate allergic airway sensitization and airway inflammation. *J Allergy Clin Immunol*. doi: 10.1016/j.jaci.2013.12.1035

Veloso, C. A., Fernandes, J. S., Volpe, C. M., Fagundes-Netto, F. S., Reis, J. S., Chaves, M. M., & Nogueira-Machado, J. A. (2011). TLR4 and RAGE: similar routes leading to inflammation in type 2 diabetic patients. *Diabetes Metab, 37*(4), 336-342. doi: 10.1016/j.diabet.2010.12.005

Vlassara, H., & Palace, M. R. (2002). Diabetes and advanced glycation endproducts. *Journal of Internal Medicine, 251*(2), 87-101. doi: 10.1046/j.1365-2796.2002.00932.x

Winden, D. R., Ferguson, N. T., Bukey, B. R., Geyer, A. J., Wright, A. J., Jergensen, Z. R., . . . Reynolds, P. R. (2013). Conditional over-expression of RAGE by embryonic alveolar epithelium compromises the respiratory membrane and impairs endothelial cell differentiation. *Respir Res, 14*, 108. doi: 10.1186/1465-9921-14-108

Xue, J., Rai, V., Singer, D., Chabierski, S., Xie, J., Reverdatto, S., . . . Shekhtman, A. (2011). Advanced glycation end product recognition by the receptor for AGEs. *Structure, 19*(5), 722-732. doi: 10.1016/j.str.2011.02.013

Xue, J., Ray, R., Singer, D., Bohme, D., Burz, D. S., Rai, V., . . . Shekhtman, A. (2014). The receptor for advanced glycation end products (RAGE) specifically recognizes methylglyoxal-derived AGEs. *Biochemistry, 53*(20), 3327-3335. doi: 10.1021/bi500046t

Yan, S. F., Ramasamy, R., & Schmidt, A. M. (2009). The receptor for advanced glycation endproducts (RAGE) and cardiovascular disease. *Expert Rev Mol Med, 11*, e9. doi: 10.1017/s146239940900101x

Yu, L., Zhao, Y., Xu, S., Ding, F., Jin, C., Fu, G., & Weng, S. (2013). Advanced Glycation End Product (AGE)-AGE Receptor (RAGE) System Upregulated Connexin43 Expression in Rat Cardiomyocytes via PKC and Erk MAPK Pathways. *Int J Mol Sci, 14*(2), 2242-2257. doi: 10.3390/ijms14022242

Zhang, Q. J., Holland, W. L., Wilson, L., Tanner, J. M., Kearns, D., Cahoon, J. M., . . . Symons, J. D. (2012). Ceramide mediates vascular dysfunction in diet-induced obesity by PP2A-mediated dephosphorylation of the eNOS-Akt complex. *Diabetes, 61*(7), 1848-1859. doi: 10.2337/db11-1399

CHAPTER 2: Cardiomyocyte Mitochondrial Respiration Is Reduced By Receptor For Advanced Glycation End-Products (Rage) Signaling In A Ceramide-Dependent Manner

[1]Michael B. Nelson, [2]Adam C. Swensen, [1]Duane R. Winden, [1]Jared S. Bodine, [1]Benjamin T. Bikman, [1]Paul R. Reynolds*

Departments of [1]Physiology and Developmental Biology and [2]Chemistry and Biochemistry, Brigham Young University, Provo, UT 84602

Address correspondence to:

Paul R. Reynolds, Ph.D.
Brigham Young University, Department of Physiology and Developmental Biology
3054 Life Sciences Building
Provo, UT 84602
TEL: (801) 422-1933
FAX: (801) 422-0700
E-mail: paul_reynolds@byu.edu

Running Title: RAGE and ceramide impact cardiomyocyte respiration

Keywords: RAGE, ceramide, cigarette-smoke, mitochondria, cardiomyopathy

Abstract

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-methyl lysine prior to mitochondrial respiration assessment. We discovered that mitochondrial respiration was significantly impaired in AGE-treated cells, but not when co-treated with myriocin, an inhibitor of *de novo* ceramide biosynthesis. Moreover, we exposed WT and RAGE KO mice to secondhand cigarette smoke and found reduced mitochondrial respiration in the left ventricle myocardium from WT mice, but the RAGE KO mice were protected from this effect. Finally, conditional over-expression of RAGE in the lungs of transgenic mice elicited a robust increase in left ventricular ceramides in the absence of smoke exposure. Altogether, these findings suggest a RAGE-ceramide axis as an important contributor to AGE-mediated disrupted cardiomyocyte mitochondrial function.

Introduction

Receptors for advanced glycation end-products (RAGE) are important mediators of numerous chronic complications. Many of these conditions, including peripheral neuropathy (Bekircan-Kurt, Uceyler, & Sommer, 2014), retinopathy (Abu El-Asrar et al., 2014; Chen, Curtis, & Stitt, 2013), nephropathy (Zhou, Wang, Zhu, & Hao, 2012), and cardiomyopathy (Bodiga, Eda, & Bodiga, 2014; Ma et al., 2009), reduce quality of life (Rodriguez-Pascual et al., 2011) and often involve macrovascular and microvascular complications (Chawla et al., 2014).

RAGE was originally characterized for its ability to bind advanced glycation end-products (AGEs) and for its role as a prominent feed-forward receptor involved in inflammation (Reynolds et al., 2008). AGEs are derived via the non-enzymatic combination of amino groups and reducing sugars (Nicholl & Bucala, 1998) and hyperglycemia-mediated induction of protein glycosylation is implicated in the production of systemic AGEs (Hodgson et al., 2014; Xue et al., 2014). However, independent of blood glucose, cigarette smoke exposure increases AGE formation through Malliard chemical reactions involving smoke components and plasma proteins (Nicholl et al., 1998). Whether through chronic hyperglycemia or secondhand cigarette smoke exposure, cellular responses provide abundant AGEs for RAGE signaling that causes enhanced expression of pro-inflammatory cytokines and deleterious inflammatory responses (Manigrasso, Juranek, Ramasamy, & Schmidt, 2014). Two AGEs often found in increased abundance with diabetics and smokers are N(ε)-carboxy-methyl-lysine (CML) (Schmitt, Linder, Standker, Hammes, & Preissner, 2008) and N(ε)-carboxy-ethyl-lysine (Xue et al., 2011).

In addition to RAGE, sphingolipids are another factor increasingly recognized to play a role in the progression of inflammatory disorders (Bikman, 2012; Maceyka & Spiegel, 2014). Ceramides, in particular, are a subset of sphingolipid that have been linked to cardiometabolic disruption (Tippetts et al., 2014), altered mitochondrial dynamics and function (Bikman & Summers, 2011; Smith et al., 2013; Summers, 2006), increasing atheroma development (Bismuth, Lin, Yao, & Chen, 2008), and mediating insulin resistance (Thatcher et al., 2014). We have previously shown that ceramides are actively synthesized in the lung with cigarette smoke exposure (Tippetts et al., 2014).

Since activation of inflammatory signaling pathways has been shown to up-regulate ceramide biosynthesis (Bikman, 2012), 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 4, which increases ceramide biosynthesis upon activation (Holland et al., 2011). 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 America Type Cell Culture (ATCC; Manassas, VA) and used at passages 9-12. CML-BSA was purchased from MBL International (Woburn, MA), and the myriocin was obtained from Sigma-Aldrich (St. Louis, MO). The cells were split into 6-well plates and grown to confluency. Myriocin-treated cells were pretreated for 1 hour with 1μL of 10 mM myriocin. Cells were subsequently co-treated with either 30 μL CML-BSA/mL growth media or normal growth media for 24 hours. 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 knock out (KO) mice lacking membrane and soluble RAGE were generated on a C57BL/6 background. Conditional RAGE transgenic mice were also generated that overexpress RAGE in alveolar epithelium when fed doxycycline (dox) (Reynolds, Stogsdill, Stogsdill, & Heimann, 2011; J. A. Stogsdill et al., 2012). Dox incorporation into murine diets caused the up-regulation of RAGE in the lungs of transgenic mice from postnatal

day 20 until sacrifice date at postnatal day 110 (M. P. Stogsdill et al., 2013). The 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 IACUC protocols at Brigham Young University.

Smoke Exposure

For one study, WT and RAGE KO mice were randomly assigned to control- and smoke-

exposure groups and acutely treated using an in-house nose-only smoke exposure system

(InExpose System, Scireq, Canada). Over the course of 1 week, the mice were restrained daily

and connected to an exposure tower for 10 minutes where they were nasally exposed to

secondhand cigarette smoke from 2 cigarettes. Computer-generated puffs resulted in 10 seconds

of secondhand exposure followed by 50 seconds of fresh air. After exposure, the mice were then

allowed to rest for 10 minutes before repeating the process two additional times until they had

been exposed to a total of 6 cigarettes per day. The smoke challenge chosen in the study was

associated with a good tolerance of mice to the smoke sessions, and an acceptable level of

particulate density concentration according to literature (Rinaldi et al., 2012; Wood et al., 2014).

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-

exposure groups and exposed to secondhand smoke. The procedure, however, included exposure

to 4 cigarettes per day for 8 weeks. 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 (Reynolds, Mucenski, Le Cras, Nichols, & Whitsett, 2004; Robinson, Johnson, Bennion, & Reynolds, 2012). The ceramide primary antibody used for immunohistochemical detection was obtained from Sigma Aldrich (C8104-50TST, 1:500). Development in 3,3-diaminobenzidone (DAB) 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 minutes on ice, then briefly vortexed. Aqueous and organic phases were separated by 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 ScientificLTQ Orbitrap XL mass spectrometer, as previously described (Hansen et al., 2014).

Mitochondrial Respiration

Oxygen consumption from H9C2 cardiomyocytes and cardiac muscle obtained from mice was determined using an O2K oxygraph (Oroboros Instruments). Cells (digitonin 1mg/ml) and tissue (saponin 50 µg/ml) were then permeabilized. Following 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). Following stabilization, ADP was added to determine oxidative phosphorylation capacity (P, 2.5 mM). Succinate (S, 10 mM) was then added to assess complex 1 + 2 electron flow. To determine full electron transport system capacity, the chemical uncoupler FCCP (carbonyl cyanide p-trifluoromethoxyphenylhydrazone,

0.05 μM) was added (E). In order to assess complex 2 electron flow, complex 1 was then inhibited by including rotenone (Rot, 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 Superscript III First-Strand Synthesis System in order to obtain cDNA for PCR. Primers for serine palmitoyltransferase 1 and 2 (SPT1, SPT2) and ceramide synthase 2 (CERS2) 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 and comparisons were made with amplified actin. Control wells lacking template or RT were included to identify primer-dimer products and to exclude possible contaminants.

Immunoblotting

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 was blotted using a mouse polyclonal antibody (RnDSystems, Pittsburg, PA, #AF1179) against RAGE (1:1000) as already outlined (Reynolds et al., 2010). 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 per group. Data are presented as the mean ± SEM. 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 serine palmitoyltransferase (SPT), the rate limiting enzyme in *de novo* ceramide biosynthesis. Compared to control cells, gene expression levels of SPT1, SPT2, and CerS2 were significantly elevated in CML-treated cells (Figure 2.1A). Similarly, compared to control conditions, cells exposed to CML-BSA showed a robust increase in ceramide production by mass spectrometry, but not when pretreated with myriocin (CML+MYR; Figure 2.1B). The increase in SPT and ceramides plausibly implicate RAGE as a factor contributing to *de novo* ceramide biosynthesis.

In order 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 addition of glutamate and malate (leak state, GM_L) (Figure 2.2A). Pre-treatment with myriocin, however, abolished the effects of CML-BSA and restored respiration to control levels. Similar results were also found upon stimulation of complex 1-mediated oxidative

phosphorylation with ADP addition (GM_P). 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 (GMS_P less GM_P; Figure 2.2B). 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. Altogether, 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 (Ramasamy & Schmidt, 2012; Wood et al., 2014), we sought to ascertain the expression profile of RAGE in cardiac tissue under both control conditions and following smoke exposure. To establish its expression in heart, WT mice were restrained and exposed to either room air or secondhand cigarette smoke for one or 8 weeks. Following the treatment, we assessed RAGE protein expression in left ventricle myocardium. Compared to control animals, RAGE expression was significantly elevated in animals after one week (not shown) or eight weeks of secondhand cigarette smoke exposure (Figure 2.3). We next analyzed ceramide levels by mass spectrometry in order to assess whether ceramides are also elevated in cardiomyocytes following secondhand smoke exposure. WT mice exposed to acute cigarette smoke showed a significant increase in ceramides (Figure 2.4A); however, this effect was diminished in RAGE KO mice after one week of exposure. In order to qualitatively

visualize these differences, we also performed immunohistochemistry on heart samples for ceramides. WT mice exposed to secondhand cigarette smoke expressed pronounced DAB-mediated staining for ceramides in heart tissue (Figure 2.4B, arrow), but ceramides were qualitatively diminished in exposed RAGE KO mice (Figure 2.4B).

RAGE Signaling Was Necessary For Cigarette Smoke-Mediated Reductions In Myocardial Mitochondrial Respiration

Following exposure to secondhand cigarette smoke, 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 (Figure 2.5A). However, RAGE deletion was protective against secondhand smoke-induced respiration defects (Figure 2.5A). As with cells, we observed reduced complex II function when analyzing the Complex II Control Factor (GMS$_P$ less GM$_P$; Figure 2.5B).

Conditional Pulmonary Up-Regulation Of RAGE Increased Biosynthesis Of Cardiomyocyte Ceramides

The experiments outlined in Figures 3-5 considered the biology of RAGE and ceramide in cardiomyocytes following controlled pulmonary exposure to secondhand smoke. To further assess the role of RAGE in promoting cardiac accumulation of ceramide, studies were designed in the absence of smoke that compare control mice and conditional transgenic mice that genetically over-express RAGE in the peripheral lung. Up-regulation of RAGE in these mouse models has been validated by both immunoblotting and qPCR (J. A. Stogsdill et al., 2012; M. P. Stogsdill et al., 2013). Lipids were purified from heart tissue obtained from RAGE transgenic mice following 90 days of RAGE up-regulation and age-matched non-transgenic controls prior to subjection to mass spectrometry analysis. Compared to the control mice, hearts from

transgenic mice showed a significant increase in ceramides (Figure 2.6), further supporting the notion that RAGE is a sufficient mediator of *de novo* ceramide biosynthesis in cardiac tissue.

Discussion

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 (Ma et al., 2009; Nozynski et al., 2012), 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 non-specific inflammatory response to a host of heterogeneous compounds, but this process can become pathological with sustained activation and chronic inflammation (Nedic, Rattan, Grune, & Trougakos, 2013). Although the scope of our paper is limited to the effects of RAGE and ceramides on cardiomyocyte mitochondrial function, both are implicated in a host of diabetic and smoke-induced cardiovascular complications. However, AGEs inhaled by smokers may be chemically and functionally different when compared to *de novo* AGE synthesis in uncontrolled diabetics. Ma et al. (Ma et al., 2009) revealed that AGE-RAGE ablation prevents diabetes-induced altered cardiovascular function, including cardiomyopathy. Moreover, Park et al. (Park, Rosebury, Kindt, Kowala, & Panek, 2008) 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 (Duncan, 2011). This is relevant given our previous findings that ceramides directly disrupt mitochondrial physiology, reducing respiration and increasing oxidative stress (Tippetts et al., 2014), through a mitochondrial fission-dependent process (Smith et al., 2013). We 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 the control- and myriocin-treated cells. Our demonstration that AGEs induce ceramide-dependent impairment in mitochondrial oxygen 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 heart stems from two observations: 1) as noted earlier, RAGE mediates cardiovascular complications (Ma et al., 2009); and 2) RAGE shares common signaling intermediates in pathways known to activate ceramide biosynthesis, namely Toll-like receptor 4 (TLR4). We reported in 2011 that TLR4 is required for lipid- and endotoxin-induced ceramide biosynthesis (Holland et al., 2011). Interestingly, not only do TLR4 and RAGE share common downstream signaling (e.g., IRAK) (Sakaguchi et al., 2011), but also common ligand activators (e.g., HMGB1) (Ding et al., 2013; Sims, Rowe, Rietdijk, Herbst, & Coyle, 2010). 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 (Reynolds, Kasteler, Schmitt, & Hoidal,

2011; Robinson, Stogsdill, Lewis, Wood, & Reynolds, 2012), 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. (Denis et al., 2002) were the first to find a ceramide-RAGE connection when they noted a roughly twofold 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 TG 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 (Talukder et al., 2011; Tonstad & Svendsen, 2005), our results provide a possible mechanism whereby smoke exposure leads to cardiovascular complications. Ceramide has been shown to mediate cardiomyopathy (Park, Hu, et al., 2008), atherosclerosis (Bismuth et al., 2008), and inhibit NO-induced vasodilation (Zhang et al., 2012). 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 myocardium on normal mitochondrial function, these results provide evidence for the utility of anti-ceramide therapies in the treatment or prevention of multiple cardiovascular complications.
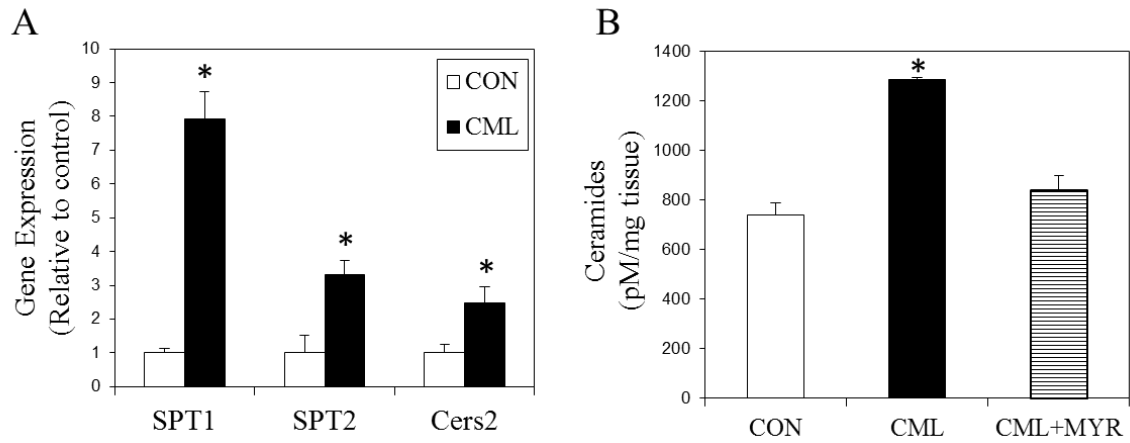
Figure 2.1: RAGE Signaling Increases De Novo Ceramide Biosynthesis In Cardiomyocytes. Cardiac myocytes (H9C2) were treated for 24 h with either normal growth media (CON) or growth media infused with carboxy-methyl lysine BSA (CML; 30 μL/ml growth media), with and without myriocin (Myr; 10 μM). Following the treatment period, expression levels of SPT1, SPT2, and CerS2 were quantified (Figure 2.1A, n=3), in addition to ceramides (Figure 2.1B, n=3). *, $p < 0.05$ for CML vs. other treatments.
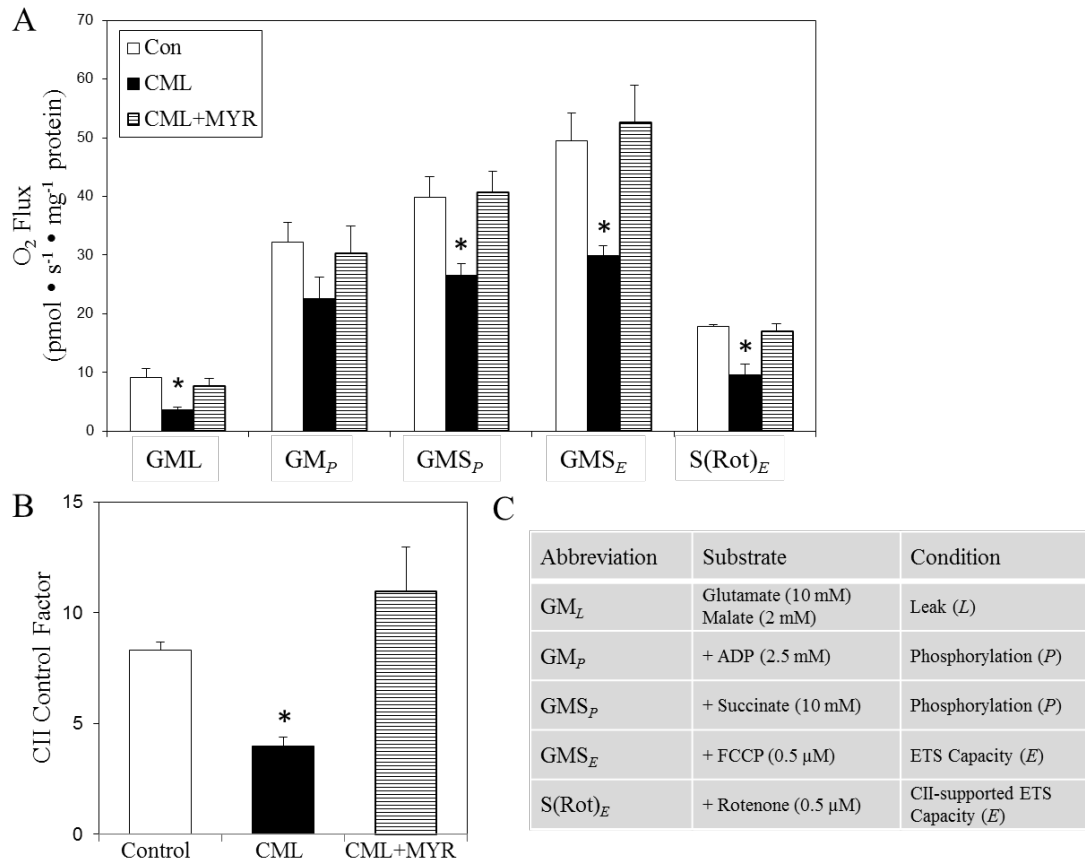
Figure 2.2: RAGE Signaling Reduces Cardiomyocyte Mitochondrial Respiration Through Ceramide Accrual. Cardiac myocytes (H9C2, n=3) were treated for 24 h with either normal growth media or growth media containing CML-BSA with and without myriocin (Con, CML, CML+Myr). Following treatment, heart cell mitochondrial oxygen consumption was assessed using a substrate-uncoupler-inhibitor titration protocol (Figure 2.2A; See Figure 2.2C for description). Complex II Control Factor was used to determine the specific effect on succinate addition on respiration (Figure 2.2B). *, $p < 0.05$ for CML vs. other treatments.
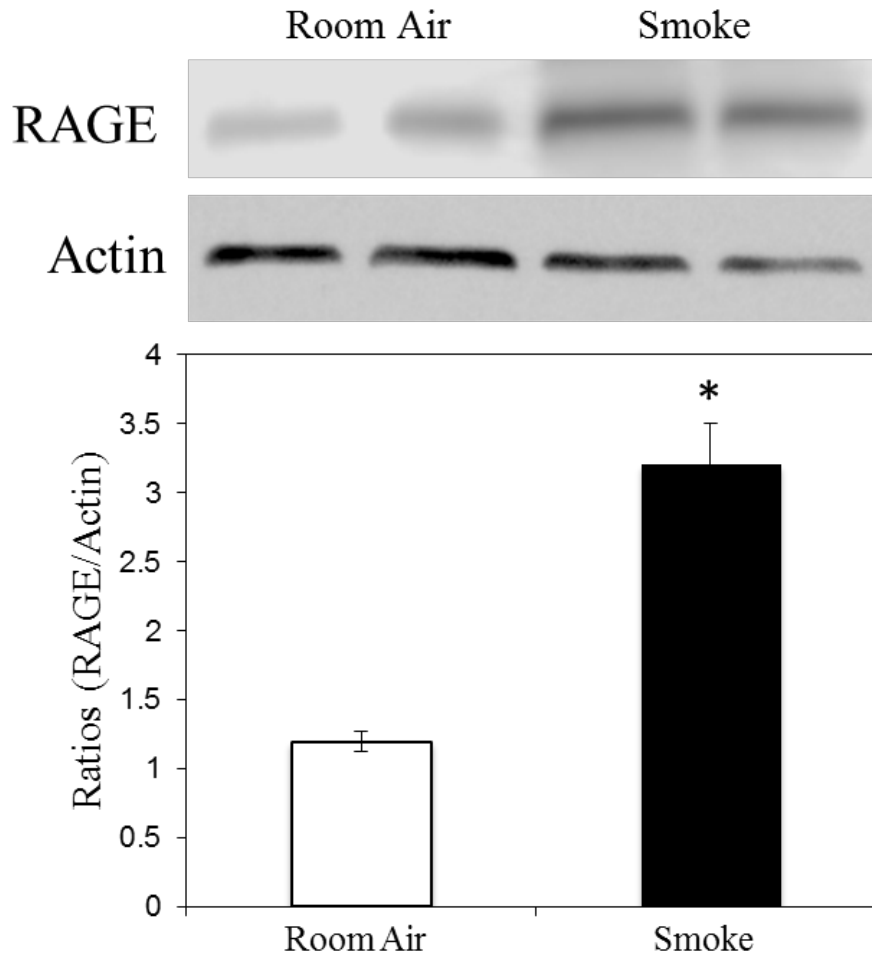
Figure 2.3: Cigarette Smoke Increases RAGE Expression In Heart With Secondhand Cigarette Smoke Exposure. WT mice were restrained and exposed to either room air (n=3) or secondhand cigarette smoke (n=3, see materials and methods) for 8 wk. Following the exposure period, western blot (Figure 2.3A) and quantification (Figure 2.3B) was performed on heart tissue lysates to determine relative expression levels of RAGE. *, $p < 0.05$ for smoke vs. restrain.
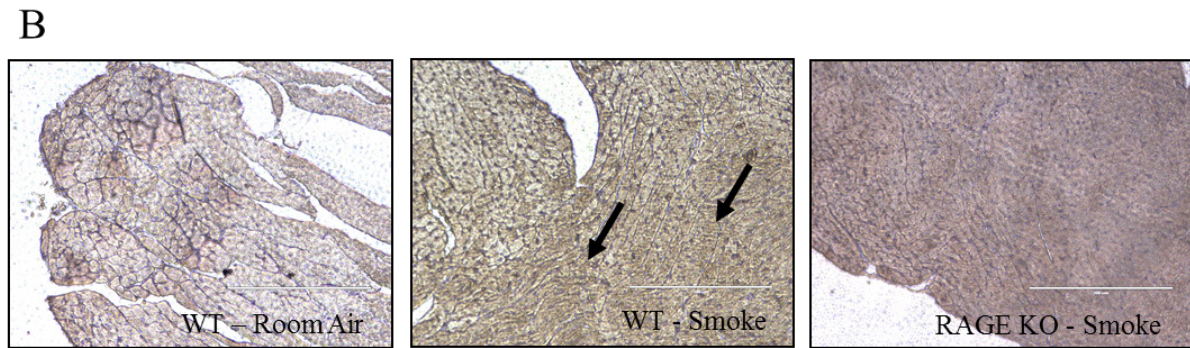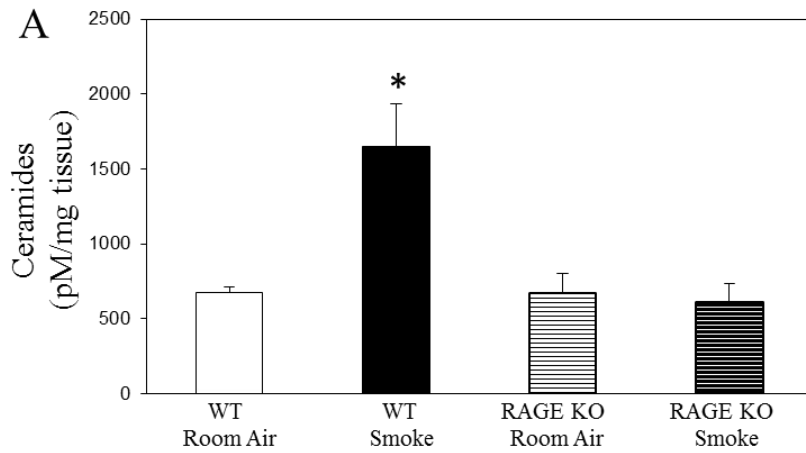
Figure 2.4: RAGE KO Prevents Heart Ceramide Accrual With Cigarette Smoke. WT and RAGE KO mice were restrained and exposed to either room air or acute secondhand cigarette smoke for 1 wk. Following the exposure period, lipids were isolated from heart tissue and analyzed for ceramides (Figure 2.4A). Immunohistochemical staining for ceramide was also performed to qualitatively depict any differences in heart tissue between WT and RAGE KO mice under the different exposure conditions (Figure 2.4B). ceramides were significantly increased in WT mice exposed to tobacco smoke (arrow) compared to exposed RAGE KO mice. Scale bars represent 200 nm and *, $p < 0.05$ for smoke vs. restrain within each group.
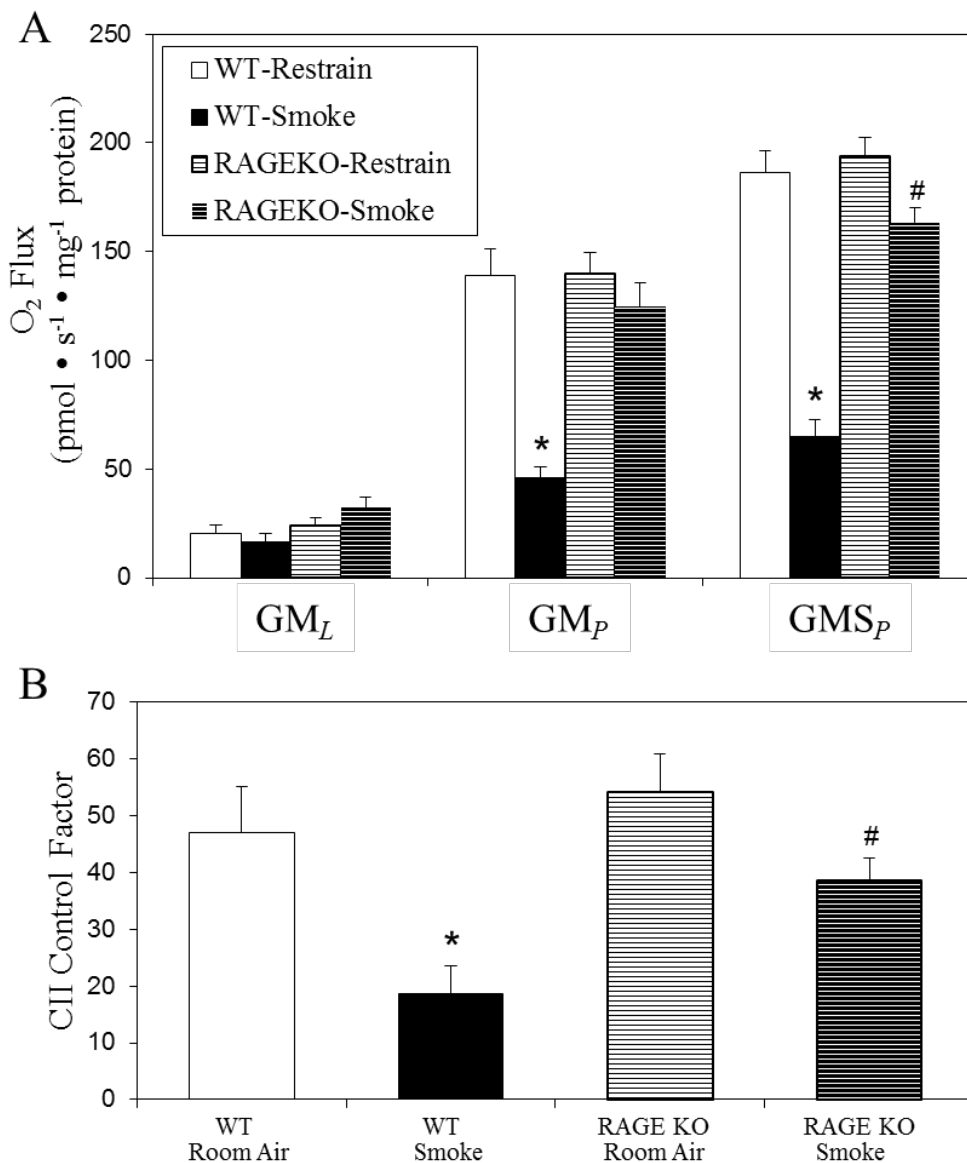
Figure 2.5: RAGE KO Prevents Cigarette Smoke-Induced Reduced Myocardial Mitochondrial Respiration. WT and RAGE KO mice were restrained and exposed to either room air or acute secondhand cigarette smoke for one wk. Following the exposure period, mitochondrial oxygen consumption of cardiac myocytes was assessed (Figure 2.5A). Complex II Control Factor was used to determine the specific effect on succinate addition on respiration (Figure 2.5B).*, $p < 0.05$ for smoke vs. other treatments. #, $p < 0.05$ for RAGEKO-Smoke vs. RAGEKO-Restrain.
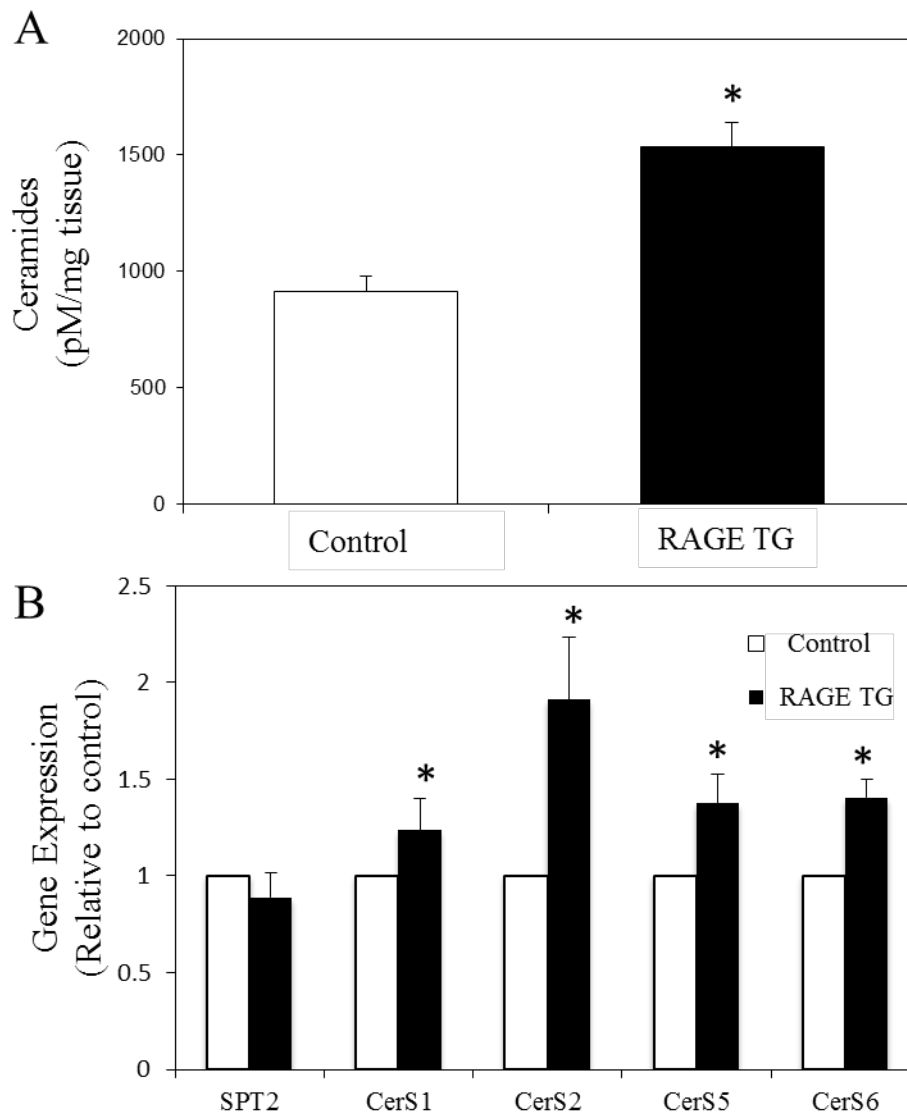
Figure 2.6: Conditional Up-Regulation of Pulmonary RAGE Expression Increases Heart Ceramides. Non-transgenic controls (Con) and Transgenic (RAGE TG) mice that overexpress RAGE were sacrificed and assessed for ceramide levels in cardiac tissue. *, $p < 0.05$ for RAGE TG vs WT.

References

Abu El-Asrar, A. M., Nawaz, M. I., De Hertogh, G., Alam, K., Siddiquei, M. M., Van den Eynde, K., . . . Opdenakker, G. (2014). S100A4 is upregulated in proliferative diabetic retinopathy and correlates with markers of angiogenesis and fibrogenesis. *Mol Vis, 20*, 1209-1224.

Bekircan-Kurt, C. E., Uceyler, N., & Sommer, C. (2014). Cutaneous activation of rage in nonsystemic vasculitic and diabetic neuropathy. *Muscle Nerve, 50*(3), 377-383. doi: 10.1002/mus.24164

Bikman, B. T. (2012). A role for sphingolipids in the pathophysiology of obesity-induced inflammation. *Cell Mol Life Sci, 69*(13), 2135-2146. doi: 10.1007/s00018-012-0917-5

Bikman, B. T., & Summers, S. A. (2011). Ceramides as modulators of cellular and whole-body metabolism. *J Clin Invest, 121*(11), 4222-4230. doi: 10.1172/jci57144

Bismuth, J., Lin, P., Yao, Q., & Chen, C. (2008). Ceramide: a common pathway for atherosclerosis? *Atherosclerosis, 196*(2), 497-504. doi: 10.1016/j.atherosclerosis.2007.09.018

Bodiga, V. L., Eda, S. R., & Bodiga, S. (2014). Advanced glycation end products: role in pathology of diabetic cardiomyopathy. *Heart Fail Rev, 19*(1), 49-63. doi: 10.1007/s10741-013-9374-y

Chawla, D., Bansal, S., Banerjee, B. D., Madhu, S. V., Kalra, O. P., & Tripathi, A. K. (2014). Role of advanced glycation end product (AGE)-induced receptor (RAGE) expression in diabetic vascular complications. *Microvasc Res, 95*, 1-6. doi: 10.1016/j.mvr.2014.06.010

Chen, M., Curtis, T. M., & Stitt, A. W. (2013). Advanced glycation end products and diabetic retinopathy. *Curr Med Chem, 20*(26), 3234-3240.

Denis, U., Lecomte, M., Paget, C., Ruggiero, D., Wiernsperger, N., & Lagarde, M. (2002). Advanced glycation end-products induce apoptosis of bovine retinal pericytes in culture: involvement of diacylglycerol/ceramide production and oxidative stress induction. *Free Radic Biol Med, 33*(2), 236-247.

Ding, H. S., Yang, J., Chen, P., Yang, J., Bo, S. Q., Ding, J. W., & Yu, Q. Q. (2013). The HMGB1-TLR4 axis contributes to myocardial ischemia/reperfusion injury via regulation of cardiomyocyte apoptosis. *Gene, 527*(1), 389-393. doi: 10.1016/j.gene.2013.05.041

Duncan, J. G. (2011). Mitochondrial dysfunction in diabetic cardiomyopathy. *Biochim Biophys Acta, 1813*(7), 1351-1359. doi: 10.1016/j.bbamcr.2011.01.014

Hansen, M. E., Tippetts, T. S., Anderson, M. C., Holub, Z. E., Moulton, E. R., Swensen, A. C., . . . Bikman, B. T. (2014). Insulin increases ceramide synthesis in skeletal muscle. *J Diabetes Res, 2014*, 765784. doi: 10.1155/2014/765784

Hodgson, K., Morris, J., Bridson, T., Govan, B., Rush, C., & Ketheesan, N. (2014). Immunological mechanisms contributing to the double burden of diabetes and intracellular bacterial infections. *Immunology*. doi: 10.1111/imm.12394

Holland, W. L., Bikman, B. T., Wang, L. P., Yuguang, G., Sargent, K. M., Bulchand, S., . . . Summers, S. A. (2011). Lipid-induced insulin resistance mediated by the proinflammatory receptor TLR4 requires saturated fatty acid-induced ceramide biosynthesis in mice. *J Clin Invest, 121*(5), 1858-1870. doi: 10.1172/jci43378

Ma, H., Li, S. Y., Xu, P., Babcock, S. A., Dolence, E. K., Brownlee, M., . . . Ren, J. (2009). Advanced glycation endproduct (AGE) accumulation and AGE receptor (RAGE) up-regulation contribute to the onset of diabetic cardiomyopathy. *J Cell Mol Med, 13*(8b), 1751-1764. doi: 10.1111/j.1582-4934.2008.00547.x

Maceyka, M., & Spiegel, S. (2014). Sphingolipid metabolites in inflammatory disease. *Nature, 510*(7503), 58-67. doi: 10.1038/nature13475

Manigrasso, M. B., Juranek, J., Ramasamy, R., & Schmidt, A. M. (2014). Unlocking the biology of RAGE in diabetic microvascular complications. *Trends Endocrinol Metab, 25*(1), 15-22. doi: 10.1016/j.tem.2013.08.002

Nedic, O., Rattan, S. I., Grune, T., & Trougakos, I. P. (2013). Molecular effects of advanced glycation end products on cell signalling pathways, ageing and pathophysiology. *Free Radic Res, 47 Suppl 1*, 28-38. doi: 10.3109/10715762.2013.806798

Nicholl, I. D., & Bucala, R. (1998). Advanced glycation endproducts and cigarette smoking. *Cell Mol Biol (Noisy-le-grand), 44*(7), 1025-1033.

Nicholl, I. D., Stitt, A. W., Moore, J. E., Ritchie, A. J., Archer, D. B., & Bucala, R. (1998). Increased levels of advanced glycation endproducts in the lenses and blood vessels of cigarette smokers. *Mol Med, 4*(9), 594-601.

Nozynski, J., Zakliczynski, M., Konecka-Mrowka, D., Zielinska, T., Zakliczynska, H., Nikiel, B., . . . Zembala, M. (2012). Advanced glycation end product accumulation in the cardiomyocytes of heart failure patients with and without diabetes. *Ann Transplant, 17*(2), 53-61.

Park, T. S., Hu, Y., Noh, H. L., Drosatos, K., Okajima, K., Buchanan, J., . . . Goldberg, I. J. (2008). Ceramide is a cardiotoxin in lipotoxic cardiomyopathy. *J Lipid Res, 49*(10), 2101-2112. doi: 10.1194/jlr.M800147-JLR200

Park, T. S., Rosebury, W., Kindt, E. K., Kowala, M. C., & Panek, R. L. (2008). Serine palmitoyltransferase inhibitor myriocin induces the regression of atherosclerotic plaques in hyperlipidemic ApoE-deficient mice. *Pharmacol Res, 58*(1), 45-51. doi: 10.1016/j.phrs.2008.06.005

Ramasamy, R., & Schmidt, A. M. (2012). Receptor for advanced glycation end products (RAGE) and implications for the pathophysiology of heart failure. *Curr Heart Fail Rep, 9*(2), 107-116. doi: 10.1007/s11897-012-0089-5

Reynolds, P. R., Kasteler, S. D., Cosio, M. G., Sturrock, A., Huecksteadt, T., & Hoidal, J. R. (2008). RAGE: developmental expression and positive feedback regulation by Egr-1 during cigarette smoke exposure in pulmonary epithelial cells. *Am J Physiol Lung Cell Mol Physiol, 294*(6), L1094-1101. doi: 10.1152/ajplung.00318.2007

Reynolds, P. R., Kasteler, S. D., Schmitt, R. E., & Hoidal, J. R. (2011). Receptor for advanced glycation end-products signals through Ras during tobacco smoke-induced pulmonary inflammation. *Am J Respir Cell Mol Biol, 45*(2), 411-418. doi: 10.1165/rcmb.2010-0231OC

Reynolds, P. R., Mucenski, M. L., Le Cras, T. D., Nichols, W. C., & Whitsett, J. A. (2004). Midkine is regulated by hypoxia and causes pulmonary vascular remodeling. *J Biol Chem, 279*(35), 37124-37132. doi: 10.1074/jbc.M405254200

Reynolds, P. R., Schmitt, R. E., Kasteler, S. D., Sturrock, A., Sanders, K., Bierhaus, A., . . . Hoidal, J. R. (2010). Receptors for advanced glycation end-products targeting protect against hyperoxia-induced lung injury in mice. *Am J Respir Cell Mol Biol, 42*(5), 545-551. doi: 10.1165/rcmb.2008-0265OC

Reynolds, P. R., Stogsdill, J. A., Stogsdill, M. P., & Heimann, N. B. (2011). Up-regulation of receptors for advanced glycation end-products by alveolar epithelium influences cytodifferentiation and causes severe lung hypoplasia. *Am J Respir Cell Mol Biol, 45*(6), 1195-1202. doi: 10.1165/rcmb.2011-0170OC

Rinaldi, M., Maes, K., De Vleeschauwer, S., Thomas, D., Verbeken, E. K., Decramer, M., . . . Gayan-Ramirez, G. N. (2012). Long-term nose-only cigarette smoke exposure induces emphysema and mild skeletal muscle dysfunction in mice. *Dis Model Mech, 5*(3), 333-341. doi: 10.1242/dmm.008508

Robinson, A. B., Johnson, K. D., Bennion, B. G., & Reynolds, P. R. (2012). RAGE signaling by alveolar macrophages influences tobacco smoke-induced inflammation. *Am J Physiol Lung Cell Mol Physiol, 302*(11), L1192-1199. doi: 10.1152/ajplung.00099.2012

Robinson, A. B., Stogsdill, J. A., Lewis, J. B., Wood, T. T., & Reynolds, P. R. (2012). RAGE and tobacco smoke: insights into modeling chronic obstructive pulmonary disease. *Front Physiol, 3*, 301. doi: 10.3389/fphys.2012.00301

Rodriguez-Pascual, C., Rodriguez-Justo, S., Garcia-Villar, E., Narro-Vidal, M., Torrente-Carballido, M., & Paredes-Galan, E. (2011). Quality of life, characteristics and metabolic control in diabetic geriatric patients. *Maturitas, 69*(4), 343-347. doi: 10.1016/j.maturitas.2011.05.001

Sakaguchi, M., Murata, H., Yamamoto, K., Ono, T., Sakaguchi, Y., Motoyama, A., . . . Huh, N. H. (2011). TIRAP, an adaptor protein for TLR2/4, transduces a signal from RAGE phosphorylated upon ligand binding. *PLoS One, 6*(8), e23132. doi: 10.1371/journal.pone.0023132

Schmitt, S., Linder, M., Standker, L., Hammes, H. P., & Preissner, K. T. (2008). Identification of CML-modified proteins in hemofiltrate of diabetic patients by proteome analysis. *Exp Clin Endocrinol Diabetes, 116*(1), 26-34. doi: 10.1055/s-2007-985370

Sims, G. P., Rowe, D. C., Rietdijk, S. T., Herbst, R., & Coyle, A. J. (2010). HMGB1 and RAGE in inflammation and cancer. *Annu Rev Immunol, 28*, 367-388. doi: 10.1146/annurev.immunol.021908.132603

Smith, M. E., Tippetts, T. S., Brassfield, E. S., Tucker, B. J., Ockey, A., Swensen, A. C., . . . Bikman, B. T. (2013). Mitochondrial fission mediates ceramide-induced metabolic disruption in skeletal muscle. *Biochem J*. doi: 10.1042/bj20130807

Stogsdill, J. A., Stogsdill, M. P., Porter, J. L., Hancock, J. M., Robinson, A. B., & Reynolds, P. R. (2012). Embryonic overexpression of receptors for advanced glycation end-products by alveolar epithelium induces an imbalance between proliferation and apoptosis. *Am J Respir Cell Mol Biol, 47*(1), 60-66. doi: 10.1165/rcmb.2011-0385OC

Stogsdill, M. P., Stogsdill, J. A., Bodine, B. G., Fredrickson, A. C., Sefcik, T. L., Wood, T. T., . . . Reynolds, P. R. (2013). Conditional overexpression of receptors for advanced glycation end-products in the adult murine lung causes airspace enlargement and induces inflammation. *Am J Respir Cell Mol Biol, 49*(1), 128-134. doi: 10.1165/rcmb.2013-0013OC

Summers, S. A. (2006). Ceramides in insulin resistance and lipotoxicity. *Prog Lipid Res, 45*(1), 42-72. doi: 10.1016/j.plipres.2005.11.002

Talukder, M. A., Johnson, W. M., Varadharaj, S., Lian, J., Kearns, P. N., El-Mahdy, M. A., . . . Zweier, J. L. (2011). Chronic cigarette smoking causes hypertension, increased oxidative stress, impaired NO bioavailability, endothelial dysfunction, and cardiac remodeling in mice. *Am J Physiol Heart Circ Physiol, 300*(1), H388-396. doi: 10.1152/ajpheart.00868.2010

Thatcher, M. O., Tippetts, T. S., Nelson, M. B., Swensen, A. C., Winden, D. R., Hansen, M. E., . . . Bikman, B. T. (2014). Ceramides mediate cigarette smoke-induced metabolic disruption in mice. *Am J Physiol Endocrinol Metab*, ajpendo.00258.02014. doi: 10.1152/ajpendo.00258.2014

Tippetts, T. S., Winden, D. R., Swensen, A. C., Nelson, M. B., Thatcher, M. O., Saito, R. R., . . . Bikman, B. T. (2014). Cigarette smoke increases cardiomyocyte ceramide accumulation and inhibits mitochondrial respiration. *BMC Cardiovasc Disord, 14*, 165. doi: 10.1186/1471-2261-14-165

Tonstad, S., & Svendsen, M. (2005). Premature coronary heart disease, cigarette smoking, and the metabolic syndrome. *Am J Cardiol, 96*(12), 1681-1685. doi: 10.1016/j.amjcard.2005.07.083

Wood, T. T., Winden, D. R., Marlor, D. R., Wright, A. J., Jones, C. M., Chavarria, M., . . . Reynolds, P. R. (2014). Acute Secondhand Smoke-Induced Pulmonary Inflammation is Diminished in RAGE Knock out Mice. *Am J Physiol Lung Cell Mol Physiol*. doi: 10.1152/ajplung.00185.2014

Xue, J., Rai, V., Singer, D., Chabierski, S., Xie, J., Reverdatto, S., . . . Shekhtman, A. (2011). Advanced glycation end product recognition by the receptor for AGEs. *Structure, 19*(5), 722-732. doi: 10.1016/j.str.2011.02.013

Xue, J., Ray, R., Singer, D., Bohme, D., Burz, D. S., Rai, V., . . . Shekhtman, A. (2014). The receptor for advanced glycation end products (RAGE) specifically recognizes methylglyoxal-derived AGEs. *Biochemistry, 53*(20), 3327-3335. doi: 10.1021/bi500046t

Zhang, Q. J., Holland, W. L., Wilson, L., Tanner, J. M., Kearns, D., Cahoon, J. M., . . . Symons, J. D. (2012). Ceramide mediates vascular dysfunction in diet-induced obesity by PP2A-mediated dephosphorylation of the eNOS-Akt complex. *Diabetes, 61*(7), 1848-1859. doi: 10.2337/db11-1399

Zhou, X., Wang, B., Zhu, L., & Hao, S. (2012). A novel improved therapy strategy for diabetic nephropathy: targeting AGEs. *Organogenesis, 8*(1), 18-21. doi: 10.4161/org.19332

CHAPTER 3: General Discussion And Future Directions

Our data from the previous chapter reinforce other studies implicating RAGE and ceramide in cardiovascular disease. RAGE signaling contributes to cardiomyopathy (Park et al., 2008), coronary heart disease (Ligthart et al., 2014), atherosclerosis (Johnson et al., 2014), and even elicits endothelial dysfunction (Wu et al., 2013). This work, however, suggests that cardiac lipotoxicity and excessive accumulation of ceramides mediate, at least in part, the effects of RAGE in diabetic- and smoke-induced cardiovascular disease. Our rationale for looking at RAGE as a mediator of ceramide lipotoxicity stems from work conducted in our lab, as well as other studies that looked at TLR4. TLR4 mediates lipid- and endotoxin-induced ceramide biosynthesis and shares many signaling pathways and ligand activators with RAGE (Schilling et al., 2013).

The first point presented in our research is that advanced glycation end-products reduce cardiomyocyte mitochondrial function in a ceramide-dependent manner. AGEs, including proteins with N (ε)-(carboxymethyl) lysine adducts, are important in a number of diabetic- and smoke-induced pathologies (Schmitt, Linder, Standker, Hammes, & Preissner, 2008) and have been previously shown to induce oxidative stress (Wang et al., 2014) and impair mitochondrial function in cardiac myocytes (L. Zhang et al., 2014). Our rationale for exposing cardiac myocytes to CML-BSA and mice to cigarette smoke stems from evidence that various factors including diet (Poulsen et al., 2013), cigarette smoke (Cerami et al., 1997), and chronic hyperglycemia increase serum levels of advanced glycation end-products (Gaens, Stehouwer, & Schalkwijk, 2013).

The finding that RAGE signaling in lung contributed to cigarette smoke-induced ceramide accrual in cardiomyocytes is novel and has broad implications for future studies

37

looking at the interactions between heart and lung. Our lab previously showed that cigarette smoke increases cardiomyocyte ceramide accumulation (Tippetts et al., 2014); however, the data presented here take it one step further by offering a mechanism to account for the increase in cardiac ceramides. While other factors certainly may contribute to cardiac lipotoxicity (van de Weijer, Schrauwen-Hinderling, & Schrauwen, 2011), the RAGE-ceramide axis is a novel area of research that merits further investigation.

Furthermore, our data showed that RAGE signaling was necessary for cigarette smoke-mediated reductions in myocardial mitochondrial respiration. A robust increase in cardiac ceramides was observed in wild type mice, but the RAGE KO mice were protected from smoke-induced reduced respiration. Cardiomyocytes, in particular, are dependent on efficient mitochondrial metabolism (Wohlgemuth, Calvani, & Marzetti, 2014) and healthy turnover by carefully regulated mitochondrial fission-fusion events. Previous research conducted by Zhu et al. suggested that AGE-RAGE interactions may interfere with proper mitochondrial dynamics leading to increased apoptosis (Zhu et al., 2011). Several studies have even reported that inhibition of mitochondrial fission attenuates the progression of cardiovascular disease (Kubli & Gustafsson, 2012; Ong et al., 2010). Likewise, ceramides have also been reported to reduce contractility (Simon et al., 2014) and stimulate apoptosis by stimulating cytochrome-c release from mitochondria (Parra et al., 2013).

Although the work here suggests a novel function for RAGE, further studies are needed to clarify signaling pathways that interconnect RAGE and ceramide. Our work demonstrated that RAGE signaling increased expression of several enzymes involved in ceramide biosynthesis; however, it's unclear which signaling pathways RAGE is acting through and whether it also results in increased activation of the *de novo* enzymes regulating ceramide synthesis. RAGE and

TLR4 both signal through the canonical NF-kβ pathway to increase expression of inflammatory cytokines (Ibrahim, Armour, Phipps, & Sukkar, 2013; Reynolds, Kasteler, Schmitt, & Hoidal, 2011) but whether the NF-kβ pathway is involved has yet to be determined. Furthermore, it would be insightful to perform studies evaluating heart and lung interactions in the context of environmental issues, including inversion, to see whether cardiac myocyte respiration is hindered in response to exposure to particulate matter in the community.

This research also has broad implications for potential cardiovascular therapies. Importantly, it suggests that anti-RAGE and anti-ceramide approaches may alleviate cardiovascular symptoms in individuals with diabetes and chronic cigarette smoke exposure. Previous studies have shown that RAGE expression is attenuated by pharmacological treatment with a wide array of drugs including metformin (T. Zhang, Hu, Cai, Yi, & Wen, 2014), calcitriol (Lee et al., 2014), atorvastatin (Feng et al., 2011), and pioglitazone. Aside from targeting of RAGE, however, emphasis should also be placed on managing serum AGE levels and finding effective ways to reduce their interaction with RAGE. Vulesevic et al. recently suggested pharmacological targeting of methylglyoxal as a means of alleviating cardiovascular disease (Vulesevic, Milne, & Suuronen, 2014). Fukami et al. also suggested that administration of soluble RAGE (sRAGE) may reduce levels of inflammation and serve a protective function (Fukami, Yamagishi, & Okuda, 2014). Regardless of the method, however, a common thread among many of these authors is that blockade of AGE/RAGE interaction and signal transduction reduces oxidative stress and attenuates myocardial injury (Daffu et al., 2013).

Finally, there is also the potential for anti-ceramide therapies in the treatment of cardiovascular disease. Indeed, numerous studies have shown that inhibition of ceramide synthesis improves vascular function (Q. J. Zhang et al., 2012) and alleviates both

atherosclerosis (Chun et al., 2011) and cardiomyopathy (Park et al., 2008). Despite the beneficial

effects of ceramide inhibitors such as myriocin and fumonisin B1 in these models of

cardiovascular disease (Park et al., 2008), however, there remains a need to discover a ceramide

blocker that is safe and effective in humans.

References

Cerami, C., Founds, H., Nicholl, I., Mitsuhashi, T., Giordano, D., Vanpatten, S., . . . Cerami, A. (1997). Tobacco smoke is a source of toxic reactive glycation products. *Proc Natl Acad Sci U S A, 94*(25), 13915-13920.

Chun, L., Junlin, Z., Aimin, W., Niansheng, L., Benmei, C., & Minxiang, L. (2011). Inhibition of ceramide synthesis reverses endothelial dysfunction and atherosclerosis in streptozotocin-induced diabetic rats. *Diabetes Res Clin Pract, 93*(1), 77-85. doi: 10.1016/j.diabres.2011.03.017

Daffu, G., del Pozo, C. H., O'Shea, K. M., Ananthakrishnan, R., Ramasamy, R., & Schmidt, A. M. (2013). Radical roles for RAGE in the pathogenesis of oxidative stress in cardiovascular diseases and beyond. *Int J Mol Sci, 14*(10), 19891-19910. doi: 10.3390/ijms141019891

Feng, B., Xu, L., Wang, H., Yan, X., Xue, J., Liu, F., & Hu, J. F. (2011). Atorvastatin exerts its anti-atherosclerotic effects by targeting the receptor for advanced glycation end products. *Biochim Biophys Acta, 1812*(9), 1130-1137. doi: 10.1016/j.bbadis.2011.05.007

Fukami, K., Yamagishi, S., & Okuda, S. (2014). Role of AGEs-RAGE system in cardiovascular disease. *Curr Pharm Des, 20*(14), 2395-2402.

Gaens, K. H., Stehouwer, C. D., & Schalkwijk, C. G. (2013). Advanced glycation endproducts and its receptor for advanced glycation endproducts in obesity. *Curr Opin Lipidol, 24*(1), 4-11. doi: 10.1097/MOL.0b013e32835aea13

Ibrahim, Z. A., Armour, C. L., Phipps, S., & Sukkar, M. B. (2013). RAGE and TLRs: relatives, friends or neighbours? *Mol Immunol, 56*(4), 739-744. doi: 10.1016/j.molimm.2013.07.008

Johnson, L. L., Tekabe, Y., Kollaros, M., Eng, G., Bhatia, K., Li, C., . . . Schmidt, A. M. (2014). Imaging RAGE expression in atherosclerotic plaques in hyperlipidemic pigs. *EJNMMI Res, 4*, 26. doi: 10.1186/s13550-014-0026-6

Kubli, D. A., & Gustafsson, A. B. (2012). Mitochondria and mitophagy: the yin and yang of cell death control. *Circ Res, 111*(9), 1208-1221. doi: 10.1161/circresaha.112.265819

Lee, T. W., Kao, Y. H., Lee, T. I., Chang, C. J., Lien, G. S., & Chen, Y. J. (2014). Calcitriol modulates receptor for advanced glycation end products (RAGE) in diabetic hearts. *Int J Cardiol, 173*(2), 236-241. doi: 10.1016/j.ijcard.2014.02.041

Ligthart, S., Sedaghat, S., Ikram, M. A., Hofman, A., Franco, O. H., & Dehghan, A. (2014). EN-RAGE: a novel inflammatory marker for incident coronary heart disease. *Arterioscler Thromb Vasc Biol, 34*(12), 2695-2699. doi: 10.1161/atvbaha.114.304306

Ong, S. B., Subrayan, S., Lim, S. Y., Yellon, D. M., Davidson, S. M., & Hausenloy, D. J. (2010). Inhibiting mitochondrial fission protects the heart against ischemia/reperfusion injury. *Circulation, 121*(18), 2012-2022. doi: 10.1161/circulationaha.109.906610

Park, T. S., Hu, Y., Noh, H. L., Drosatos, K., Okajima, K., Buchanan, J., . . . Goldberg, I. J. (2008). Ceramide is a cardiotoxin in lipotoxic cardiomyopathy. *J Lipid Res, 49*(10), 2101-2112. doi: 10.1194/jlr.M800147-JLR200

Parra, V., Moraga, F., Kuzmicic, J., Lopez-Crisosto, C., Troncoso, R., Torrealba, N., . . . Lavandero, S. (2013). Calcium and mitochondrial metabolism in ceramide-induced cardiomyocyte death. *Biochim Biophys Acta, 1832*(8), 1334-1344. doi: 10.1016/j.bbadis.2013.04.009

Poulsen, M. W., Hedegaard, R. V., Andersen, J. M., de Courten, B., Bugel, S., Nielsen, J., . . . Dragsted, L. O. (2013). Advanced glycation endproducts in food and their effects on health. *Food Chem Toxicol, 60*, 10-37. doi: 10.1016/j.fct.2013.06.052

Reynolds, P. R., Kasteler, S. D., Schmitt, R. E., & Hoidal, J. R. (2011). Receptor for advanced glycation end-products signals through Ras during tobacco smoke-induced pulmonary inflammation. *Am J Respir Cell Mol Biol, 45*(2), 411-418. doi: 10.1165/rcmb.2010-0231OC

Schilling, J. D., Machkovech, H. M., He, L., Sidhu, R., Fujiwara, H., Weber, K., . . . Schaffer, J. E. (2013). Palmitate and lipopolysaccharide trigger synergistic ceramide production in primary macrophages. *J Biol Chem, 288*(5), 2923-2932. doi: 10.1074/jbc.M112.419978

Schmitt, S., Linder, M., Standker, L., Hammes, H. P., & Preissner, K. T. (2008). Identification of CML-modified proteins in hemofiltrate of diabetic patients by proteome analysis. *Exp Clin Endocrinol Diabetes, 116*(1), 26-34. doi: 10.1055/s-2007-985370

Simon, J. N., Chowdhury, S. A., Warren, C. M., Sadayappan, S., Wieczorek, D. F., Solaro, R. J., & Wolska, B. M. (2014). Ceramide-mediated depression in cardiomyocyte contractility through PKC activation and modulation of myofilament protein phosphorylation. *Basic Res Cardiol, 109*(6), 445. doi: 10.1007/s00395-014-0445-6

Tippetts, T. S., Winden, D. R., Swensen, A. C., Nelson, M. B., Thatcher, M. O., Saito, R. R., . . . Bikman, B. T. (2014). Cigarette smoke increases cardiomyocyte ceramide accumulation and inhibits mitochondrial respiration. *BMC Cardiovasc Disord, 14*, 165. doi: 10.1186/1471-2261-14-165

van de Weijer, T., Schrauwen-Hinderling, V. B., & Schrauwen, P. (2011). Lipotoxicity in type 2 diabetic cardiomyopathy. *Cardiovasc Res, 92*(1), 10-18. doi: 10.1093/cvr/cvr212

Vulesevic, B., Milne, R. W., & Suuronen, E. J. (2014). Reducing methylglyoxal as a therapeutic target for diabetic heart disease. *Biochem Soc Trans, 42*(2), 523-527. doi: 10.1042/bst20130254

Wang, X., Yu, S., Wang, C. Y., Wang, Y., Liu, H. X., Cui, Y., & Zhang, L. D. (2014). Advanced glycation end products induce oxidative stress and mitochondrial dysfunction in SH-SY5Y cells. *In Vitro Cell Dev Biol Anim*. doi: 10.1007/s11626-014-9823-5

Wohlgemuth, S. E., Calvani, R., & Marzetti, E. (2014). The interplay between autophagy and mitochondrial dysfunction in oxidative stress-induced cardiac aging and pathology. *J Mol Cell Cardiol, 71*, 62-70. doi: 10.1016/j.yjmcc.2014.03.007

Wu, F., Feng, J. Z., Qiu, Y. H., Yu, F. B., Zhang, J. Z., Zhou, W., . . . Luo, H. D. (2013). Activation of receptor for advanced glycation end products contributes to aortic remodeling and endothelial dysfunction in sinoaortic denervated rats. *Atherosclerosis, 229*(2), 287-294. doi: 10.1016/j.atherosclerosis.2013.04.033

Zhang, L., Huang, D., Shen, D., Zhang, C., Ma, Y., Babcock, S. A., . . . Ren, J. (2014). Inhibition of protein kinase C betaII isoform ameliorates methylglyoxal advanced glycation endproduct-induced cardiomyocyte contractile dysfunction. *Life Sci, 94*(1), 83-91. doi: 10.1016/j.lfs.2013.11.011

Zhang, Q. J., Holland, W. L., Wilson, L., Tanner, J. M., Kearns, D., Cahoon, J. M., . . . Symons, J. D. (2012). Ceramide mediates vascular dysfunction in diet-induced obesity by PP2A-mediated dephosphorylation of the eNOS-Akt complex. *Diabetes, 61*(7), 1848-1859. doi: 10.2337/db11-1399

Zhang, T., Hu, X., Cai, Y., Yi, B., & Wen, Z. (2014). Metformin protects against hyperglycemia-induced cardiomyocytes injury by inhibiting the expressions of receptor for advanced glycation end products and high mobility group box 1 protein. *Mol Biol Rep, 41*(3), 1335-1340. doi: 10.1007/s11033-013-2979-3

Zhu, Y., Shu, T., Lin, Y., Wang, H., Yang, J., Shi, Y., & Han, X. (2011). Inhibition of the receptor for advanced glycation endproducts (RAGE) protects pancreatic beta-cells. *Biochem Biophys Res Commun, 404*(1), 159-165. doi: 10.1016/j.bbrc.2010.11.085

CURRICULUM VITAE

NAME: Michael Bruce Nelson

ADDRESS: 166 West 640 North
                 American Fork, UT
                 (801)736-1614
                 michaelbrucenelson@gmail.com

EDUCATION: B.S., Physiology and Developmental Biology (Jun 2013)
                 Brigham Young University, Dept. of Physiology and Developmental Biol.
                 Provo, UT 84602

                 M.S., Physiology and Developmental Biology (Expected April 2015)
                 Brigham Young University, Dept. of Physiology and Developmental Biol.
                 Provo, UT 84602

                 Thesis: The Role of Receptors for Advanced Glycation End-Products
                 (RAGE) and Ceramide in Cardiovascular Disease
                 Advisor: Dr. Paul R. Reynolds

PROFESSIONAL EXPERIENCE:

                 Research Assistant and Undergraduate Mentor (April 2011-present)
                 Brigham Young University, Dept. of Physiology and Developmental Biol.
                 Provo, UT 84602

                 Supervisor: Dr. Paul R. Reynolds
                                3054 LSB
                                (801) 422-1933
                                paul_reynolds@byu.edu

                 Duties:
                    • Design and conduct hypothesis-based research on
                      RAGE and ceramide in the context of diabetes and
                      cigarette-smoke exposure
                    • Manage data and present findings at professional
                      research conferences
                    • Maintain and operate research lab equipment
                    • Train, mentor and assist undergraduates on laboratory
                      protocols and procedures

Teaching Assistant & Laboratory Instructor (January 2011-present)
Brigham Young University, Departments of Physiology and
Developmental Biology and Microbiology and Molecular Biology

Molecular and Microbiology (MMBIO 240); January 2012-April2012
Cellular Biology (PDBIO 360); April 2012-Jun 2013
Tissue Biology Lab (PDBIO 325); January 2011-April 2015
Life Sciences Tutor; January 2013-April 2013

Duties:
- Teach, tutor and manage weekly histology classes
- Perform maintenance work and service lab equipment
- Build and maintain course website
- Supervise and mentor other teaching assistants
- Grade assignments and exams
- Hold regular office hours

SERVICE AND OUTREACH:

Missionary
Church of Jesus Christ of Latter-day Saints
Bangkok, Thailand

- Performed humanitarian work
- Taught tenets of the LDS faith in the Thai language

Hospital Volunteer; January 2011 - Present
Intermountain Healthcare
American Fork, UT 84003

- Assisted hospital staff and patients in Med/Surg and the Emergency
  Room

LABORATORY SKILLS:

Cell Culture
- Maintaining immortalized cell lines

Laboratory Equipment
- Spectrophotometer, light microscope, centrifuge, incubator/water bath,
  vacuum, microtome

Molecular Laboratory Techniques
- DNA Extraction: DNA extraction from tissue and cells

- RNA Extraction: Extraction from tissues and cells
- Gene Amplification: PCR, cDNA amplification, RT-PCR, gel electrophoresis
- Protein Analysis: Isolation from tissue and cells, immunoprecipitation, Western blotting

Immunohistochemistry and Immunofluorescence
- Tissue fixation and processing
- Tissue sectioning
- Tissue staining

PUBLICATIONS:

Peer reviewed manuscripts

Thatcher MO, Smith ME, Tippetts TS, Brassfield ES, Nelson MB, Thacker B, Swensen AC, Winden DR, Prince JT, Reynolds PR, Bikman BT. Cigarette smoke exposure leads to metabolic disruption in skeletal muscle via ceramide accumulation. *American Journal of Physiology. Endocrinology and Metabolism EPub* Sep 30, 2014.

Tippetts TS, Winden DR, Swensen, AC, Nelson MB, Thatcher MO, Saito RR, Condie TB, Simmons KJ, Judd AM, Prince JT, Reynolds PR, Bikman BT. Cigarette smoke increases cardiomyocyte ceramide accumulation and disrupts mitochondrial function. *BioMed Central Cardiovascular Disorders* EPub Nov 22, 2014

Submitted manuscripts

Nelson MB, Swensen AC, Winden DR, Bodine JS, Bikman BT, Reynolds PR. Cardiomyocyte mitochondrial respiration is reduced by receptor for advanced glycation end-products (RAGE) signaling in a ceramide-dependent manner *(AJP,submitted)*.

ABSTRACTS:

Nelson MB, Tippetts T, Winden DR, Reynolds PR, and Bikman B. RAGE activation reduces cardiomyocyte mitochondrial function in a ceramide-dependent manner. ADA Meeting 2014.

Thatcher M, Tippetts T, Johnson I, Holub Z, Nelson MB, Winden DR, Reynolds PR, and Bikman B. Ceramide mediates cigarette smoke-induced skeletal muscle metabolic disruption. ADA Meeting 2014.

Thatcher M.O., Brassfield E.S., Smith M.E., Nelson M.B., Reynolds P.R. and Bikman B.T. 2013. Smoking mediates smoking-induced insulin resistance. FASEB J 27:874.27.

AWARDS AND RECOGNITIONS

BYU Teaching Assistantships: 2
BYU Research Assistantships: 2
Fall 2013 Dept. of Physiology and Developmental Biol. Scholarship
Winter 2014 Dept. of Physiology and Developmental Biol. Scholarship
Fall 2014 Dept. of Physiology and Developmental Biol. Scholarship
Winter 2015 Dept. of Physiology and Developmental Biol. Scholarship
June 2013 Magna cum Laude, Brigham Young University, Provo, UT
Mar 2012 Member of Honor Society of Phi Kappa Phi

REFERENCES:

Dr. Paul R. Reynolds, Ph.D.
Department of Physiology and Developmental Biology
Brigham Young University, 3054 LSB
Provo, UT 84602
(801) 422-1933
paul_reynolds@byu.edu

Dr. Benjamin T. Bikman, Ph.D.
Department of Physiology and Developmental Biology
Brigham Young University, 3017 LSB
Provo, UT 84602
(801) 422-1798
benjamin_bikman@byu.edu

Dr. Steven M. Johnson
Department of Microbiology and Molecular Biology
Brigham Young University, 3132 LSB
Provo, UT 84602
(801) 422-9170
stevenj@byu.edu

Dr. Juan A. Arroyo
Department of Physiology and Developmental Biology
Brigham Young University, 3052 LSB
Provo, UT 84602
(801) 422-3221
jarroyo@byu.edu